Molecular mechanisms of disease in hereditary red blood cell enzymopathies

Richard van Wijk

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The cover photograph shows the central spiral staircase at the chateau at Chambord (France). Like DNA, it is a double helix, with two entrances; two people can walk up the opposing spiral stairways and never meet. The staircase was probably designed by Leonardo da Vinci. (Photograph kindly provided by Joop Opijnen).

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Molecular mechanisms of disease in hereditary red blood cell enzymopathies

Moleculaire pathogenese van erfelijke enzymopathieën van de rode bloedcel

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door

Henricus Anthonius van Wijk

geboren op 1 juni 1966, te Nijkerk.

Promotores: Prof. Dr. W.W. van Solinge
Faculteit Farmaceutische Wetenschappen
Prof. Dr. J.J.M. Marx
Faculteit Geneeskunde

Co-promoter: Dr. G. Rijksen
Universitair Medisch Centrum Utrecht

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Please don't let me fear
Anything I cannot explain
I can't believe
I'll never believe in anything again

(Declan Patrick Aloysius MacManus)

Voor Petra, Noah, en Aron

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CHAPTER 1

The energy-less red blood cell is lost – General introduction and outline of the thesis

The moment the mature red blood cell leaves the bone marrow, it is optimally adapted to perform the binding and transport of oxygen, and its delivery to all tissues. This is the most important task of the erythrocyte during its estimated 120-day journey in the blood stream. The membrane, hemoglobin, and proteins involved in metabolic pathways of the red blood cell interact to modulate oxygen transport, protect hemoglobin from oxidant-induced damage, and maintain the osmotic environment of the cell. The biconcave shape of the red blood cell provides an optimal area for respiratory exchange. The latter requires passage through microcapillaries, which is achieved by a drastic modification of its biconcave shape, made possible only by the loss of the nucleus and cytoplasmic organelles and, consequently, the ability to synthesize proteins.[1]

During their intravascular lifespan, erythrocytes require energy to maintain a number of vital cell functions. These include (1) maintenance of glycolysis; (2) maintenance of the electrolyte gradient between plasma and red cell cytoplasm through the activity of adenosine triphosphate (ATP)-driven membrane pumps; (3) synthesis of glutathione and other metabolites; (4) purine and pyrimidine metabolism; (5) maintenance of hemoglobin's iron in its functional, reduced, ferrous state; (6) protection of metabolic enzymes, hemoglobin, and membrane proteins from oxidative denaturation; and (7) preservation of membrane phospholipid asymmetry. Because of the lack of nuclei and mitochondria, mature red blood cells are incapable of generating energy via the (oxidative) Krebs cycle. Instead, erythrocytes depend on the anaerobic conversion of glucose by the Embden-Meyerhof Pathway and the oxidative Hexose Monophosphate Shunt for the generation and storage of high-energy phosphates (in particular ATP) and reductive potential in the form of glutathione and purine nucleotides: reduced nicotinamide adenine dinucleotide (NADH), and nicotinamide adenine dinucleotide phosphate (NADPH) (Figure 1).

In addition, erythrocytes possess a unique glycolytic bypass for the production of 2,3-diphosphoglycerate (2,3-DPG): the Rapoport-Luebering Shunt. This shunt circumvents the phosphoglycerate kinase (PGK) step and accounts for the synthesis and regulation of 2,3-diphosphoglycerate (2,3-DPG) levels that decreases hemoglobin's affinity for oxygen.[2] In addition, 2,3-DPG constitutes an energy buffer.

Numerous enzymes are involved in the above-mentioned pathways and many red blood cell enzymopathies have been described in the Embden-Meyerhof Pathway, the Hexose Monophosphate Shunt, the Rapoport-Luebering Shunt, the glutathione pathway, purine–pyrimidine metabolism and methemoglobin reduction.[3-14] These enzymopathies disturb the erythrocyte's integrity and shorten its cellular survival, resulting in hemolytic anemia.[15] According to their clinical consequences, two groups of enzymopathies can be distinguished. The first concerns deficiencies in enzymes involved in the Embden-Meyerhof

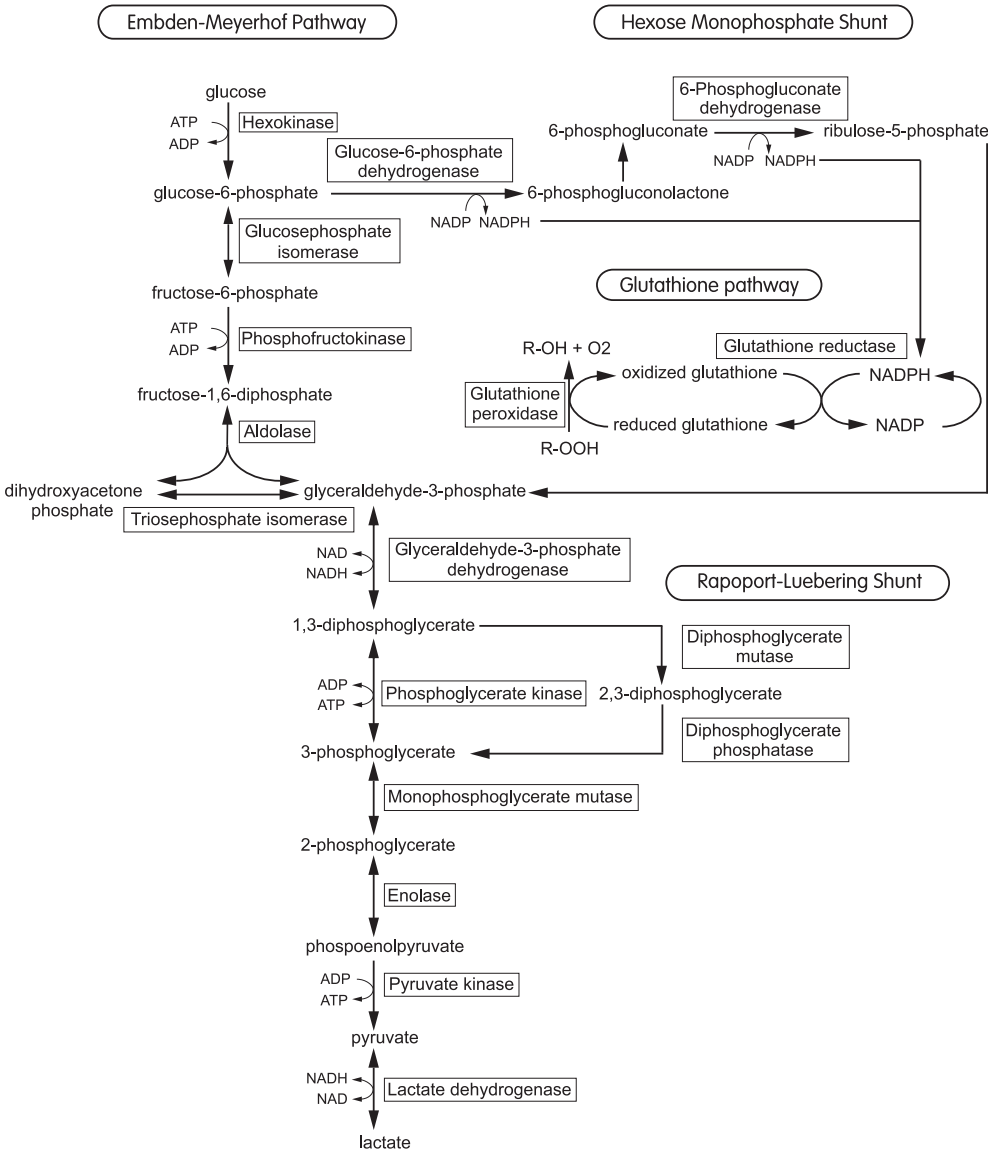

Figure 1. Schematic overview of the Embden-Meyerhof Pathway, the Hexose Monophosphate Shunt, the Rapoport-Luebering Shunt, and the Glutathione Pathway.

Pathway and purine–pyrimidine metabolism. Ultimately, these deficiencies impair ATP production, leading to chronic nonspherocytic hemolytic anemia (CNSHA). The most

common cause of CNSHA is pyruvate kinase (PK) deficieny. Second, disorders concerning the Hexose Monophosphate Shunt, which maintains adequate levels of reduced glutathione (GSH). This group of disorders is associated with hemolytic episodes, induced by oxidative stress, drugs, or infections. Deficiency of glucose-6-phosphate dehydrogenase (G6PD) is the most frequently encountered enzymopathy in this group. In general, the degree of hemolysis is dependent on the relative importance of the affected enzyme and the properties of the mutant enzyme with regard to functional abnormalities or instability, or both. The ability to compensate for the enzyme deficiency by overexpressing isozymes or using alternative pathways contribute to the clinical picture of patients with red blood cell enzymopathies. A frequently observed response to compensate for anemia is increased erythrocyte production (reticulocytosis). Reticulocytosis (reticulocytes normally comprise 0.5 to 2.5% of total erythrocytes) is an important sign of hemolysis. Reticulocytes still preserve cytoplasmic organelles, including ribosomes and mitochondria, and are thus capable of protein synthesis and the production of ATP by oxidative phosphorylation. Several enzymes including hexokinase (HK), PK, G6PD, aldolase, and pyrimidine 5'-nucleotidase (P5'N) display much higher activity in reticulocytes and are often referred to as the age-related enzymes.[16]

A number of red blood cell enzymopathies concern enzymes expressed in other tissues as well. The deficiency is, however, more pronounced in red blood cells, when compared to other cells, because of the long life span of the mature erythrocyte after the loss of protein synthesis.[17] Therefore, once an enzyme in red blood cells is degraded or has otherwise become nonfunctional, it cannot be replaced by newly synthesized proteins. Some enzyme deficiencies (*e.g.* triosephosphate isomerase deficiency) may involve cells other than the red blood cell as well. By far the majority of red cell enzymopathies are hereditary in nature, although acquired deficiencies have also been described, mainly in malignant hematological disorders.[18-21]

In this chapter, a summary is made of the major features regarding the biochemical, structural and genetic basis of clinically relevant red blood cell enzymopathies involved in the Embden-Meyerhof Pathway, the Rapoport-Luebering Shunt, the Hexose Monophosphate Shunt, and the Glutathione Pathway.

The Embden-Meyerhof Pathway

Glucose is the energy source of the red blood cell. Under normal physiological circumstances (*i.e* no excessive oxidative stress), 90% of glucose is catabolized anaerobically to pyruvate or lactate by the Embden-Meyerhof Pathway, or glycolysis (Figure

1). Although one mole of ATP is used by HK, and an additional mole of ATP by phosphofructokinase (PFK), the net gain is two moles of ATP per mole of glucose, because a total of four moles of ATP are generated by PGK and PK. In addition, reductive potential is generated in the form of NADH, in the step catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GA3PD). This reducing energy can be used to reduce methemoglobin to hemoglobin by NADH-cytochrome b5 reductase (cytb5r). If this reaction takes place, the end product of the glycolysis is pyruvate. However, if NADH is not reoxidized here, it is used in reducing pyruvate to lactate by lactate dehydrogenase (LD) in the last step of the glycolysis. The Embden-Meyerhof Pathway is subjected to a complex mechanism of inhibiting and stimulating factors. The overall velocity of red blood cell glycolysis is regulated by three rate-limiting enzymes, HK, PFK, and PK, and by the availability of nicotinamide adenine dinucleotide (NAD) and ATP. Some glycolytic enzymes are allosterically stimulated (*e.g.* fructose-1,6-diphosphate (FBP) for PK) or inhibited (*e.g.* glucose-6-phosphate (G6P) for HK) by intermediate products of the pathway.

In general, enzymopathies of the Embden-Meyerhof Pathway cause CNSHA. The continous lack of sufficient energy and other metabolic impairments results in a shortened lifespan of the mature red blood cell, albeit with variable clinical severity. The chronic nature of hemolysis contrasts with the periodic hemolytic episodes that characterize most cases of G6PD deficiency (see below). The lack of characteristic changes in red blood cell morphology differentiates the glycolytic enzymopathies from erythrocyte membrane defects and most hemoglobinopathies.

Hexokinase

Hexokinase catalyzes the phosphorylation of glucose to G6P, using ATP as a phosphoryl donor (Figure 1). As the initial step of glycolysis, HK is one of the rate-limiting enzymes of this pathway. The activity of hexokinase is significantly higher in reticulocytes compared to mature red cells, in which it is very low. In fact, of all glycolytic enzymes, HK has the lowest enzymatic activity *in vitro*.[14]

In mammalian tissues four isozymes of HK with different enzymatic properties exist, HK-I to III, with a molecular mass of 100 kDa, and HK-IV (or glucokinase), with a molecular mass of 50 kDa. HK-I to III are considered to be evolved from an ancestral 50 kDa HK by gene duplication and fusion.[22] Consequently, both the C- and N-terminal halves of HK-I to III show extensive internal sequence similarity but only in case of HK-II is catalytic function maintained in both the C- and N-terminal halves. HK-I and HK-III have further evolved into enzymes with catalytic (C-terminal) and regulatory (N-terminal) halves, respectively. HK-I is the predominant isozyme in human tissues that depend strongly on glucose utilisation for

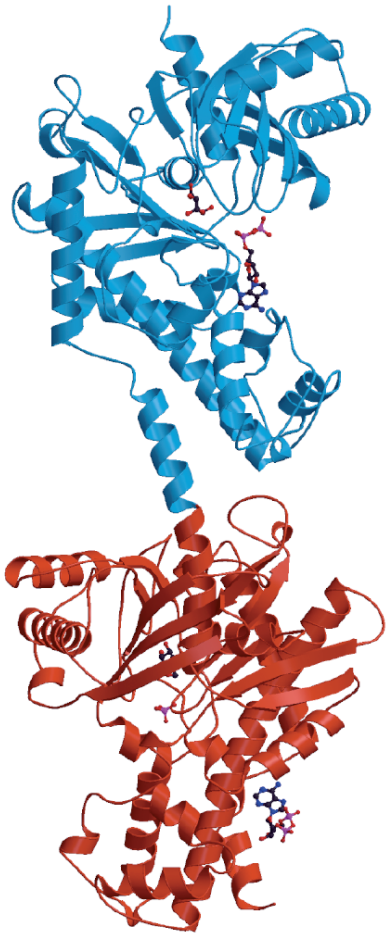

Figure 2. Hexokinase I monomer in complex with glucose, phosphate, and ADP. The C- and N-terminal halves are colored blue and red, respectively.

their physiological functioning, such as brain, muscle and erythrocytes. HK-I displays unique regulatory properties in its sensitivity to inhibition by physiological levels of the product G6P and, moreover, relief of this inhibition by inorganic phosphate (P*i*).[23,24]

The recent determination of the structures of the human and rat HK-I isozymes have provided substantial insight into ligand binding sites and subsequent modes of interaction of these ligands (Figure 2).[25-28] The mode of inhibition by G6P remains subject to debate.[25-34] Erythrocytes contain a specific subtype of HK (HK-R)[35] that is encoded by the HK-I gene (*HK1)*, localized on chromosome 10q22[36] and spanning more than 100 kb.[37] The structure

of *HK1* is complex. It encompasses 25 exons, which, by tissue-specific transcription, generate multiple transcripts by alternative splicing of different 5' exons.[37] Erythroid-specific transcriptional control results in a unique red blood cell-specific mRNA that differs from HK-I transcripts at the 5' untranslated region (5'-UTR) and at the first 63 nucleotides of the coding region.[38-40] Consequently, HK-R lacks the porin-binding domain that mediates HK-I binding to mitochondria.[38]

Hexokinase deficiency (MIM 235700) is a rare autosomal, recessively inherited disease with CNSHA as the predominant clinical feature. As with most glycolytic red cell enzyme deficiencies the severity of hemolysis is variable, ranging from severe neonatal hemolysis and death to a fully compensated chronic hemolytic anemia. Splenectomy is in general beneficial. Seventeen families with hexokinase deficiency have been described to date[41] and only three patients have been characterized at the molecular level.[42-45] One compound heterozygous patient carried a missense mutation that encoded a leucine to serine substitution at residue 529. An as yet unidentified mutation on the other allele caused skipping of the sixth exon, as detected in this patient's cDNA.[42,43] One other patient was homozygous for a missense mutation that predicted the substitution of a highly conserved threonine by serine at residue 680 in the enzyme's active site, where it interacted with phosphate moieties of adenosine diphosphate (ADP), ATP and G6P.[45] The last case constituted a lethal out-of-frame deletion of exons 5 to 8 of *HK1*. This deletion was identified in the homozygous state in a fetus who died *in utero*.[44]

Glucose-6-Phosphate Isomerase

Glucose 6-phosphate isomerase (GPI) catalyzes the interconversion of G6P into fructose-6-phosphate (F6P) in the second step of the Embden-Meyerhof Pathway (Figure 1). As a result of this reversible reaction, products of the Hexose Monophosphate Shunt can be recycled to G6P. Unlike HK and other age-related enzymes, the GPI activity in reticulocytes is only slightly higher than that of mature erythrocytes. Apart from its role in glycolysis, GPI exerts cytokine properties outside the cell and is involved in several extracellular processes.[46] Because GPI knock-out mice die in the embryological state, GPI is considered to be a crucial enzyme.[47,48]

Recently, the crystal structure of human GPI was resolved. The enzyme is a homodimer, composed of two 63-kDa subunits of 558 amino acids each. The active site is composed of polypeptide chains from both subunits. Thus, formation of the dimer is a prerequisite for catalytic activity.[49,50] The structural gene coding for GPI (*GPI*) is located on chromosome 19q13.1.[51] *GPI* spans at least 50 kb and consists of 18 exons that are transcribed into a cDNA of 1.9 kb in length.[52]

GPI deficiency (MIM 172400) is an autosomal recessive disease and is second to PK deficiency in frequency. Approximately 50 families with GPI deficiency have been described worldwide.[53] Homozygous or compound heterozygous patients have chronic hemolytic anemia of variable severity and display enzymatic activities of less than 25% of normal. Splenectomy is often beneficial. Hemolytic crises may be triggered by viral or bacterial infections but, in contrast to G6PD deficieny, drug-induced hemolysis is rare.[54,55] A few patients displayed hemolytic crises after oxidative stress.[56-59] Hydrops fetalis appears more common in GPI deficiency than in other enzyme deficiencies.[58,59] In rare cases, GPI deficiency also affected non-erythroid tissues, causing neurological symptoms and granulocyte dysfunction.[60] Normally, GPI is very stable but a striking feature of nearly all GPI mutants is their thermolability, whereas kinetic properties are more or less unaffected. Twenty-nine mutations have been detected in *GPI*, 24 of which were missense mutations, three were nonsense mutations, and two mutations affected splice sites.[53] Mapping of these mutations to the crystal structure of human GPI has provided insight in the molecular mechanisms causing hemolytic anemia in this disorder. In accordance with the three-dimensional structure, mutations could be categorized into three distinct groups that affected (1) the overall structure, (2) the dimer interface and (3) the active site.[50]

Phosphofructokinase

Phosphofructokinase catalyzes the, rate-limiting, ATP-mediated phosphorylation of F6P to FBP (Figure 1). PFK is a homo- or heterotetramer with a molecular mass of approximately 380 kDa, and the enzyme is allosterically regulated, among other metabolites, by 2,3-DPG.[61] Three different subunits have been identified in humans: PFK-M (muscle), PFK-L (liver) and PFK-P (platelet).[62] The subunits are expressed in a tissue-specific manner and, in erythrocytes, five isoenzymes of varying subunit composition (M_4, M_3L_1, M_2L_2, ML_3, and L_4) can be identified.[63] The cDNAs for all three subunits have been cloned.[64-66] The gene encoding the M subunit (*PFKM*) has been reassigned to chromosome 12q13.3[67] and spans 30 kb. It contains 24 exons and contains at least two promoter regions.[68] The L-subunit encoding gene (*PFKL*) contains 22 exons and spans more than 28 kb.[69] It is located on chromosome 21q22.3.[70]

PFK deficiency (MIM 171850) is a rare autosomal, recessively inherited disorder. Because red blood cells contain both M- and L-subunits, mutations affecting either of both genes will affect enzyme activity. Thus, mutations concerning the L subunit will render red blood cells that contain only M_4 and, consequently, they are PFK deficient. In such cases, patients display a mild hemolytic disorder without myopathy. Alternatively, a deficiency of the M subunit results in the absence of muscle PFK and, in addition, causes a partial PFK

deficiency in erythrocytes. Accordingly, deficiency of the M subunit causes myopathy and a mild hemolytic disorder. Erythrocytes that express only the L_4 PFK also show a metabolic block at the PFK step in glycolysis and lowered 2,3-DPG levels. To date, 15 PFK deficient *PFKM* alleles from more than 30 families have been characterized.[71] One-third of the reported patients are of Jewish origin and in this population an intronic splice site mutation, IVS5+1G>A,[72] and a single base-pair deletion in exon 22, c.2003delC,[73] are among the most frequently encountered mutations. Patients usually have only a mild, well compensated hemolytic anemia with an enzyme activity of approximately half normal.

Aldolase

Aldolase catalyzes the reversible conversion of fructose-1,6-biphosphate to glyceraldehyde-3-phosphate (GA3P) and dihydroxyacetone phosphate (DHAP) (Figure 1). Aldolase is a tetramer of identical subunits of 40 kDa each, and three distinct isoenzymes have been identified, aldolase A, B and C. Erythrocytes, muscle and brain express the 364 amino acids long aldolase A subunits. The gene for aldolase A (*ALDOA*) is located on chromosome 16q22–q24.[74] It spans 7.5 kb and consists of 12 exons. Multiple transcription-initiation sites have been determined and *ALDOA* pre-mRNA is spliced in a tissue-specific manner.[75]

Aldolase deficiency (MIM 103850) is a very rare disorder and only five patients from four families have been described.[76-79] They all displayed moderate chronic hemolytic anemia. Enzyme stability was decreased in a boy described by Beutler *et al.* In addition to hemolytic anemia, this patient also displayed mental retardation and dysmorphic features.[76] The two patients reported by Miwa *et al.*[77] suffered from severe hemolytic anemia, exacerbated by infection, but none of the features described by Beutler *et al*. In one of these patients the causative mutation concerned a homozygous substitution of aspartic acid to glycine at residue 128.[80] The patient reported by Kreuder *et al*. suffered from hemolytic anemia, myopathy and psychomotor retardation. In this case, aldolase deficiency was caused by an *ALDOA* missense mutation that predicted a glutamate to lysine substitution at residue 206.[78] Recently, a patient was described with CNSHA and severe rhabdomyolysis but normal cognitive function. This patient showed severe clinical symptoms and, ultimately, died at the age of four years. She was compound heterozygous for a null mutation, Arg303Stop, and a missense mutation, Cys338Tyr, near a critical region in aldolase A.[79]

Triosephosphate Isomerase

Triosephosphate isomerase (TPI) is the glycolytic enzyme with the highest activity *in vitro*.[14] TPI catalyzes the interconversion of GA3P and DHAP. It consists of a dimer with two identical subunits of 248 amino acids (27 kDa).[81] No TPI isozymes are known but three

distinct electrophoretic forms can be distinguished as a result of post-translational modifications.[82,83] In red blood cells, TPI activity is not maturation dependent.
TPI deficiency (MIM 190450) is a rare autosomal recessive disorder, characterized by hemolytic anemia at onset, often accompanied by neonatal hyperbilirubinemia requiring exchange transfusion. In addition, patients display progressive neurological dysfunction, increased susceptibility to infection, and cardiomyopathy. Affected individuals often die in childhood.[84] Patients show an 20- to 60-fold increased DHAP concentration in their erythrocytes,[85] consistent with a metabolic block at the TPI step.
TPI is transcribed from a single gene (*TPI1*) on chromosome 12p13[86] that consists of seven exons and spans 3.5 kb.[87] Three processed pseudogenes have been identified.[87] Fourteen mutations have been described in *TPI1* and the most frequently ocurring mutation (74%) causes a glutamine to aspartic acid change at residue 104.[88] This substitution likely perturbs the local structure of the active site.[81] Haplotype analysis strongly suggested a single origin for this mutation whereby the common ancestor originated from Northern Europe.[89] This is in agreement with the European origin of almost all TPI-deficient individuals.

Phosphoglycerate Kinase

Phosphoglycerate kinase generates one molecule of ATP by catalyzing the reversible conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate (Figure 1). This reaction can be bypassed by the Rapoport-Luebering shunt (see below) with the consequent loss of one ATP molecule. Two isozymes of PGK exist, PGK-1, ubiquitously expressed in all somatic cells, and PGK-2, expressed only in spermatozoa.[90] PGK-1 is a 48-kDa monomer consisting of 417 amino acids.[91,92] The three-dimensional structure of horse muscle PGK, highly homologous to the human enzyme, revealed that PGK consists of two domains. These domains are connected by a conserved hinge, allowing for conformational freedom.[93] The ADP/ATP binding site is located on the C-terminal domain while phosphoglycerates bind the N-terminal domain. A conformational rearrangement involving bending of the hinge occurs upon binding of both substrates, bringing them in position for phosphate transfer.[94]
The gene encoding PGK-1 (*PGK1*) is located on Xq13.3,[95] thereby rendering PGK deficiency an X-linked disorder. *PGK1* spans 23 kb and is composed of 11 exons.[96]
PGK deficiency (MIM 311800) is characterized by chronic hemolytic anemia (often fully compensated), dysfunction of the central nervous system, and myopathy. However, the phenotype of PGK-1 deficiency is highly variable because patients usually do not display all three clinical features.[97] PGK activity varies between 0 and 20% of normal but there is no correlation of residual enzymatic activity with clinical severity. Fourteen different mutations in *PGK1* have been described in association with PGK deficiency.[71] Most of these mutations

are missense mutants of which one, in addition to encoding a non-conserved Glu252Ala substitution, resulted in aberrant splicing with a consequent 90% reduction in mRNA levels.[98]

Pyruvate Kinase

Pyruvate kinase catalyzes the irreversible phosphorylgroup transfer from phosphoenolpyruvate (PEP) to ADP, yielding pyruvate and the second mole of ATP in glycolysis (Figure 1). PK is a key regulatory enzyme of glycolysis, and pyruvate is crucial for several metabolic pathways. The enzyme is active as a tetramer, and four different isozymes are expressed in mammals. The M1 isozyme is expressed in skeletal muscle, heart, and brain. It is the only isozyme that is not subjected to allosteric regulation. The M2 isozyme is expressed in early fetal tissues, but also in most adult tissues, including leucocytes and platelets. L-type PK is predominately expressed in the liver whereas R-type PK expression is confined to the red blood cell.[99-101] In basophilic erythroblasts, both PK-R and PK-M2 are expressed. During further erythroid differentiation and maturation, a switch in isozymes occurs whereby progressively increased PK-R expression gradually replaces PK-M2.[102-105] This is in part due to changes in protein synthesis and degradation rates.[106] Human red blood cell PK consists of two distinct species, R1 and R2. R1 PK predominates in reticulocytes and young erythrocytes, whereas mature red blood cells mainly possess R2 PK.[107-109] R1 PK is a homotetramer composed of four PK-R, also called L', subunits (L'_4). Limited proteolytic degradation of the 63 kDa PK-R subunit renders a 57-58 kDa PK-L subunit that is incorporated in the heterotetramer R2 PK ($L_2L'_2$).[108,110,111] The enzymatic activity of PK decreases with increasing cell age of the erythrocyte. PK is allosterically activated by PEP and FBP, and negatively regulated by its product ATP.[112,113] Furthermore, PK has an absolute requirement for cations, normally Mg^{2+} and K^+.[114,115]

Recently, the three-dimensional structure of human erythrocyte PK was elucidated.[116] Each PK-R subunit can be divided into four domains (Figure 3), the N domain (residues 1 to 84), A domain (residues 85–159 and 263–431), B domain (residues 160–262) and C domain (residues 432–574).[116] Domain A is the most highly conserved whereas the B and C domain are more variable.[117] The active site lies in a cleft between the A domain and the flexible B domain. The B domain is capable of rotating away from the A domain generating either the 'open' or 'closed' conformation. The C domain contains the binding site for FBP. The allosteric transition from the inactive T-state to the active R-state involves the simultaneous and concerted rotations of entire domains of each subunit, in such a way that all subunit and domain interfaces are modified.[118,119] The allosteric and catalytic sites are able to communicate with each other across the relatively long distance that separates the FBP

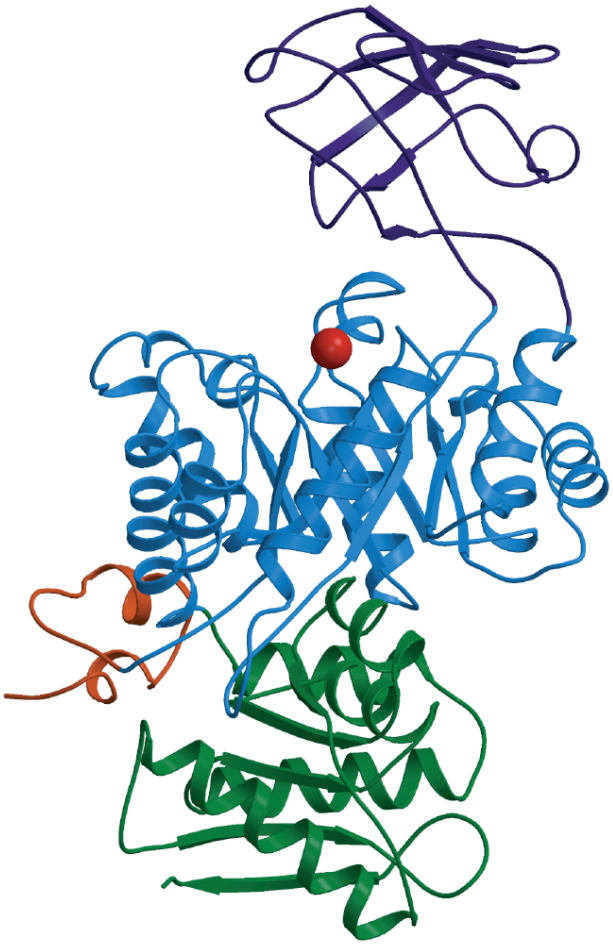

Figure 3. Pyruvate kinase monomer, The A, B, C, and N domains are colored blue, purple, green, and red, respectively. The position of bound phosphoenolpyruvate is indicated by a red sphere.

binding site from the catalytic center. The A/A' and C/C' subunit interface interactions and the A/B interdomain interactions are considered key determinants of the allosteric response.[116,119-124]

The gene encoding the PK-M1 and PK-M2 subunits is located on chromosome 15q22[125] and the respective subunits of 531 amino acids each are produced by alternative splicing.[126,127] The PK-R and PK-L subunits are transcribed from a single gene (*PKLR*), located on chromosome 1q21,[128,129] by the use of alternative promoters.[99,130-133] The gene consists of 12 exons and spans 9.5 kb.[134,135] Exon 1 is erythroid-specific and exon 2 is liver specific. Exons 3 to 12 are

included in both mRNAs and encode a PK-R subunit of 574 amino acids, whereas the PK-L subunit comprises 531 amino acids.

PK deficiency (MIM 266200) is the most common cause of nonspherocytic hemolytic anemia due to defective glycolysis and is inherited in an autosomal recessive manner. The estimated prevalence is 51 cases (*i.e.* homozygous or compound heterozygous patients) per million in the white population.[136] The continous lack of sufficient energy for normal functioning shortens the life-span of the mature PK-deficient erythrocyte and results in non-spherocytic hemolytic anemia. 2,3-DPG levels are generally increased, thus ameliorating the anemia by lowering the oxygen-affinity of hemoglobin.[137] Phenotypically, the clinical picture varies from severe hemolysis causing neonatal death to a well compensated hemolytic anemia. Some PK-deficient patients present with hydrops fetalis.[138] Reticulocytosis is almost always observed. Splenectomy often ameliorates the hemolysis, especially in severe cases, and increases the reticulocyte counts even further. To date, more than 150 mutations in *PKLR* have been reported to be associated with pyruvate kinase deficiency.[139,140] A schematic overview is presented in Figure 4. Most (70%) mutations in *PKLR* are missense mutations concerning conserved residues in structurally and functionally important domains of PK. In the European and North-American population, the most frequently detected mutations are missense mutants c.1456C>T (Arg486Trp),[141] c.1529G>A (Arg510Gln),[141] c.994G>A (Gly332Ser),[142] and nonsense mutant c.721G>T (Glu241Stop).[141] One large, in-frame deletion, has been described in *PKLR*.[143] There appears to be no direct relationship between the nature and location of the substituted amino acid and the type of molecular perturbation.[116,144] One transcriptional mutant is of particular interest because it silences erythroid-specific *PKLR* transcription completely.[145] The causative single-base change disrupts a putative binding domain for an as yet unidentified *trans*-acting factor that mediates the effects of factors necessary for regulation of PK gene expression during red cell differentation and maturation.[145]

Glyceraldehyde-3-phosphate Dehydrogenase, Monophosphoglycerate Mutase, Enolase, and Lactate Dehydrogenase

Red blood cell deficiencies of glyceraldehyde-3-phosphate dehydrogenase[146,147] and enolase[148] have been described in association with hemolytic anemia but a causal relationship was not established.

Monophosphoglycerate mutase (MPGM) in erythrocytes is a homodimer, composed of two subunits BB. Deficiency of this subunit, that is expressed in many other tissues as well, has not been reported.

Deficiencies of either the H (B) or M (A) subunits of lactate dehydrogenase have been reported. However, neither defiency causes hemolytic anemia, despite strongly reduced

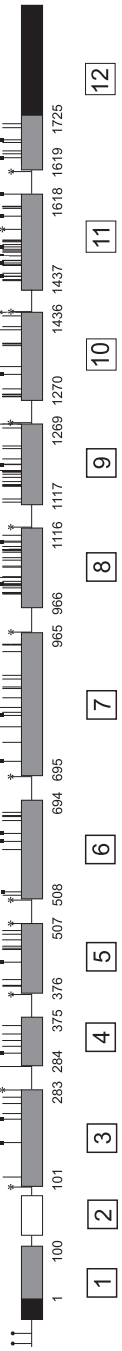

Figure 4. Schematic representation of the *PKLR* gene and its erythroid-specific promoter. Exons, but not introns, are drawn to scale. Exons are numbered and depicted as grey rectangles with 5' and 3' non-coding sequences in black. The open rectangle represents the liver-specific exon 2. Nucleotides are numbered starting from the ATG in red blood cell-specific exon 1. The location of the more than 150 mutations associated with PK deficiency is indicated (reference 139, and Chapter 4). Vertical lines: missense mutants, ●: transcriptional mutants, ✷: frameshift and nonsense mutants, ■: mutations affecting pre-mRNA splicing. Double-sized vertical lines indicate multiple base changes at the same nucleotide position. The horizontal double arrow denotes a deletion of exon 11, as identified on the cDNA level.

enzymatic activity.[149] Interestingly, homozygous LDH M subunit deficiency in mice is associated with chronic hemolytic anemia.[150]

Rapoport-Luebering Shunt

In the erythrocyte, 2,3-DPG is synthesized and dephosphorylated in the Rapoport-Luebering Shunt. Both reactions are catalyzed by one multifunctional protein that posesses diphosphoglycerate mutase (DPGM) and diphosphoglycerate phosphatase (DPGP) enzymatic activities (Figure 1).[151,152] This erythroid-specific enzyme[153] regulates 2,3-DPG levels in the red blood cell and is very important in regulating the oxygen affinity of hemoglobin. DPGM is a homodimer, with 30-kDa subunits consisting of 258 amino acids.

The Rapoport-Luebering Shunt is unique to mammalian red blood cells and represents the principal physiological means to regulate oxygen affinity of hemoglobin.[154-156] From an oxygen-transport point of view, the Embden-Meyerhof pathway serves mainly the generation of 2,3-DPG because this is quantitatively the major glycolytic intermediate in the red blood cell and its levels are about equal to the sum of the other glycolytic intermediates. The oxygen affinity of hemoglobin is also influenced by slight changes of blood pH, and a corresponding sensitivity for the pH exists in the Rapoport-Luebering Shunt that, again, permits changes in 2,3-DPG contents to fine-tune the oxygen affinity of hemoglobin. In PK deficiency 2,3-DPG is increased as a result of the metabolic block at the PK step and of a retrograde accumulation of products of glycolysis. The increased 2,3-DPG levels result in a decreased oxygen affinity of hemoglobin so that oxygen is more readily transferred to tissue. This beneficial circumstance is absent in those glycolytic enzyme defects that cause a decrease in 2,3-DPG levels, *e.g.* HK and GPI deficiency.

The gene for DPGM (*DPGM*) has been mapped to chromosome 7q31-34[157] and it consists of three exons, spanning more than 22 kb.[158] DPGM deficiency (MIM 222800) is a very rare autosomal recessive disorder and only a few cases have been described, all belonging to one French family.[159] Patients had severely reduced 2,3-DPG levels and increased ATP levels. They were clinically normal and displayed no hemolytic anemia. Instead, they presented with erythrocytosis that likely resulted from the reduced 2,3-DPG levels and, consequently, the increased oxygen affinity of hemoglobin. These patients were compound heterozygous for a single-nucleotide change in *DPGM* that predicted the replacement of the highly conserved arginine at residue 89 by cysteine (DPGM Créteil I), and a single-nucleotide deletion that introduces a premature stopcodon and, consequently, encodes a truncated peptide (DPGM Créteil II).[160]

Hexose Monophosphate Shunt (Pentose Phosphate Shunt)

Approximately 10% of glucose in red blood cells is normally catabolized by the Hexose Monophosphate Shunt (or Pentose Phosphate Shunt). However, in case of oxidative stress, *e.g.* during infection or evoked by the use of certain drugs, this fraction may be markedly increased. The principal function of the Hexose Monophosphate Shunt is to produce two NADPH molecules from NADP *via* the oxidation of G6P. NADPH is required for a variety of biosynthetic pathways, for the stability of catalase, and the regeneration and preservation of the reduced form of glutathione (Figure 1). These processes are crucial to protect the cell against oxidative stress. Because of the lack of other sources of NADPH, the erythrocyte relies strongly on the key enzyme of this reaction, glucose-6-phosphate dehydrogenase.
Each of two enzymes, G6PD (see below) and 6-phosphogluconate dehydrogenase (6PGD), generate one molecule of NADPH in the Hexose Monophosphate Shunt (Figure 1). In contrast to G6PD deficiency, hereditary 6PGD deficiency does not cause hemolytic anemia, even in the homozygous condition.[161]

Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase is expressed in all cells and catalyzes the first step in the Hexose Monophosphate Shunt that involves the conversion of glucose-6-phosphate to 6-phosphogluconate (Figure 1). This step generates one mole of NADPH. G6PD is an essential enzyme because total lack of G6PD has been shown to be a lethal condition at an early stage

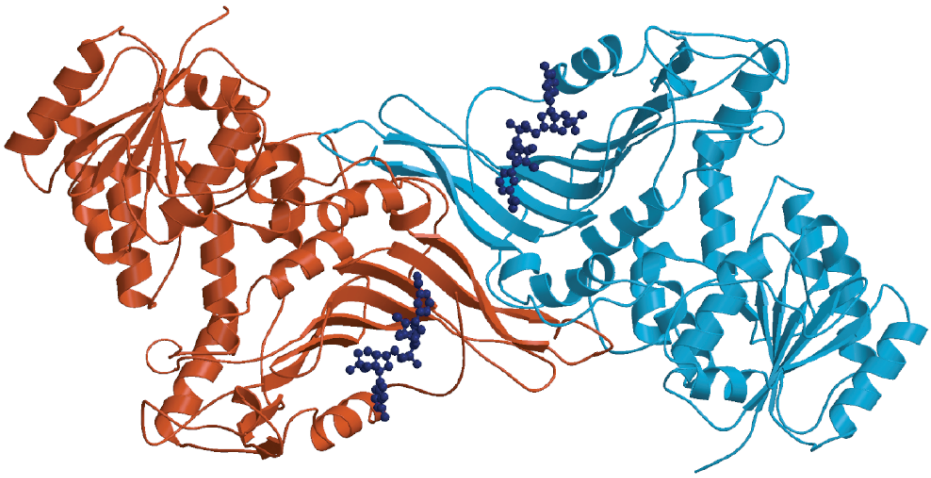

Figure 5. A dimer of human glucose-6-phosphate deydrogenase. Subunits A and B are colored red and blue. Structural NADP+ molecules are drawn in ball-and-stick mode and colored dark blue.

in embryonic development.[162] G6PD activity is higher in reticulocytes than in mature erythrocytes. Under physiological conditions, the active human enzyme exists in a dimer-tetramer equilibrium. Lowering the pH causes a shift towards the tetrameric form.[163]
The crystal structure of human G6PD was recently elucidated.[164] G6PD consists of identical 59 kDa subunits of 515 amino acids long. Each subunit is comprised of two domains, an N-terminal domain and a large β/α domain with an antiparallel nine-stranded sheet (Figure 5). The extensive interface between the two monomers is of crucial importance for enzymatic stability and activity.[164]
The gene encoding G6PD (*G6PD*) is located on the X-chromosome (Xq28),[165] spans 18 kb and consists of 13 exons of which exon 1 is noncoding. The promoter shares many features common to other housekeeping genes.[166]
G6PD deficiency (MIM 305900) is the most common enzymopathy, affecting more than 400 million people worldwide. Five different syndromes can be distinguished: (1) drug-induced hemolysis, (2) infection-induced hemolysis, (3) favism, (4) neonatal jaundice, and (5) CNSHA. The majority of G6PD-deficient individuals develop hemolysis only in response to oxidative stress, after intake of certains drugs, during infections, or after consumption of fava beans. Apart from these acute hemolytic episodes these patients are usually asymptomatic. In contrast, the most severe form of G6PD deficiency causes mild to severe chronic hemolysis, exacerbated by oxidative stress. More than 400 different G6PD variants have been described, associated with a wide range of biochemical characteristics and phenotypes.[167] Accordingly, five classes of G6PD-deficient variants can be distinguished.[168]

Class I	severe deficiency, associated with CNSHA
Class II	severe deficiency (< 10% residual activity) but no CNSHA
Class III	moderate to mild deficiency (10–60% residual activity) (*e.g.* G6PD A–)
Class IV	very mild or no deficiency (*e.g.* G6PD A)
Class V	increased enzymatic activity (only one known variant, G6PD Hektoen)

Nearly half of the mutations encoding class I G6PD variants directly affect the subunit interface of the G6PD dimer.[164,169,170] However, identical variants may still lead to different phenotypes.[171,172] One well-established genetic determinant accounting for the phenotypic variability in G6PD deficiency, in particular with regard to neonatal jaundice, is represented by the co-inheritance of Gilbert's syndrome.[173,174] The genetic basis of Gilbert's syndrome constitutes a polymorphic dinucleotide insertion repeat in the promoter of *UGT1*, the gene that codes for UDP glucuronosyltransferase 1. This enzyme catalyzes bilirubin conjugation

by the liver and the presence of a variant promoter is actively involved in the pathogenesis of G6PD deficiency-associated hyperbilirubinemia.[175,176]
For further reading regarding G6PD (deficiency) we refer to excellent reviews by Beutler,[177] Dacie,[178] Luzzatto,[179] and Vulliamy.[167]

The Glutathione Pathway

The sulfhydryl-containing tripeptide reduced glutathione protects hemoglobin and other essential proteins in the red blood cell from peroxidative injury. In this process, which involves the reduction of peroxides or oxidized protein sulfhydryl groups, GSH is converted to oxidized glutathione (GSSG).

GSH synthesis – γ-Glutamylcysteine Synthetase, and Glutathione Synthetase
The first step in glutathione biosynthesis involves the synthesis of glutamylcysteine from L-glutamate, L-cysteine, and ATP. This rate-limiting step in glutathione synthesis is ATP-dependent and catalyzed by γ-glutamylcysteine synthetase (GC-S). There is a feedback inhibition by reduced glutathione. GC-S is a heterodimer composed of a catalytic 73 kDa heavy chain (GC-S_h) and a regulatory 31 kDa light chain (GC-S_l).[180,181] These subunits are encoded by separate genes, located on chromosome 6p12 (*GCLC*)[182] and 1p21 (*GCLM*),[183] respectively.
Hereditary GC-S deficiency (MIM 606857) is a very rare autosomal recessive disorder. It is associated with mild CNSHA that may be fully compensated. Drug- and infection-induced hemolytic crises may occur. Only 10 patients have been described, five of which were characterized at the molecular level.[184-186] One mutation predicted the substitution of histidine by leucine at residue 370 of GC-S_h, probably resulting in an unstable protein.[184] Two other, related, patients were homozygous for a GC-S_h variant that harboured a leucine instead of proline at residue 158.[185] Recently, two other related patients were reported that carried a missense mutation, coding for arginine instead of cysteine at residue 127 of GC-S_h. This variant was associated with decreased GSH production and markedly decreased enzymatic activity.[186]
The second step in GSH synthesis involves the addition of glycine to the dipeptide γ-glutamylcysteine. This irreversible and ATP-dependent reaction is mediated by glutathione synthetase (GSH-S). GSH-S is a homodimer of 52 kDa[187] and the 23 kb gene coding for GSH-S (*GSS*) is located on chromosome 20q11.2.[188]
Only a few cases of GSH-S deficiency (MIM 601002) have been reported and these were

associated with mild chronic hemolytic anemia.[188,189] GSH-S deficiency is inherited in an autosomal recessive manner and patients may be susceptible to more severe, acute hemolytic crises when exposed to 'oxidant' substances. Two distinct types of GSH-S deficiency can be distinguished. Both types share the existence of mild chronic hemolysis and this is the predominant clinical feature of the first type of GSH-S deficiency. However, in patients with the second and more severe form, the major features are mental retardation, severe generalized muscle weakness, tremors, incoordination, hemolytic anemia, and metabolic acidosis. This type of GSH-S deficiency is also known as 5-oxoprolinuria or pyroglutamic aciduria.[190] The difference in severity reflects the fact that in the mild form, GSH-S deficiency is confined to the red blood cells. GSH-S activity is present in adequate amounts in young erythrocytes but because of unstable GSH-S variants, enzymatic activity declines rapidly when the cell ages, due to the lack of protein synthesis. Other cells of the body that contain nuclei and ribosomes are capable of compensating for the loss of GSH-S by increasing protein synthesis capacity. With regard to the severe type of GSH-S deficiency, all cells of the body have low GSH-S enzymatic activity because no functional enzyme can be produced in adequate amounts. In both types of GSH-S deficiency, the red blood cell exhibits markedly reduced GSH levels. Several causative mutations have been identified, associated with the disease.[188,189,191]

Generating oxidized glutathione – Glutathione Reductase and Glutathione Peroxidase

Glutathione reductase (GSR) links the glutathione pathway to the Hexose Monophophate Shunt by means of the reversible oxidation and reduction of NADP (Figure 1). Thereby, GSR maintains high levels of reduced glutathione in the red blood cell. GSR requires flavin adenine dinucleotide (FAD) as a cofactor. A mitochondrial and a cytoplasmic GSR isozyme are both produced from the same mRNA, most likely by alternative initiation of translation.[192] The enzyme is a homodimer, linked by a disulfide bridge. Each 56-kDa subunit contains four domains, of which domains 1 and 2 bind FAD and NADPH, respectively. Domain 4 constitutes the interface.[193,194] The subunit is encoded by the GSR gene (*GSR*), located on chromosome 8p21.1.[195] *GSR* spans 50 kb and contains 13 exons.[192] GSR deficiency (MIM 138300) is an autosomal recessive disease. Because of the extremely large GSR pool in human erythrocytes,[196] even the almost complete absence of GSR is associated with relatively mild clinical expression, without chronic hemolysis.[197]

Glutathione peroxidase (GSH-Px) is a homotetrameric enzym, consisting of 21-kDa subunits. The enzyme converts hydrogen peroxide to water, thus reducing the peroxidative stress for cellular proteins. Several GSH-Px isozymes are known but only GSH-Px1 is active in the erythrocytes. Red blood cells also display high catalase activity. Catalase also converts hydrogen

peroxide to water, rendering GSH-Px activity redundant. Therefore, deficiencies of either catalase or GSH-Px are without significant consequences for the red blood cell. GSH-Px activity shows ethnic variation[198] and although a partial GSH-Px deficiency has been documented in association with hemolytic anemia,[199] a clear causal relationship has not been established.

Outline of the thesis

The phenotype of hereditary red blood cell enzymopathies is highly variable. One of the important frontiers in the interaction between laboratory medicine and clinical practice is to establish the correlation between a patient's genotype and his phenotype. The phenotype, however, does not depend solely on the molecular properties of mutant proteins but, rather, reflects a complex interplay between physiological, environmental, and other (genetic) factors. Moreover, the ultimate outcome of (mutant) gene expression is subjected to several control mechanisms during transcription, pre-mRNA processing, and translation (Figure 6). Therefore, the obligatory first step towards a comprehensive understanding of the disease phenotype in red blood cell enzymopathies comprises the elucidation of its molecular mechanisms of disease. It is evident that this requires not only the identification of the causative mutation on the DNA level but, instead, the combination of genetic information with clinical, biochemical and structural data. In this way, the identified mutation on the DNA level and the mechanisms *via* which these mutations exert their effects render a causal relationship that, ultimately, may answer the question: "Why is the patient ill?"

In **chapter 2** we describe how a single-base change in the erythroid-specific promoter of *PKLR* silenced expression of this otherwise normal allele completely and, ultimately, led to the identification of a novel transcriptional regulatory element in *PKLR* gene.

In **chapter 3**, a novel approach, using RNA from *ex vivo* produced nucleated erythroid cells from the patient, was employed to study the effects of two splice site mutations, encountered in one compound heterozygous patient with severe PK deficiency. The spectrum of identified aberrant transcripts was correlated to the PK-deficient phenotype.

Chapter 4 describes the molecular diagnosis of PK deficiency in a large group of patients. To better understand the mechanisms by which the novel amino acid substitutions could impair enzymatic function and lead to PK deficiency, we evaluated their position and the nature of substitution in the three-dimensional model of the recently elucidated crystal structure of human erythrocyte PK.

Due to an irony of fate, a Danish mother gave birth to two patients with severe PK deficiency, each from a different father. One of the children was homozygous for a novel missense

mutation and this proved to be fatal. In **chapter 5** we describe the effect of these mutations by molecular modeling of the two mutated residues.

Chapter 6 describes two patients with severe glucose-6-phosphate dehydrogenase deficiency. Two *de novo* mutations were identified, one of which was novel. The predicted amino acid substitutions both affected the same crucial region in the enzyme and although perturbation of this region is usually associated with severe clinical features, both patients displayed distinct clinical symptoms.

In **chapter 7** we have established the molecular basis of hexokinase deficiency in a patient who was diagnosed more than 20 years ago. Previously determined biochemical data could now be attributed to the obtained genetic and structural data of this rare glycolytic enzyme defect.

Finally, in **chapter 8** the combined findings of these investigations are discussed and placed into a broader perpsective. Possible aims for further research are discussed.

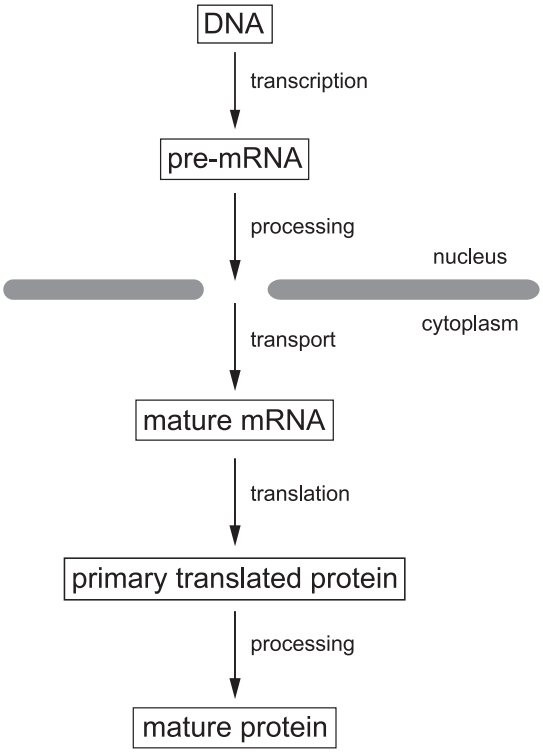

Figure 6. Schematic representation of human gene expression.

References

1. Surgenor DM, ed. The Red Blood Cell. New York: Academic Press; 1974.
2. Benesch R, Benesch RE. The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. Biochem Biophys Res Commun. 1967;26:162-167.
3. Miwa S. Hereditary disorders of red cell enzymes in the Embden-Meyerhof pathway. Am J Hematol. 1983;14:381-391.
4. Dacie J. Hereditary enzyme-deficiency haemolytic anemias I: Introduction and pyruvate-kinase deficiency. In: Dacie J, ed. The Haemolytic Anaemias. London: Churchill Livingstone; 1985:282-320.
5. Dacie J. Hereditary enzyme-deficiency haemolytic anemias II: Deficiencies of enzymes of the Embden-Meyerhoff (EM) pathway other than pyruvate kinase and of enzymes involved in purine and pyrimidine metabolism. In: Dacie J, ed. The Haemolytic Anaemias. London: Churchill Livingstone; 1985:321-363.
6. Miwa S, Fujii H. Molecular aspects of erythroenzymopathies associated with hereditary hemolytic anemia. Am J Hematol. 1985;19:293-305.
7. Valentine WN, Tanaka KR, Paglia DE. Hemolytic anemias and erythrocyte enzymopathies. Ann Intern Med. 1985;103:245-257.
8. Mentzer WC, Glader BE. Disorders of erythrocyte metabolism. In: Mentzer WC, Wagner GM, eds. The Hereditary Hemolytic Anemias. New York: Churchill Livingstone; 1989:267-318.
9. Beutler E. The molecular biology of G6PD variants and other red cell enzyme defects. Annu Rev Med. 1992;43:47-59.
10. Arya R, Layton DM, Bellingham AJ. Hereditary red cell enzymopathies. Blood Rev. 1995;9:165-175.
11. Miwa S, Fujii H. Molecular basis of erythroenzymopathies associated with hereditary hemolytic anemia: tabulation of mutant enzymes. Am J Hematol. 1996;51:122-132.
12. McMullin MF. The molecular basis of disorders of red cell enzymes. J Clin Pathol. 1999;52:241-244.
13. Hirono A, Kanno H, Miwa M, Beutler E. Pyruvate kinase deficiency and other enzymopathies of the erythrocyte. In: Scriver C, Beaudet AL, Valle D, Sly WS, eds. The Metabolic and Molecular Bases of Inherited Disease.; 2001:4637-4664
14. Eber SW. Disorders of erythrocyte glycolysis and nucleotide metabolism. In:

Handin RI, Lux SE, IV, P. ST, eds. Blood. Principles and Practice of Hematology. Philadelphia: Lippincott Williams & Wilkins; 2003.

15. Valentine WN, Paglia DE. The primary cause of hemolysis in enzymopathies of anaerobic glycolysis: a viewpoint. Blood Cells. 1980;6:819-829.
16. Beutler E. The relationship of red cell enzymes to red cell life-span. Blood Cells. 1988;14:69-91.
17. Mason PJ. New insights into G6PD deficiency. Br J Haematol. 1996;94:585-591.
18. Boivin P, Galand C, Hakim J, Kahn A. Acquired erythroenzymopathies in blood disorders: study of 200 cases. Br J Haematol. 1975;31:531-543.
19. Gherardi M, Bierme R, Corberand J, Vergnes H. Heterogeneity of erythrocyte pyruvate kinase deficiency and related metabolic disorders in patients with hematological diseases. Clin Chim Acta. 1977;78:465-471.
20. Kahn A. Abnormalities of erythrocyte enzymes in dyserythropoiesis and malignancies. Clin Haematol. 1981;10:123-138.
21. Kornberg A, Goldfarb A. Preleukemia manifested by hemolytic anemia with pyruvate-kinase deficiency. Arch Intern Med. 1986;146:785-786.
22. Griffin LD, Gelb BD, Wheeler DA, Davison D, Adams V, McCabe ER. Mammalian hexokinase 1: evolutionary conservation and structure to function analysis. Genomics. 1991;11:1014-1024.
23. Wilson JE. Hexokinases. Rev Physiol Biochem Pharmacol. 1995;126:65-198.
24. Cárdenas ML, Cornish-Bowden A, Ureta T. Evolution and regulatory role of the hexokinases. Biochim Biophys Acta. 1998;1401:242-264.
25. Aleshin AE, Zeng C, Bourenkov GP, Bartunik HD, Fromm HJ, Honzatko RB. The mechanism of regulation of hexokinase: new insights from the crystal structure of recombinant human brain hexokinase complexed with glucose and glucose-6-phosphate. Structure. 1998;6:39-50.
26. Mulichak AM, Wilson JE, Padmanabhan K, Garavito RM. The structure of mammalian hexokinase-1. Nat Struct Biol. 1998;5:555-560.
27. Sebastian S, Wilson JE, Mulichak A, Garavito RM. Allosteric regulation of type I hexokinase: A site-directed mutational study indicating location of the functional glucose 6-phosphate binding site in the N-terminal half of the enzyme. Arch Biochem Biophys. 1999;362:203-210.
28. Aleshin AE, Kirby C, Liu X, et al. Crystal structures of mutant monomeric hexokinase I reveal multiple ADP binding sites and conformational changes relevant to allosteric regulation. J Mol Biol. 2000;296:1001-1015.
29. Magnani M, Bianchi M, Casabianca A, et al. A recombinant human 'mini'-

hexokinase is catalytically active and regulated by hexose 6-phosphates. Biochem J. 1992;285:193-199.

30. Arora KK, Filburn CR, Pedersen PL. Structure/function relationships in hexokinase. Site-directed mutational analyses and characterization of overexpressed fragments implicate different functions for the N- and C-terminal halves of the enzyme. J Biol Chem. 1993;268:18259-18266.
31. Tsai HJ, Wilson JE. Functional organization of mammalian hexokinases: characterization of chimeric hexokinases constructed from the N- and C-terminal domains of the rat type I and type II isozymes. Arch Biochem Biophys. 1995;316:206-214.
32. Zeng C, Fromm HJ. Active site residues of human brain hexokinase as studied by site-specific mutagenesis. J Biol Chem. 1995;270:10509-10513.
33. Fang T-Y, Alechina O, Aleshin AE, Fromm HJ, Honzatko RB. Identification of a phosphate regulatory site and a low affinity binding site for glucose 6-phosphate in the N-terminal half of human brain hexokinase. J Biol Chem. 1998;273:19548-19553.
34. Hashimoto M, Wilson JE. Kinetic and regulatory properties of HK I^+, a modified form of the type I isozyme of mammalian hexokinase in which interactions between the N- and C-Terminal halves have been disrupted. Arch Biochem Biophys. 2002;399:109-115.
35. Murakami K, Blei F, Tilton W, Seaman C, Piomelli S. An isozyme of hexokinase specific for the human red blood cell (HK_R). Blood. 1990;75:770-775.
36. Shows TB, Eddy RL, Byers MG, et al. Localization of the human hexokinase I gene (HK1) to chromosome 10q22. Cytogenet Cell genet. 1989;51:1079.
37. Andreoni F, Ruzzo A, Magnani M. Structure of the 5' region of the human hexokinase type I (HKI) gene and identification of an additional testis-specific HKI mRNA. Biochim Biophys Acta. 2000;1493:19-26.
38. Murakami K, Piomelli S. Identification of the cDNA for human red blood cell-specific hexokinase isozyme. Blood. 1997;89:762-766.
39. Ruzzo A, Andreoni F, Magnani M. Structure of the human hexokinase type I gene and nucleotide sequence of the 5' flanking region. Biochem J. 1998;331:607-613.
40. Murakami K, Kanno H, Miwa S, Piomelli S. Human HK_R isozyme: organization of the hexokinase I gene, the erythroid-specific promoter, and transcription initiation site. Mol Genet Metab. 1999;67:118-130.
41. Kanno H. Hexokinase: gene structure and mutations. Baillieres Best Pract Res Clin Haematol. 2000;13:83-88.

42. Bianchi M, Magnani M. Hexokinase mutations that produce nonspherocytic hemolytic anemia. Blood Cells Mol Dis. 1995;21:2-8.
43. Bianchi M, Crinelli R, Serafini G, Giammarini C, Magnani M. Molecular bases of hexokinase deficiency. Biochim Biophys Acta. 1997;1360:211-221.
44. Kanno H, Murakami K, Hariyama Y, Ishikawa K, Miwa S, Fujii H. Homozygous intragenic deletion of type I hexokinase gene causes lethal hemolytic anemia of the affected fetus. Blood. 2002;100:1930.
45. van Wijk R, Rijksen G, Huizinga EG, Nieuwenhuis HK, van Solinge WW. HK Utrecht: missense mutation in the active site of human hexokinase associated with hexokinase deficiency and severe nonspherocytic hemolytic anemia. Blood. 2003;101:345-347.
46. Jeffery CJ. Moonlighting proteins. Trends Biochem Sci. 1999;24:8-11.
47. West JD, Flockhart JH, Peters J, Ball ST. Death of mouse embryos that lack a functional gene for glucose phosphate isomerase. Genet Res. 1990;56:223-236.
48. West JD. A genetically defined animal model of anembryonic pregnancy. Hum Reprod. 1993;8:1316-1323.
49. Cordeiro AT, Godoi PH, Delboni LF, Oliva G, Thiemann OH. Human phosphoglucose isomerase: expression, purification, crystallization and preliminary crystallographic analysis. Acta Crystallogr D Biol Crystallogr. 2001;57:592-595.
50. Read J, Pearce J, Li X, Muirhead H, Chirgwin J, Davies C. The crystal structure of human phosphoglucose isomerase at 1.6 Å resolution: implications for catalytic mechanism, cytokine activity and haemolytic anaemia. J Mol Biol. 2001;309:447-463.
51. McMorris FA, Chen TR, Ricciuti F, Tischfield J, Creagan R, Ruddle F. Chromosome assignments in man of the genes for two hexosephosphate isomerases. Science. 1973;179:1129-1131.
52. Xu W, Lee P, Beutler E. Human glucose phosphate isomerase: exon mapping and gene structure. Genomics. 1995;29:732-739.
53. Kugler W, Lakomek M. Glucose-6-phosphate isomerase deficiency. Baillieres Best Pract Res Clin Haematol. 2000;13:89-101.
54. Paglia DE, Paredes R, Valentine WN, Dorantes S, Konrad PN. Unique phenotypic expression of glucosephosphate isomerase deficiency. Am J Hum Genet. 1975;27:62-70.
55. Van Biervliet JP. Glucosephosphate isomerase deficiency in a Dutch family. Acta Paediatr Scand. 1975;64:868-872.
56. Hutton JJ, Chilcote RR. Glucose phosphate isomerase deficiency with hereditary nonspherocytic hemolytic anemia. J Pediatr. 1974;85:494-497.

57. Whitelaw AG, Rogers PA, Hopkinson DA, et al. Congenital haemolytic anaemia resulting from glucose phosphate isomerase deficiency: genetics, clinical picture, and prenatal diagnosis. J Med Genet. 1979;16:189-196.
58. Matthay KK, Mentzer WC. Erythrocyte enzymopathies in the newborn. Clin Haematol. 1981;10:31-55.
59. Ravindranath Y, Paglia DE, Warrier I, Valentine W, Nakatani M, Brockway RA. Glucose phosphate isomerase deficiency as a cause of hydrops fetalis. N Engl J Med. 1987;316:258-261.
60. Schröter W, Eber SW, Bardosi A, Gahr M, Gabriel M, Sitzmann FC. Generalised glucosephosphate isomerase (GPI) deficiency causing haemolytic anaemia, neuromuscular symptoms and impairment of granulocytic function: a new syndrome due to a new stable GPI variant with diminished specific activity (GPI Homburg). Eur J Pediatr. 1985;144:301-305.
61. Layzer RB, Rowland LP, Bank WJ. Physical and kinetic properties of human phosphofructokinase from skeletal muscle and erythrocytes. J Biol Chem. 1969;244:3823-3831.
62. Vora S. Isozymes of human phosphofructokinase: biochemical and genetic aspects. Isozymes Curr Top Biol Med Res. 1983;11:3-23.
63. Vora S, Durham S, de Martinville B, George DL, Francke U. Assignment of the human gene for muscle-type phosphofructokinase (PFKM) to chromosome 1 (region cen leads to q32) using somatic cell hybrids and monoclonal anti-M antibody. Somatic Cell Genet. 1982;8:95-104.
64. Nakajima H, Noguchi T, Yamasaki T, Kono N, Tanaka T, Tarui S. Cloning of human muscle phosphofructokinase cDNA. FEBS Lett. 1987;223:113-116.
65. Levanon D, Danciger E, Dafni N, et al. The primary structure of human liver type phosphofructokinase and its comparison with other types of PFK. DNA. 1989;8:733-743.
66. Eto K, Sakura H, Yasuda K, et al. Cloning of a complete protein-coding sequence of human platelet-type phosphofructokinase isozyme from pancreatic islet. Biochem Biophys Res Commun. 1994;198:990-998.
67. Howard TD, Akots G, Bowden DW. Physical and genetic mapping of the muscle phosphofructokinase gene (PFKM): reassignment to human chromosome 12q. Genomics. 1996;34:122-127.
68. Yamasaki T, Nakajima H, Kono N, et al. Structure of the entire human muscle phosphofructokinase-encoding gene: a two-promoter system. Gene. 1991;104:277-282.

69. Elson A, Levanon D, Brandeis M, et al. The structure of the human liver-type phosphofructokinase gene. Genomics. 1990;7:47-56.
70. Van Keuren M, Drabkin H, Hart I, Harker D, Patterson D, Vora S. Regional assignment of human liver-type 6-phosphofructokinase to chromosome 21q22.3 by using somatic cell hybrids and a monoclonal anti-L antibody. Hum Genet. 1986;74:34-40.
71. Fujii H, Miwa S. Other erythrocyte enzyme deficiencies associated with non-haematological symptoms: phosphoglycerate kinase and phosphofructokinase deficiency. Baillieres Best Pract Res Clin Haematol. 2000;13:141-148.
72. Raben N, Sherman J, Miller F, Mena H, Plotz P. A 5' splice junction mutation leading to exon deletion in an Ashkenazic Jewish family with phosphofructokinase deficiency (Tarui disease). J Biol Chem. 1993;268:4963-4967.
73. Sherman JB, Raben N, Nicastri C, et al. Common mutations in the phosphofructokinase-M gene in Ashkenazi Jewish patients with glycogenesis VII—and their population frequency. Am J Hum Genet. 1994;55:305-313.
74. Kukita A, Yoshida MC, Fukushige S, et al. Molecular gene mapping of human aldolase A (ALDOA) gene to chromosome 16. Hum Genet. 1987;76:20-26.
75. Izzo P, Costanzo P, Lupo A, Rippa E, Paolella G, Salvatore F. Human aldolase A gene. Structural organization and tissue-specific expression by multiple promoters and alternate mRNA processing. Eur J Biochem. 1988;174:569-578.
76. Beutler E, Scott S, Bishop A, Margolis N, Matsumoto F, Kuhl W. Red cell aldolase deficiency and hemolytic anemia: a new syndrome. Trans Assoc Am Physicians. 1973;86:154-166.
77. Miwa S, Fujii H, Tani K, et al. Two cases of red cell aldolase deficiency associated with hereditary hemolytic anemia in a Japanese family. Am J Hematol. 1981;11:425-437.
78. Kreuder J, Borkhardt A, Repp R, et al. Brief report: inherited metabolic myopathy and hemolysis due to a mutation in aldolase A. N Engl J Med. 1996;334:1100-1104.
79. Yao DC, Tolan DR, Murray MF, et al. Hemolytic anemia and severe rhabdomyolysis due to compound heterozygous mutations of the gene for erythrocyte/muscle isozyme of aldolase: ALDOA(Arg303X/Cys338Tyr). Blood. Prepublished online November 13, 2003; DOI 10.1182/blood-2003-09-3160.
80. Kishi H, Mukai T, Hirono A, Fujii H, Miwa S, Hori K. Human aldolase A deficiency associated with a hemolytic anemia: thermolabile aldolase due to a single base mutation. Proc Natl Acad Sci U S A. 1987;84:8623-8627.
81. Mande SC, Mainfroid V, Kalk KH, Goraj K, Martial JA, Hol WG. Crystal structure

of recombinant human triosephosphate isomerase at 2.8 Å resolution. Triosephosphate isomerase-related human genetic disorders and comparison with the trypanosomal enzyme. Protein Sci. 1994;3:810-821.

82. Peters J, Hopkinson DA, Harris H. Genetic and non-genetic variation of triose phosphate isomerase isozymes in human tissues. Ann Hum Genet. 1973;36:297-312.
83. Yuan PM, Talent JM, Gracy RW. Molecular basis for the accumulation of acidic isozymes of triosephosphate isomerase on aging. Mech Ageing Dev. 1981;17:151-162.
84. Weatherall DJ, Provan AB. Red cells I: inherited anaemias. Lancet. 2000;355:1169-1175.
85. Rosa R, Prehu MO, Calvin MC, Badoual J, Alix D, Girod R. Hereditary triose phosphate isomerase deficiency: seven new homozygous cases. Hum Genet. 1985;71:235-240.
86. Law ML, Kao FT. Induced segregation of human syntenic genes by 5-bromodeozyuridine + near-visible light. Somatic Cell Genet. 1978;4:465-476.
87. Brown JR, Daar IO, Krug JR, Maquat LE. Characterization of the functional gene and several processed pseudogenes in the human triosephosphate isomerase gene family. Mol Cell Biol. 1985;5:1694-1706.
88. Daar IO, Artymiuk PJ, Phillips DC, Maquat LE. Human triose-phosphate isomerase deficiency: a single amino acid substitution results in a thermolabile enzyme. Proc Natl Acad Sci U S A. 1986;83:7903-7907.
89. Schneider A, Westwood B, Yim C, et al. The 1591C mutation in triosephosphate isomerase (TPI) deficiency. Tightly linked polymorphisms and a common haplotype in all known families. Blood Cells Mol Dis. 1996;22:115-125.
90. McCarrey JR, Thomas K. Human testis-specific PGK gene lacks introns and possesses characteristics of a processed gene. Nature. 1987;326:501-505.
91. Huang IY, Fujii H, Yoshida A. Structure and function of normal and variant human phosphoglycerate kinase. Hemoglobin. 1980;4:601-609.
92. Michelson AM, Markham AF, Orkin SH. Isolation and DNA sequence of a full-length cDNA clone for human X chromosome-encoded phosphoglycerate kinase. Proc Natl Acad Sci U S A. 1983;80:472-476.
93. Banks RD, Blake CC, Evans PR, et al. Sequence, structure and activity of phosphoglycerate kinase: a possible hinge-bending enzyme. Nature. 1979;279:773-777.
94. Bernstein BE, Michels PA, Hol WG. Synergistic effects of substrate-induced

conformational changes in phosphoglycerate kinase activation. Nature. 1997;385:275-278.

95. Willard HF, Goss SJ, Holmes MT, Munroe DL. Regional localization of the phosphoglycerate kinase gene and pseudogene on the human X chromosome and assignment of a related DNA sequence to chromosome 19. Hum Genet. 1985;71:138-143.

96. Michelson AM, Blake CC, Evans ST, Orkin SH. Structure of the human phosphoglycerate kinase gene and the intron-mediated evolution and dispersal of the nucleotide-binding domain. Proc Natl Acad Sci U S A. 1985;82:6965-6969.

97. Tsujino S, Shanske S, DiMauro S. Molecular genetic heterogeneity of phosphoglycerate kinase (PGK) deficiency. Muscle Nerve. 1995;3:S45-49.

98. Ookawara T, Dave V, Willems P, et al. Retarded and aberrant splicings caused by single exon mutation in a phosphoglycerate kinase variant. Arch Biochem Biophys. 1996;327:35-40.

99. Kanno H, Fujii H, Miwa S. Structural analysis of human pyruvate kinase L-gene and identification of the promoter activity in erythroid cells. Biochem Biophys Res Commun. 1992;188:516-523.

100. Lacronique V, Boquet D, Lopez S, Kahn A, Raymondjean M. *In vitro* and *in vivo* protein - DNA interactions on the rat erythroid-specific L' pyruvate kinase gene promoter. Nucleic Acids Res. 1992;20:5669-5676.

101. Max-Audit I, Eleouet JF, Roméo P-H. Transcriptional regulation of the pyruvate kinase erythroid-specific promoter. J Biol Chem. 1993;268:5431-5437.

102. Takegawa S, Fujii H, Miwa S. Change of pyruvate kinase isozymes from M_2- to L-type during development of the red cell. Br J Haematol. 1983;54:467-474.

103. Max-Audit I, Testa U, Kechemir D, Titeux M, Vainchenker W, Rosa R. Pattern of pyruvate kinase isozymes in erythroleukemia cell lines and in normal human erythroblasts. Blood. 1984;64:930-936.

104. Nijhof W, Wierenga PK, Staal GE, Jansen G. Changes in activities and isozyme patterns of glycolytic enzymes during erythroid differentiation in vitro. Blood. 1984;64:607-613.

105. Takegawa S, Shinohara T, Miwa S. Hemin-induced conversion of pyruvate kinase isozymes in K562 cells. Blood. 1984;64:754-757.

106. Max-Audit I, Kechemir D, Mitjavila MT, Vainchenker W, Rotten D, Rosa R. Pyruvate kinase synthesis and degradation by normal and pathologic cells during erythroid maturation. Blood. 1988;72:1039-1044.

107. Nakashima K, Miwa S, Oda S, Tanaka T, Imamura K. Electrophoretic and kinetic

studies of mutant erythrocyte pyruvate kinases. Blood. 1974;43:537-548.

108. Kahn A, Marie J, Garreau H, Sprengers ED. The genetic system of the L-type pyruvate kinase forms in man. Subunit structure, interrelation and kinetic characteristics of the pyruvate kinase enzymes from erythrocytes and liver. Biochim Biophys Acta. 1978;523:59-74.
109. Kahn A, Marie J. Pyruvate kinases from human erythrocytes and liver. Methods Enzymol. 1982;90:131-140.
110. Marie J, Kahn A. Proteolytic processing of human erythrocyte pyruvate kinase: study of normal and deficient enzymes. Biochem Biophys Res Commun. 1979;91:123-129.
111. Wang C, Chiarelli LR, Bianchi P, et al. Human erythrocyte pyruvate kinase: characterization of the recombinant enzyme and a mutant form (R510Q) causing nonspherocytic hemolytic anemia. Blood. 2001;98:3113-3120.
112. Black JA, Henderson MH. Activation and inhibition of human erythrocyte pyruvate kinase by organic phosphates, amino acids, peptides and anions. Biochim Biophys Acta. 1972;284:115-127.
113. Staal GE, Koster JF, Kamp H, van Milligen-Boersma L, Veeger C. Human erythrocyte pyruvate kinase. Its purification and some properties. Biochim Biophys Acta. 1971;227:86-96.
114. Nowak T, Mildvan AS. Nuclear magnetic resonance studies of the function of potassium in the mechanism of pyruvate kinase. Biochemistry. 1972;11:2819-2828.
115. Gupta RK, Oesterling RM. Dual divalent cation requirement for activation of pyruvate kinase; essential roles of both enzyme- and nucleotide-bound metal ions. Biochemistry. 1976;15:2881-2887.
116. Valentini G, Chiarelli LR, Fortin R, et al. Structure and function of human erythrocyte pyruvate kinase. Molecular basis of nonspherocytic hemolytic anemia. J Biol Chem. 2002;17:23807-23814.
117. Enriqueta Muñoz M, Ponce E. Pyruvate kinase: current status of regulatory and functional properties. Comp Biochem Physiol B Biochem Mol Biol. 2003;135:197-218.
118. Mattevi A, Bolognesi M, Valentini G. The allosteric regulation of pyruvate kinase. FEBS Lett. 1996;389:15-19.
119. Valentini G, Chiarelli L, Fortin R, Speranza ML, Galizzi A, Mattevi A. The allosteric regulation of pyruvate kinase. J Biol Chem. 2000;275:18145-18152.
120. Mattevi A, Valentini G, Rizzi M, Speranza ML, Bolognesi M, Coda A. Crystal structure of *Escherichia coli* pyruvate kinase type I: molecular basis of the allosteric transition. Structure. 1995;3:729-741.

121. Jurica MS, Mesecar A, Heath PJ, Shi W, Nowak T, Stoddard BL. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. Structure. 1998;6:195-210.
122. Rigden DJ, Phillips SE, Michels PA, Fothergill-Gilmore LA. The structure of pyruvate kinase from *Leishmania mexicana* reveals details of the allosteric transition and unusual effector specificity. J Mol Biol. 1999;291:615-635.
123. Fenton AW, Blair JB. Kinetic and allosteric consequences of mutations in the subunit and domain interfaces and the allosteric site of yeast pyruvate kinase. Arch Biochem Biophys. 2002;397:28-39.
124. Wooll JO, Friesen RHE, White MA, et al. Structural and functional linkages between subunit interfaces in mammalian pyruvate kinase. J Mol Biol. 2001;312:525-540.
125. Tani K, Yoshida MC, Satoh H, et al. Human M2-type pyruvate kinase: cDNA cloning, chromosomal assignment and expression in hepatoma. Gene. 1988;73:509-516.
126. Noguchi T, Inoue H, Tanaka T. The M_1- and M_2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. J Biol Chem. 1986;261:13807-13812.
127. Takenaka M, Yamada K, Lu T, Kang R, Tanaka T, Noguchi T. Alternative splicing of the pyruvate kinase M gene in a minigene system. Eur J Biochem. 1996;235:366-371.
128. Tani K, Fujii H, Tsutsumi H, et al. Human liver type pyruvate kinase: cDNA cloning and chromosomal assignment. Biochem Biophys Res Commun. 1987;143:431-438.
129. Satoh H, Tani K, Yoshida MC, Sasaki M, Miwa S, Fujii H. The human liver-type pyruvate kinase (PKL) gene is on chromosome 1 at band q21. Cytogenet Cell Genet. 1988;47:132-133.
130. Marie J, Simon MP, Dreyfus JC, Kahn A. One gene, but two messenger RNAs encode liver L and red cell L' pyruvate kinase subunits. Nature. 1981;292:70-72.
131. Noguchi T, Yamada K, Inoue H, Matsuda T, Tanaka T. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. J Biol Chem. 1987;262:14366-14371.
132. Tremp GL, Boquet D, Ripoche MA, et al. Expression of the rat L-type pyruvate kinase gene from its dual erythroid- and liver-specific promoter in transgenic mice. J Biol Chem. 1989;264:19904-19910.
133. Kanno H, Fujii H, Hirono A, Miwa S. cDNA cloning of human R-type pyruvate

kinase and identification of a single amino acid substitution (Thr^{384}→Met) affecting enzymatic stability in a pyruvate kinase variant (PK Tokyo) associated with hereditary hemolytic anemia. Proc Natl Acad Sci U S A. 1991;88:8218-8221.

134. Tani K, Fujii H, Nagata S, Miwa S. Human liver type pyruvate kinase: complete amino acid sequence and the expression in mammalian cells. Proc Natl Acad Sci U S A. 1988;85:1792-1795.
135. Lenzner C, Nürnberg P, Jacobasch G, Thiele B-J. Complete genomic sequence of the human PK-L/R-gene includes four intragenic polymorphisms defining different haplotype backgrounds of normal and mutant PK-genes. DNA Seq. 1997;8:45-53.
136. Beutler E, Gelbart T. Estimating the prevalence of pyruvate kinase deficiency from the gene frequency in the general white population. Blood. 2000;95:3585-3588.
137. Delivoria-Papadopoulos M, Oski FA, Gottlieb AJ. Oxygen-hemoglobulin dissociation curves: effect of inherited enzyme defects of the red cell. Science. 1969;165:601-602.
138. Ferreira P, Morais L, Costa R, et al. Hydrops fetalis associated with erythrocyte pyruvate kinase deficiency. Eur J Pediatr. 2000;159:481-482.
139. Bianchi P, Zanella A. Hematologically important mutations: red cell pyruvate kinase (third update). Blood Cells Mol Dis. 2000;26:47-53.
140. van Wijk R, Huizinga EG, Rijksen G, Marx JJM, van Solinge WW. Fourteen novel mutations in *PKLR* associated with pyruvate kinase deficiency. (*manuscript in preparation*)
141. Baronciani L, Beutler E. Analysis of pyruvate kinase-deficiency mutations that produce nonspherocytic hemolytic anemia. Proc Natl Acad Sci U S A. 1993;90:4324-4327.
142. Lenzner C, Nürnberg P, Thiele BJ, et al. Mutations in the pyruvate kinase L gene in patients with hereditary hemolytic anemia. Blood. 1994;83:2817-2822.
143. Baronciani L, Beutler E. Molecular study of pyruvate kinase deficient patients with hereditary nonspherocytic hemolytic anemia. J Clin Invest. 1995;95:1702-1709.
144. Lenzner C, Nurnberg P, Jacobasch G, Gerth C, Thiele BJ. Molecular analysis of 29 pyruvate kinase-deficient patients from central Europe with hereditary hemolytic anemia. Blood. 1997;89:1793-1799.
145. van Wijk R, van Solinge WW, Nerlov C, et al. Disruption of a novel regulatory element in the erythroid-specific promoter of the human *PKLR* gene causes severe pyruvate kinase deficiency. Blood. 2003;101:1596-1602.
146. Harkness DR. A new erythrocytic enzyme defect with hemolytic anemia: glyceraldehyde-3-phosphate dehydrogenase deficiency. J Lab Clin Med. 1966;68:879-880.

147. Oski FA. Hemolytic anemia and red cell glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) deficiency. Clin Res. 1969;17
148. Lachant NA, Jennings MA, Tanaka KR. Partial erythrocyte enolase deficiency: a hereditary disorder with variable clinical expression. Blood. 1986;68:55a.
149. Miwa S, Nishina T, Kakehashi Y, Kitamura M, Hiratsuka A. Studies on erythrocyte metabolism in a case with hereditary deficiency of H-subunit of lactate dehydrogenase. Acta Haematol Jpn. 1971;34:228-232.
150. Kremer JP, Datta T, Pretsch W, Charles DJ, Dormer P. Mechanisms of compensation of hemolytic anemia in a lactate dehydrogenase mouse mutant. Exp Hematol. 1987;15:664-670.
151. Rosa R, Gaillardon J, Rosa J. Diphosphoglycerate mutase and 2,3-diphosphoglycerate phosphatase activities of red cells: comparative electrophoretic study. Biochem Biophys Res Commun. 1973;51:536-542.
152. Ikura K, Sasaki R, Narita H, Sugimoto E, Chiba H. Multifunctional enzyme, bisphosphoglyceromutase/2,3-bisphosphoglycerate phosphatase/phosphoglycero-mutase, from human erythrocytes. Evidence for a common active site. Eur J Biochem. 1976;66:515-522.
153. Rapoport S, Luebering J. The formation of 2,3-diphosphoglycerate in rabbit erythrocytes: the existence of a diphosphoglycerate mutase. J Biol Chem. 1950;183:507-516.
154. Bellingham AJ, Huehns ER. Compensatory mechanisms in haemolytic anaemias. Proc R Soc Med. 1968;61:1315-1316.
155. Benesch RE, Benesch R, Yu CI. The oxygenation of hemoglobin in the presence of 2,3-diphosphoglycerate. Effect of temperature, pH, ionic strength, and hemoglobin concentration. Biochemistry. 1969;8:2567-2571.
156. Torrance J, Jacobs P, Restrepo A, Eschbach J, Lenfant C, Finch CA. Intraerythrocytic adaptation to anemia. N Engl J Med. 1970;283:165-169.
157. Barichard F, Joulin V, Henry I, et al. Chromosomal assignment of the human 2,3-bisphosphoglycerate mutase gene (BPGM) to region 7q34-7q22. Hum Genet. 1987;77:283-285.
158. Joulin V, Peduzzi J, Romeo PH, et al. Molecular cloning and sequencing of the human erythrocyte 2,3-bisphosphoglycerate mutase cDNA: revised amino acid sequence. EMBO J. 1986;5:2275-2283.
159. Rosa R, Prehu MO, Beuzard Y, Rosa J. The first case of a complete deficiency of diphosphoglycerate mutase in human erythrocytes. J Clin Invest. 1978;62:907-915.
160. Lemarchandel V, Joulin V, Valentin C, et al. Compound heterozygosity in a

complete erythrocyte bisphosphoglycerate mutase deficiency. Blood. 1992;80:2643-2649.

161. Parr CW, Fitch LI. Inherited quantitative variations of human phosphogluconate dehydrogenase. Ann Hum Genet. 1967;30:339-353.
162. Longo L, Vanegas OC, Patel M, et al. Maternally transmitted severe glucose 6-phosphate dehydrogenase deficiency is an embryonic lethal. EMBO J. 2002;21:4229-4239.
163. Cohen P, Rosemeyer MA. Subunit interactions of glucose-6-phosphate dehydrogenase from human erythrocytes. Eur J Biochem. 1969;8:8-15.
164. Au SWN, Gover S, Lam VMS, Adams MJ. Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural $NADP^+$ molecule and provides insights into enzyme deficiency. Structure Fold Des. 2000;8:293-303.
165. Szabo P, Purrello M, Rocchi M, et al. Cytological mapping of the human glucose-6-phosphate dehydrogenase gene distal to the fragile-X site suggests a high rate of meiotic recombination across this site. Proc Natl Acad Sci U S A. 1984;81:7855-7859.
166. Martini G, Toniolo D, Vulliamy T, et al. Structural analysis of the X-linked gene encoding human glucose 6-phosphate dehydrogenase. EMBO J. 1986;5:1849-1855.
167. Vulliamy TJ, Luzzatto L. Glucose-6-phosphate dehydrogenase deficiency and related disorders. In: Handin RI, Lux SE, IV, P. ST, eds. Blood. Principles and Practice of Hematology. Philadelphia: Lippincott Williams & Wilkins; 2003:1921-1950
168. Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group. Bull World Health Organ. 1989;67:601-611.
169. Rowland P, Basak AK, Gover S, Levy HR, Adams MJ. The three-dimensional structure of glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* refined at 2.0 Å resolution. Structure. 1994;2:1073-1087.
170. Naylor CE, Rowland P, Basak AK, et al. Glucose 6-phosphate dehydrogenase mutations causing enzyme deficiency in a model of the tertiary structure of the human enzyme. Blood. 1996;87:2974-2982.
171. Jablonska-Skwiecinska E, Lewandowska I, Plochocka D, et al. Several mutations including two novel mutations of the glucose-6-phosphate dehydrogenase gene in Polish G6PD deficient subjects with chronic nonspherocytic hemolytic anemia, acute hemolytic anemia, and favism. Hum Mutat. 1999;14:477-484.
172. van Wijk R, Huizinga EG, Prins I, et al. Distinct phenotypic expression of two de novo missense mutations affecting the dimer interface of glucose-6-phosphate dehydrogenase. Blood Cells Mol Dis. 2004;32:112-117.

173. Bosma PJ, Chowdhury JR, Bakker C, et al. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. N. Engl. J. Med. 1995;333:1171-1175.
174. Monaghan G, Ryan M, Seddon R, Hume R, Burchell B. Genetic variation in bilirubin UPD-glucuronosyltransferase gene promoter and Gilbert's syndrome. Lancet. 1996;347:578-581.
175. Kaplan M, Renbaum P, Levy-Lahad E, Hammerman C, Lahad A, Beutler E. Gilbert syndrome and glucose-6-phosphate dehydrogenase deficiency: a dose-dependent genetic interaction crucial to neonatal hyperbilirubinemia. Proc Natl Acad Sci U S A. 1997;94:12128-12132.
176. Kaplan M, Hammerman C, Beutler E. Heterozygosity for a polymorphism in the promoter region of the UGT1A1 gene. J Hepatol. 2001;35:148-150.
177. Beutler E. G6PD deficiency. Blood. 1994;84:3613-3636.
178. Dacie J. Hereditary enzyme-deficiency haemolytic anemias III: Deficiency of glucose-6-phosphate dehydrogenase. In: Dacie J, ed. The Haemolytic Anaemias. London: Churchill Livingstone; 1985:364-418.
179. Luzzatto L, Mehta A. Glucose 6-phosphate dehydrogenase deficiency. In: Scriver C, Beaudet AL, Sly WS, Valle D, eds. The Metabolic and Molecular Basis of Inherited Disease. New York: McGraw Hill; 1995:3367-3398.
180. Gipp JJ, Chang C, Mulcahy RT. Cloning and nucleotide sequence of a full-length cDNA for human liver γ-glutamylcysteine synthetase. Biochem Biophys Res Commun. 1992;185:29-35.
181. Gipp JJ, Bailey HH, Mulcahy RT. Cloning and sequencing of the cDNA for the light subunit of human liver γ-glutamylcysteine synthetase and relative mRNA levels for heavy and light subunits in human normal tissues. Biochem Biophys Res Commun. 1995;206:584-589.
182. Sierra-Rivera E, Summar ML, Dasouki M, Krishnamani MR, Phillips JA, Freeman ML. Assignment of the gene (*GLCLC*) that encodes the heavy subunit of gamma-glutamylcysteine synthetase to human chromosome 6. Cytogenet Cell Genet. 1995;70:278-279.
183. Sierra-Rivera E, Dasouki M, Summar ML, et al. Assignment of the human gene (*GLCLR*) that encodes the regulatory subunit of gamma-glutamylcysteine synthetase to chromosome 1p21. Cytogenet Cell Genet. 1996;72:252-254.
184. Beutler E, Gelbart T, Kondo T, Matsunaga AT. The molecular basis of a case of γ-glutamylcysteine synthetase deficiency. Blood. 1999;94:2890-2894.
185. Ristoff E, Augustson C, Geissler J, et al. A missense mutation in the heavy subunit

of γ-glutamylcysteine synthetase gene causes hemolytic anemia. Blood. 2000;95:2193-2196.

186. Hamilton D, Wu JH, Alaoui-Jamali M, Batist G. A novel missense mutation in the γ-glutamylcysteine synthetase catalytic subunit gene causes both decreased enzymatic activity and glutathione production. Blood. 2003;102:725-730.
187. Gali RR, Board PG. Sequencing and expression of a cDNA for human glutathione synthetase. Biochem J. 1995;310:353-358.
188. Shi ZZ, Habib GM, Rhead WJ, et al. Mutations in the glutathione synthetase gene cause 5-oxoprolinuria. Nat Genet. 1996;14:361-365.
189. Dahl N, Pigg M, Ristoff E, et al. Missense mutations in the human glutathione synthetase gene result in severe metabolic acidosis, 5-oxoprolinuria, hemolytic anemia and neurological dysfunction. Hum Mol Genet. 1997;6:1147-1152.
190. Spielberg SP, Garrick MD, Corash LM, et al. Biochemical heterogeneity in glutathione synthetase deficiency. J Clin Invest. 1978;61:1417-1420.
191. Corrons JL, Alvarez R, Pujades A, et al. Hereditary non-spherocytic haemolytic anaemia due to red blood cell glutathione synthetase deficiency in four unrelated patients from Spain: clinical and molecular studies. Br J Haematol. 2001;112:475-482.
192. Kelner MJ, Montoya MA. Structural organization of the human glutathione reductase gene: determination of correct cDNA sequence and identification of a mitochondrial leader sequence. Biochem Biophys Res Commun. 2000;269:366-368.
193. Karplus PA, Schulz GE. Refined structure of glutathione reductase at 1.54 Å resolution. J Mol Biol. 1987;195:701-729.
194. Thieme R, Pai EF, Schirmer RH, Schulz GE. Three-dimensional structure of glutathione reductase at 2 Å resolution. J Mol Biol. 1981;152:763-782.
195. Sinet PM, Bresson JL, Couturier J, et al. [Possible localization of the glutathione reductase (EC 1.6.4.2) on the 8p21 band]. Ann Genet. 1977;20:13-17.
196. Frischer H, Ahmad T. Consequences of erythrocytic glutathione reductase deficiency. J Lab Clin Med. 1987;109:583-588.
197. Loos H, Roos D, Weening R, Houwerzijl J. Familial deficiency of glutathione reductase in human blood cells. Blood. 1976;48:53-62.
198. Beutler E, Matsumoto F. Ethnic variation in red cell glutathione peroxidase activity. Blood. 1975;46:103-110.
199. Gondo H, Ideguchi H, Hayashi S, Shibuya T. Acute hemolysis in glutathione peroxidase deficiency. Int J Hematol. 1992;55:215-218.

CHAPTER 2

Disruption of a novel regulatory element in the erythroid-specific promoter of the human *PKLR* gene causes severe pyruvate kinase deficiency

Richard van Wijk[1], Wouter W. van Solinge[1], Claus Nerlov[2], Ernest Beutler[3], Terri Gelbart[3], Gert Rijksen[4], and Finn C. Nielsen[5]

[1]Department of Clinical Chemistry, and [4]Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands; [2]EMBL Mouse Biology Programme, Monterotondo - Scalo (RM), Italy; [3]Department of Molecular & Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA; [5]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark.

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Abstract

We established the molecular basis for pyruvate kinase (PK) deficiency in a white male patient with severe nonspherocytic hemolytic anemia. The paternal allele exhibited the common *PKLR* cDNA sequence (c.) 1529G>A mutation, known to be associated with PK-deficiency. On the maternal allele, three *in cis* mutations were identified in the erythroid-specific promoter region of the gene: one deletion of thymine –248 and two single nucleotide substitutions, nucleotide (nt) –324T>A and nt –83G>C. Analysis of the patient's RNA demonstrated the presence of only the 1529A allele, indicating severely reduced transcription from the allele linked to the mutated promoter region. Transfection of promoter constructs into erythroleukemic K562 cells showed that the most upstream –324T>A and –248delT mutations were non-functional polymorphisms. In contrast, the –83G>C mutation strongly reduced promoter activity. Site directed mutagenesis of the promoter region revealed the presence of a putative regulatory element (PKR-RE1) whose core binding motif, CTCTG, is located between nt –87 and –83. Electrophoretic mobility shift assay using K562 nuclear extracts indicated binding of an, as-yet-unidentified *trans*-acting factor. This novel element mediates the effects of factors necessary for regulation of pyruvate kinase gene expression during red cell differentiation and maturation.

Introduction

Pyruvate kinase (PK) catalyzes the final step of glycolysis in which phosphoenolpyruvate is converted to pyruvate with the concomitant generation of adenosine triphosphate (ATP). Pyruvate kinase deficiency is the most common cause of nonspherocytic hemolytic anemia due to defective glycolysis. The consequent lack of sufficient energy, which is required for normal functioning and cellular survival, shortens the life span of the mature PK-deficient erythrocyte. Consequently, PK deficient patients display a phenotype of non-spherocytic hemolytic anemia albeit with variable clinical severity.[1] PK deficiency is transmitted as an autosomal recessive disease and to date, more than 130 mutations in *PKLR* have been reported to be associated with pyruvate kinase deficiency.[2] Most (70%) of these mutations are missense mutations affecting conserved residues in structurally and functionally important domains of PK.

The human gene for liver and red blood cell-specific PK (*PKLR*) is located on chromosome 1q21[3] where it directs tissue-specific transcription for both the liver-specific isozymes PK-L and the red blood cell-specific isozyme PK-R[4-6] by the use of alternate promoters.[7-9]

Functional analysis of the rat PK erythroid-specific promoter has indicated that nucleotides (nts) from –870 to +54, relative to the cap site, confer erythroid specificity to a reporter gene.[10] Within this region, a minimal promoter (nts –62 to +54), including a putative –50 CCACC/Sp1 element and a –20 GATA element, displayed erythroid-specific activity.[10] Studies on the human promoter have, moreover, indicated that a region from –120 to –270, relative to the translational initiation codon, functions as a powerful enhancer.[9] DNA sequence comparison between the rat and human erythroid-specific promoter of *PKLR* reveals four well-conserved elements, indicated in Figure 1. Furthermore, two CAC boxes

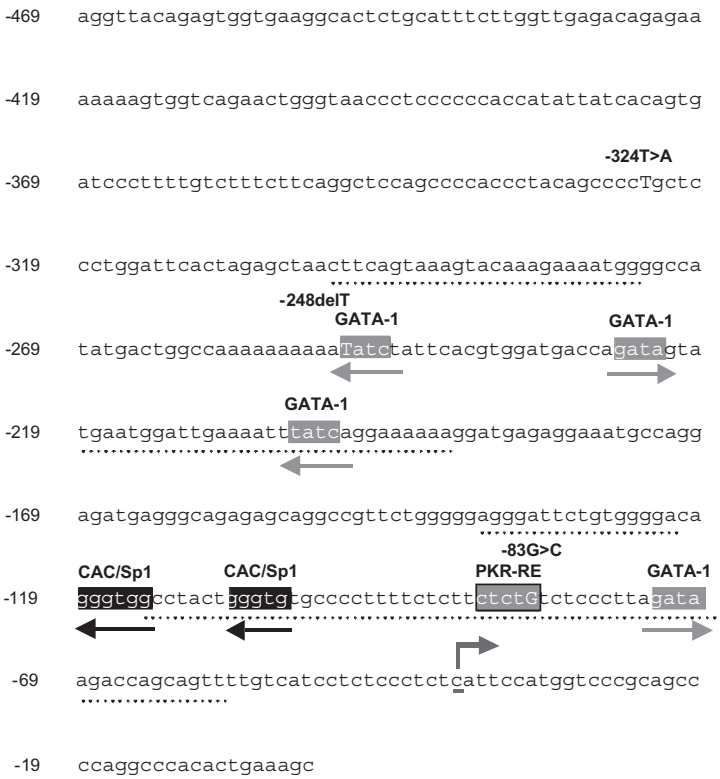

Figure 1. Partial DNA sequence of the erythroid-specific promoter of *PKLR*. A 469-bp region comprising the upstream regulatory domain and exon 1 down to the ATG codon as used in this study. Conserved elements (from Kanno *et al*.[9]) between the human and rat PK-R promoter are depicted by dotted lines. The cytosine identified as the PK-R transcriptional start site[5] is underlined. GATA-1, CAC/Sp1 motifs, and the novel regulatory element PKR-RE1, as reported in this study, in the upstream 270-bp region are shown in boxes (orientation indicated by arrows). The three *in cis* mutations, as identified in our patient, are indicated above their corresponding nucleotides (in capital letters) in the promoter sequence.

and four GATA motifs are present within the first 250 bp upstream region.[9] So far only one mutation in the PK-R promoter has proven to be associated with pyruvate kinase deficiency – a single-base substitution at nt –72 (–72A>G). The downregulation of expression by this mutated promoter has been attributed to disruption of the consensus binding motif for GATA-1 at nts –69 to –74.[11]

Previously, we reported two *in cis* mutations in a severely PK deficient Danish patient.[12] We now report on the functional analysis of these mutations and show that the most proximal one, –83G>C, constitutes part of a core binding motif of a novel regulatory element (PKR-RE1) in the erythroid-specific promoter of *PKLR*, in close proximity to a regulatory GATA-1 binding site.

Patient, materials, and methods

The patient is a six-year-old Danish boy suffering from severe, transfusion-dependent hemolytic anemia since birth. PK deficiency was diagnosed at the age of 1 year. Because of the continuous presence of transfused donor erythrocytes, we used a density gradient to separate reticulocytes from mature erythrocytes, in order to obtain an as-representative-as-possible patient-specific red cell population.[13] PK activity and activity measurements of the red blood cell age-related enzymes glucose-6-phosphate dehydrogenase (G6PD) and hexokinase (HK), were determined according to standardized procedures.[14]

DNA sequence analysis of PKLR

The coding region of *PKLR* was amplified using primers as previously described.[15] Additional primers were used for part of the putative erythroid-specific promoter (GenBank accession number AB015984 D13232) and 3'-untranslated region (3'-UTR; GenBank accession number D13243). Sense primer PKRP-ESF 5'-AGGTTACAGAGTGGTGAAGGC-3' (nts –469 to –449, relative to the start codon) and antisense primer PKRP-ESR: 5'-GCTTTCAGTGTGGGCCTGG-3' (nts –20 to –1) amplify a 469 bp region immediately upstream of the initiator methionine. Sense primer PKRU-F: 5'-TCTACGTTCTCCAGCCCACAC-3' (nts + 58 to +78, relative to the termination codon) and antisense primer PKRU-R: 5'-GAGTGGGAAGGAATTTCTGGG-3' (nts +689 to +669) amplify a 669 bp region of the 3'-UTR. Polymerase chain reactions (PCRs) were carried out with 200 ng DNA in 50-µL volumes containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.2 mM of each deoxynucleotide triphosphate (dNTP),

0.3 µM of each primer, and 2.5 U AmpliTaq Gold DNA polymerase. All reagents were obtained from Applied Biosystems (Roche Molecular Systems, Branchburg, NJ). After initial incubation for 10 minutes at 95°C, samples were subjected to 35 cycles of amplification with denaturation at 94°C for 30 seconds, annealing for 30 seconds at 64°C (60°C for exon 9 and 3'-UTR), and extension at 72°C for 45 seconds, followed by an elongated extension time of 10 minutes after the last cycle. Automated DNA sequence analysis was performed with the ABI Prism dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Sequencing reactions were all carried out in forward and reverse direction, and samples were analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems). The PCR products were purified prior to DNA sequence analysis using the QIAquick PCR purification kit (Qiagen, Valencia, CA).

Restriction enzyme analysis

Mutations were confirmed by restriction enzyme analysis on newly amplified PCR product. The cDNA sequence (c.) 1529G>A mutation in exon 11 was confirmed by *Sty*I digestion as described.[15] The two promoter mutations, –324T>A and –83G>C, were confirmed by digestion of the PCR product with *Bst*XI and *Bsm*AI, respectively. The 469-bp PCR product from the wild-type allele, as amplified with PKRP-ESF and PKRP-ESR, contains one recognition site for *Bst*XI that yields fragments of 206 bp and 263 bp after digestion. The –324T>A mutation creates a second recognition sequence for this enzyme, resulting in additional fragments of 152 bp and 54 bp. The –83G>C mutation abolishes one of two recognition sequences for *Bsm*AI normally present in this PCR product. Consequently, fragments of 35 bp and 434 bp are produced after digestion of the mutant allele with *Bsm*AI whereas 35-bp, 357-bp, and 77-bp fragments are the result of digestion of the wild-type allele with this restriction enzyme. All enzymes were purchased from New England Biolabs, Beverly, MA.

Reverse transcription-PCR

Reverse transcription-PCR (RT-PCR) to detect the c.1529G>A mutation was performed using the GeneAmp RNA PCR Core Kit from Applied Biosystems (Roche Molecular Systems) according to the instructions of the manufacturer.

Briefly, 1.0 µg of the patient's reticulocyte RNA was reverse transcribed using random hexamers as primers. After addition of 30 pmol of primers CDPK-11 (5'-CTCAGCCCAGCTTCTGTCTCG-3', exon 11 nts 1437 to 1457) and PKr-6 (5'-GTGTGGGCTGGAGAACGTAGA-3', exon 12 nts +78 to +58), the samples were subjected to 35 cycles of amplification with denaturation at 94°C for 30 seconds (5 minutes at 95°C

prior to the first cycle), annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds, followed by an elongated extension time of 10 minutes after the last cycle. Total liver RNA was used as a positive control and controls without RNA as well as controls in which the reverse transcription step was omitted were included.

Allelic frequency determination

Allelic frequency of –248delT and population evidence regarding its physiologic effect were obtained by screening the DNA of 241 anonymized white control subjects and heterozygotes for the c.1529G>A mutation, respectively, for the –248delT mutation by allele-specific oligonucleotide hybridization (ASOH). Genomic DNA (100 ng) was amplified in the region of nt –248T in the PK-R promoter by PCR. The 25-μL system contained 34 mM Tris-HCl, pH 8.8, 8.3 mM NH_4SO_4, 1.5 mM $MgCl_2$, 85 g/mL bovine serum albumin, 0.2 mM of each dNTP, 100 ng of sense (5'-CTCCCTGGATTCACTAGAGC-3', nts –322 to –303) and antisense (5'-AGGATGGACTTTGCTAAGT-3', nts 65 to 83) primers, and 1.5 U *Taq* DNA polymerase. After a 5-minute denaturation step at 98°C, 30 cycles of 93°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds were performed followed by a 7-minute 72°C incubation. The 405-bp PCR product (4 μL) was then spotted on Nytran SuPerCharge membranes (Schleicher and Schuell, Keene, NH). The membranes were denatured, neutralized, and UV-crosslinked prior to hybridization. The membranes were hybridized with wild-type (5'-AAATATCTATTCACGTG-3') and mutant (5'-AAAAATCTATTCACGTG-3') ^{32}P-labeled oligonucleotide probes for the –248T position of the PK-R promoter. After hybridization the membranes were washed in 6 × SSC, 0.1% sodium dodecyl sulphate (SDS) at 50°C and developed with a Cyclone storage phosphor autoradiography system (Packard Instrument, Meriden, CT).

Promoter constructs and site-directed mutagenesis

The human PK promoter constructs from the patient and healthy controls were generated from a 469-bp PCR fragment comprising the upstream regulatory domain and exon 1, down to the ATG codon (Figure 1). The blunt-end PCR fragment was cloned into the pCR-Blunt vector (Invitrogen, Paisley, United Kingdom) before it was excised and inserted into the *Mlu*I and *Xho*I sites of pGL3-Basic (Promega, Madison, WI). Site-directed mutagenesis was performed with splicing by overlap extension as described.[16] Using PK-R promoter reporter plasmid pGL3_PKRWT as the wild-type template, we generated the following mutants: pGL3_PKR91A (nt –91T>A), pGL3_PKR90T (nt –90C>T), pGL3_PKR89G (nt –89T>G), pGL3_PKR88G (nt –88T>G), pGL3_PKR87A (nt –87C>A), pGL3_PKR86G (nt –86T>G), pGL3_PKR85A (nt –85C>A), pGL3_PKR84G (nt –84T>G), pGL3_PKR83C (nt –83G>C), pGL3_PKR82G (nt –82T>G), pGL3_PKR81A (nt –81C>A), pGL3_PKR80G (nt –80T>G),

pGL3_PKR79T (nt –79C>T), and pGL3_PKR78T (nt –78C>T). Briefly, two PCR products were generated that harbored the desired mutation using primers PKRP-ESF and PKRP-ESR in combination with the applicable mutant antisense primers and sense primers, respectively (primer sequences are available on request). Fragments were electrophoresed and purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen). Subsequently, for each mutant promoter construct 12.5 µL of each of both fragments obtained by the first PCR reaction were combined and subjected to a second round of amplification with primers PKRP-ESF and PKRP-ESR. Finally, the blunt-end mutated PCR fragment was cloned into the pCR-Blunt vector (Invitrogen), and subcloned into the *Xho*I and *Mlu*I sites of the pGL3-Basic vector, as described above. All constructs were verified by DNA sequence analysis. There were three additional mutant promoter constructs prepared as described that harbored the –248delT polymorphism (pGL3_PKR248delT), the –324T>A mutation (pGL3_PKR324A) and, by using the patient's DNA as a template, both the –324T>A and –83G>C mutations *in cis* (pGL3_PKR324A/83C).

Cell culture and transient DNA transfections

K562 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 1% streptomycin, and 1% penicillin in 10% CO_2 at 37°C. Cells were transiently transfected with Lipofectamine (Life Technologies, Paisley, United Kingdom) according to the manufacturer's instructions. Briefly, 30,000 cells/cm^2 were seeded in 24-well plates 24 hours prior to transfection. The cells were transfected with 2 µg reporter plasmid DNA and 100 ng RL-SV40 plasmid (Promega) that was used as internal control. After 48 hours, luciferase activity was measured with the Dual Luciferase Assay kit (Promega) and normalized to renilla luciferase activity. The promoterless pGL3-Basic Luciferase Reporter Vector (Promega) was used as a negative control.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed essentially as described[17] with K562 nuclear extracts, prepared according to Dignam.[18] Wild-type and mutant double-stranded oligonucleotide probes were obtained by annealing the following single-stranded primers: PKWT: sense 5'-TTCTCTTCTCTGTCTCCCTT-3' and antisense 5'AAGGGAGACAGAGAAGAGAA-3'; and PKmut: sense 5'-TTCTCTTCTCgGTC-TCCCTT-3' and antisense 5'-AAGGGAGACcGAGAAGAGAA-3', respectively. PKmut contains the –84T>G mutation (in lower case). Competitors (excess of unlabelled probe oligonucleotide or corresponding mutant oligonucleotide) were included as described in the figure legends.

Results

Glycolytic enzyme activities

The results from the measurement of peripheral blood glycolytic enzyme activities in the patient and his parents are summarized in Table 1. In the patient, PK activity was only just

Table 1. Glycolytic enzyme activities in the patient and his parents

	PK (U/gHb)	G6PD (U/gHb)	HK (U/gHb)
Peripheral blood			
Control	8.4–14.4	9.5–15.0	1.05–1.81
Patient	7.1	21.4	4.30
Father	14.2	21.0	1.90
Mother	8.3	18.0	2.30
Reticulocyte-rich fraction			
Patient	3.8	21.7	4.22

below the lower reference value, whereas the HK and G6PD values were high, indicating that the red cell population was relatively young. Consequently, we interpreted the PK activity as too low. To exclude the interference of donor cells, we isolated the low-density, reticulocyte-rich fraction of the patient by Percoll-density centrifugation. Subsequent glycolytic enzyme activity measurements in this fraction showed an even lower PK activity. In contrast, the G6PD and HK activity remained unaltered, thus underscoring the presence of PK deficiency in the patient's red blood cells. The PK activity measured in peripheral blood of the father was normal, whereas the erythrocyte PK activity of the mother was low, relative to that of G6PD and HK.

DNA sequence analysis of PKLR

By DNA sequence analysis of *PKLR*, the patient was found to be heterozygous for the common c.1529G>A mutation in exon 11 (Figure 2A). This mutation was confirmed by *Sty*I digestion and subsequent restriction enzyme analysis of his parents revealed that the patient had inherited this allele from his father (data not shown). Apart from heterozygosity for the well established polymorphisms[2] c.1705A>C, c.1738C>T, and c.1992T>C in exon 12, no

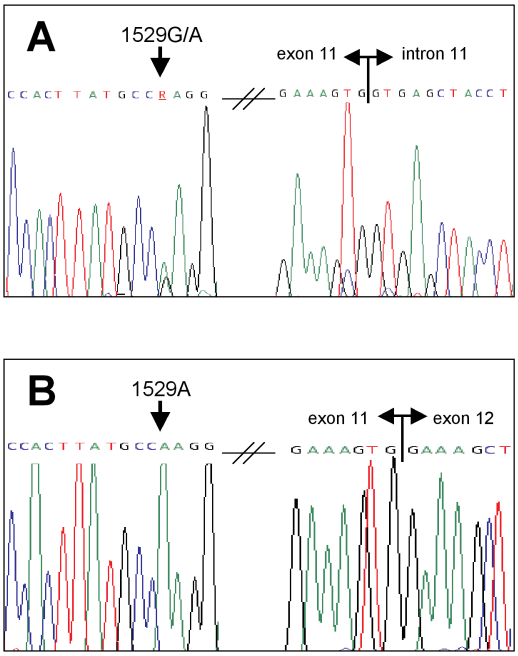

Figure 2. Heterozygous *PKLR* c.1529G>A missense mutation and sole expression of the 1529A allele in the patient. (A) *PKLR* DNA sequence analysis of exon 11 in the patient shows a heterozygous G>A substitution (arrow). The splice donor site of intron 11 is indicated. (B) RT-PCR analysis of the patient's RNA yielded only one transcript that contained the 1529A mutation (arrow), thereby indicating a severely reduced transcription of the *in trans* 1529G allele. Double horizontal arrows denote boundaries between exons.

other mutations were detected in *PKLR* exons and splice junctions. However, three previously undescribed base alterations were indentified in the PK-R promoter compared with the healthy control individual. Of these, two were single nucleotide substitutions of, respectively, thymine to adenine at nt –324 (–324T>A) and guanine to cytosine at nt –83 (–83G>C). Both mutations were confirmed in the patient by *Bst*XI and *Bsm*AI digestion, respectively, and also found to be present in the patient's mother, whereas they were absent in the patient's father (Figure 3), thereby demonstrating that both mutations were present *in cis*. Neither allele was detected in a healthy control population (n = 100). A third sequence variation was observed around nt –248 in both the patient and his mother but not in the patient's father and the control. Its characterization, however, was hampered because of an apparent concomitant variation in the number of adenines between nts –249 and –258 in all subjects. Since the latter was likely to be a PCR artifact due to slippage of *Taq* DNA

polymerase at this homonucleotide run,[19] we cloned this promoter fragment and characterized its DNA sequence context (see "Polymorphic deletion of thymine –248").

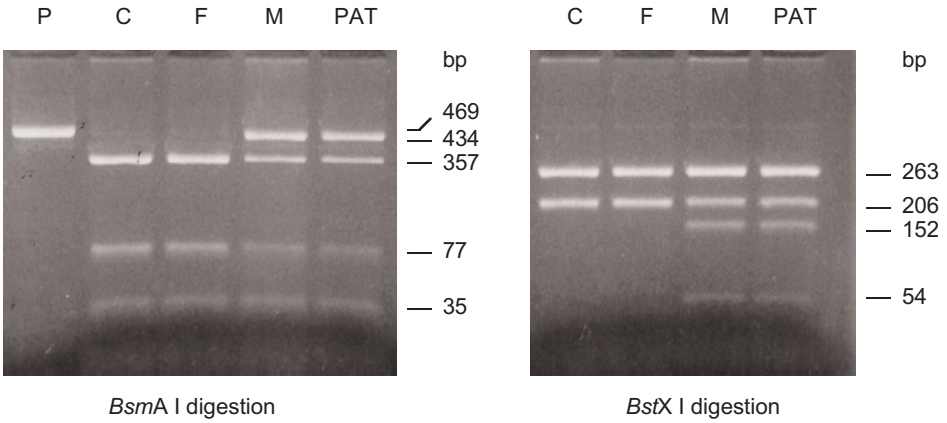

Figure 3. Heterozygosity for two novel mutations in the PK-R promoter at nt –83 (–83G>C) and nt –324 (–324T>A) in the patient and his mother. A 469-bp fragment was amplified as described and subjected to restriction enzyme digestion. The obtained pattern for each reaction is indicated by arrows (see "Patient, materials, and methods"). Heterozygosity for both the –83G>C mutation (*Bsm*AI digestion) and the –324T>A mutation (*Bst*XI digestion) was confirmed in the patient (PAT) and also detected in his mother (M), whereas they were absent in the patient's father (F).
P indicates uncut PCR product; and C, healthy control.

Polymorphic deletion of thymine –248

DNA sequence analysis of a number of cloned promoter fragments from the patient and the control confirmed the presence of the –324T>A and –83G>C mutations *in cis* in the patient. The third mutation in the patient constituted the deletion of thymine –248 (–248delT).

Since nt –248T constitutes part of an inverted consensus binding site for GATA-1, (A/T)GATA(A/G),[20] the –248delT mutation potentially disrupts binding of GATA-1 (Figure 1). Therefore, constructs containing the wild-type (pGL3_PKRWT) and the –248delT polymorphic allele (pGL3_PKR248delT) were transiently transfected in K562 cells to determine the effect of the –248delT mutation on promoter activity. A comparison between the two alleles showed a statistically significant ($P < .05$) increase in promoter activity upon –248delT (Figure 4A).

In vivo evidence regarding the functional consequences on transcriptional activity of the –248delT deletion was obtained from a study of carriers of c.1529G>A and –248delT *in trans*. If the –248T mutation abolished or severely impaired transcription, such compound heterozygous patients should be PK-deficient and anemic or at least have macrocytosis

because of increased erythropoiesis. In a previous study,[21] several thousand DNA samples from a general population were screened for the c.1529G>A mutation and 11 heterozygotes were detected. Among these, four individuals also carried the –248delT mutation. Since 37 out of 37 c.1529G>A alleles carried the wild-type promoter (data not shown), it is reasonable to assume that these individuals were compound heterozygotes for c.1529G>A and –248delT. If the –248delT mutation prevented transcription, then the patient should be PK deficient. Enzyme activities were not available, but we compared the average hemoglobin of three female compound heterozygotes (12.6 g/dL), which was no different than the 12.1 g/dL of the three female single heterozygotes for c.1529G>A. The male patient had a hemoglobin of 15.2 g/dL. All had normal mean corpuscular volume (MCV) values. We infer that the

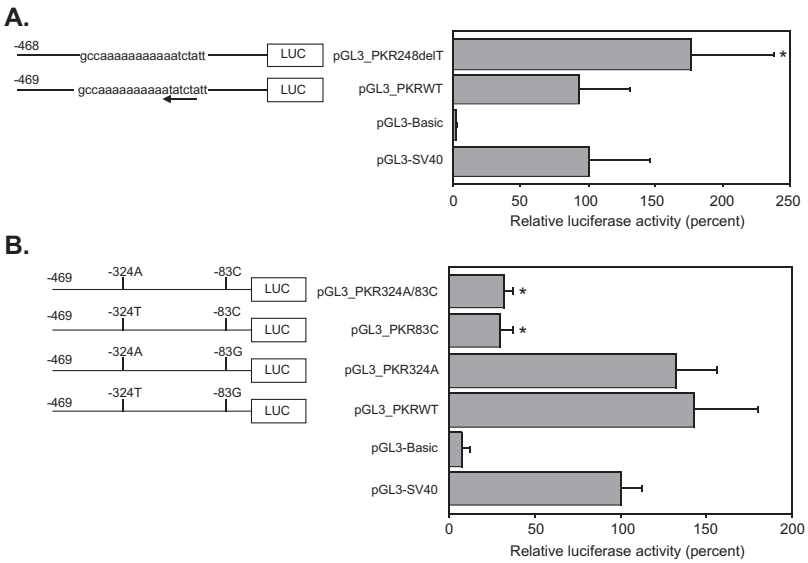

Figure 4. The –83G>C mutation in the PK-R promoter strongly down-regulates promoter activity *in vitro*. Luciferase reporter gene constructs containing 469 bp of the wild-type or mutated PK-R promoter were transiently transfected in K562 erythroleukemic cells. Luciferase activities were expressed relative to control pGL3-SV40 and pGL3-Basic was included as a negative (promoterless) control. (A) Constructs pGL3_PKRWT and pGL3_PKR248delT contained the wild-type or polymorphic –248delT allele, respectively. The latter mutation disrupts an inverted GATA-1 binding site (arrow) but no down-regulation of promoter strength is observed. In contrast, an increase in promoter activity was observed upon removal of thymine –248. (B) Individual and combined effects of the –83G>C and –324T>A missense mutations were studied using constructs that contained only the –83G>C mutation (pGL3_PKR83C) or the –324T>A mutation (pGL3_PKR324A), or both mutations *in cis* (pGL3_PKR324A/83C). The –324T>A mutation had no effect on promoter activity as compared to the wild-type (pGL3_PKRWT). In contrast, the –83G>C mutation is capable of strongly reducing *in vitro* promoter activity and is unaffected by the concomitant presence of the –324T>A substitution *in cis*. *Statistically significant ($P < .05$).

–248delT mutation is a benign polymorphism and is not associated with reduced promoter activity and, consequently, with PK-deficiency. Subsequent determination of the allelic frequency of this mutation was performed by screening 241 control subjects of a general white population for this mutation by allele-specific oligonucleotide hybridization. There were 206 wild-type subjects (–248T/T), 34 heterozygous subjects (–248T/delT), and one homozygous control (–248delT/delT). Consequently, the allelic frequency for this novel polymorphism in the PK-R promoter is 0.075.

Functional characterization of nt –324T>A and nt –83G>C promoter mutations

To asses the functional consequences of the mutated PK-R promoter region, we first performed RT-PCR analysis on the patient's RNA with primers spanning exons 11 and 12 to determine the relative expression of the two alleles. As shown in Figure 2B, only the 1529A allele could be detected in the patient, strongly indicating that transcription is severely reduced by the mutated promoter.

To study the individual and combined effects of the –324T>A and –83G>C mutations on PK-R promoter activity, we transfected constructs pGL3_PKRWT (wild-type), pGL3_PKR324A (nt –324T>A), pGL3_PKR83C (nt -83G>C) and pGL3_PKR324A/83C (nt –324T>A and nt –83G>C *in cis*) in K562 cells and compared their relative luciferase activities. Figure 4B shows that the –83G>C substitution is capable of down-regulating promoter activity (pGL3_PKR83C and pGL3_PKR324A/83C). This effect was achieved either with (pGL3_PKR324A/83C) or without (pGL3_PKR83C) the concomitant presence of the –324T>A mutation. In contrast, promoter activity was not altered, with regard to the wild-type promoter, in case only the –324T>A mutation was present (pGL3_PKR324A). We infer that the –83G>C mutation disrupts transcription.

Delineation of a putative regulatory element comprising the nt –83G>C mutation

Since the proximal erythroid-specific promoter region of *PKLR* previously was reported to dictate basal promoter activity[9], we hypothesized nt –83 to be part of a previously unrecognized *trans*-acting factor binding element. To unravel the sequence of the putative *cis*-element we generated a series of consecutive promoter mutants from nts –91 to –78 and determined their activity in K562 cells. Figure 5 shows that substitution of nts –87 to –83 leads to a decreased promoter activity. In particular, the –84T>G mutation exhibited a profound reduction in transcription. Substitutions further upstream (nts –88 to –91) or downstream (nts –82 to –78) did not significantly alter promoter activity. Thus, we defined the existence of a novel regulatory element in the PK-R promoter PKR-RE1 whose core binding motif is confined to nts –87 to –83.

To further explore the involvement of PKR-RE1 in binding *trans*-acting factors, we performed EMSA with K562 nuclear extract and oligonucleotide probes designed according to the native core binding motif (PKWT) and mutated PKR-RE1 (Pkmut; –84T>G). Figure 6 demonstrates the formation of three distinct DNA-protein complexes upon incubation with labeled PKWT (lane 1).

One of these bands (arrow) could be competed off succesfully by increasing amounts of excess unlabeled PKWT (lanes 2-4), but remained unaffected by the addition of increasing amounts of excess unlabeled PKmut as a competitor (lanes 5-7), thereby establishing its specificity. This band was absent when lysates were incubated with radiolabeled PKmut (lane 8), thereby suggesting that the particular protein-DNA interaction was abolished in

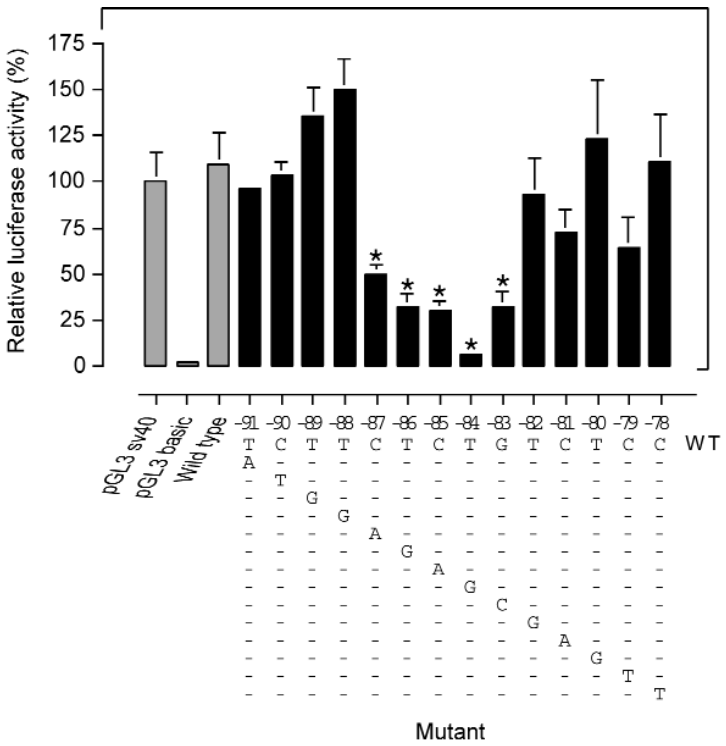

Figure 5. Site-directed mutagenesis of a region of the PK-R promoter spanning nts –91 to –78 identifies the core motif CTCTG of a novel PK-R regulatory element (PKR-RE1). PK-R promoter reporter gene constructs harboring mutations from nts –91 to –78 were expressed in K562 cells. The applicable mutation is depicted in the lower part of the figure. Luciferase activities were calculated relative to control pGL3-SV40. pGL3-Basic was included as a negative (promoterless) control. The decreased promoter activity as a result of the introduced mutations at nts –87 to –83 revealed a single pentanucleotide motif CTCTG. *Statistically significant ($P < .05$).

case PKR-RE1 was disrupted. Instead, another band appeared that could, however, be competed off succesfully by increasing amounts of both unlabeled PKWT and PKmut (lanes 9-11 and 12-14, respectively). We infer that the –83C is part of a putative *trans*-acting factor binding element, characterized by a CTCTG core motif.

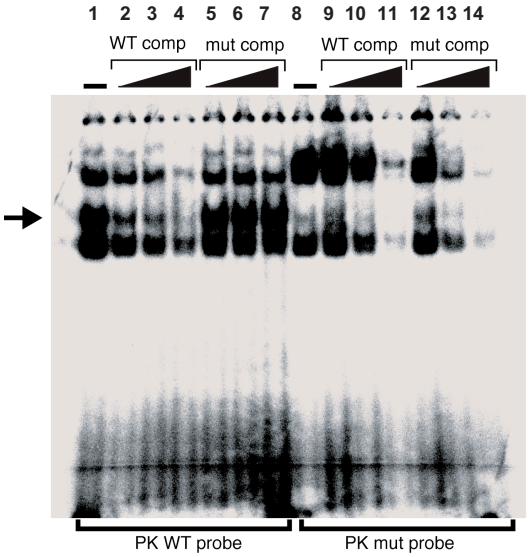

Figure 6. PKR-RE1 is involved in DNA-protein interaction. Electrophoretic mobility shift assay was performed with K562 nuclear extract and oligonucleotide probes designed according to the native core binding motif (PKWT; lanes 1-7) and mutated PKR-RE1 (PKmut; lanes 8-14). Absence (–) and increasing amounts of unlabeled wild-type and mutant competitor is indicated. The figure shows three distinct DNA-protein complexes upon incubation with labeled PKWT (lane 1). One of these bands (arrow) could be competed off succesfully by increasing amounts of excess unlabeled PKWT (lanes 2-4), but remained unaffected by the addition of increasing amounts of excess unlabeled PKmut as a competitor (lanes 5-7). In contrast, this band was absent when lysates were incubated with radiolabeled PKmut (lane 8), whereas the extra band that appeared upon incubation with PKmut could be competed off succesfully by increasing amounts of both unlabeled PKWT and PKmut (lanes 9-11 and 12-14, respectively)

Discussion

We established the molecular basis for PK deficiency in a six-year-old boy of Danish ancestry who suffered from severe hemolytic anemia. On the paternal allele of this patient we detected a guanine-to-adenine substitution at nt 1529 in *PKLR*. The Arg510Gln encoded by this frequently occuring mutation and the consequent structural changes in PK that lead

to PK deficiency have been well documented.[15,22] The fact that the father had normal PK activity (Table 1), in spite of being heterozygous, emphasizes the difficulty of accurately identifying heterozygotes based on enzyme activity alone. On the maternal allele of the patient, we detected three novel mutations in the erythroid-specific promoter of *PKLR*. Two of these mutations were single-base substitutions, –324T>A and –83G>C, relative to the initiator adenine. The third change consisted of the deletion of thymine at nt –248.

RT-PCR analysis of the patient's RNA demonstrated sole expression of the 1529A allele (Figure 2B). Therefore, it was conceivable that the mutated promoter caused effective downregulation of transcription from the affected allele. Analysis of the various promoter mutations in K562 cells showed that –83G>C alone was capable of inducing a drastically reduced promoter activity *in vitro*, whereas the –324T>A and –248delT mutations exerted no such effect (Figure 4). The latter mutation represents a nonfunctional polymorphic substitution (allele frequency 0.075), whereas –324T>A is a nonfunctional mutation.

In vivo evidence regarding the lack of functional consequences on transcriptional activity of the –248delT deletion came from a study among individuals who were compound heterozygous for the c.1529G>A and –248delT *in trans*. If transcription was hampered by the –248delT mutation, such patients should be PK deficient and anemic or at least have macrocytosis because of increased erythropoiesis. However, the average hemoglobin level of three female compound heterozygotes (12.6 g/dL) showed no differences when compared with the average hemoglobin level of three female single heterozygotes for c.1529G>A (12.1 g/dL). One male patient had a hemoglobin level of 15.2 g/dL and all individuals had normal MCV values.

Because –248delT disrupts the GATA-1 binding motif at nts –244 to –249, we also investigated whether this mutation was able to influence promoter activity *in vitro*. Luciferase activities reflecting the relative promoter strength of these constructs showed no decline in promoter activity as a result of –248delT (Figure 4A). As this would have been suggestive of a functional stimulatory GATA-1 binding site *in vitro*, it is unlikely that the latter is the case. Thus, both *in vitro* and *in vivo* evidence support the presence of a non-functional GATA-1 binding site at nts –244 to –249 in the erythroid-specific promoter of *PKLR*. Interestingly, we found that the wild-type allele confered an approximately two-fold increase in promoter activity upon deletion of thymine –248 (Figure 4A). Although the effect is relatively small, it is possible that –248delT modulates the phenotypic expression of pyruvate kinase deficiency, as previously shown for a polymorphic dinucleotide repeat at the UDP-glucuronosyltransferase 1 promoter[23] that contributes to the clinical phenotype in G6PD deficient neonates.[24]

Only one mutation in *PKLR* is known to date that is associated with a reduced transcription

from its erythroid-specific promoter and consequent quantitative reduction in PK-R. A markedly reduced amount of PK-R mRNA was detected, 20% by semi-quantitative RT-PCR analysis, as a result of a single nucleotide substitution at nt –72.[11] The involvement of GATA-1 was presumed since the mutation was located in the core of the binding motif of this erythroid-specific transcription factor.[20,25,26] Initially, a –249delA mutation in the PK-R promoter was reported in association with PK deficiency.[27] However, re-investigation of the patient's DNA revealed the presence of the same three *in cis* mutations we report here.[27] Kugler has allowed us to examine a DNA sample from his patient, and we have confirmed his results (data not shown).

Most current data on the function of the *PKLR* gene indicate that the proximal promotor region is essential for transcriptional initiation. The distal region upstream position –300, where nucleotide –324T is located, appears to be dispensable for transcriptional regulation as shown by Kanno *et al.*, who found that removal of nts –513 to –304, had no effect on promoter activity.[9] In the same study, the proximal 120 bp, containing two CAC boxes and one GATA binding motif, were shown to direct a basal promoter activity, whereas the 150 bp upstream acted as an enhancer.[9] From studies on the rat PK-R promoter, this region was also found to be functionally important.[10,28,29] Lacronique *et al*. demonstrated that a proximal 320-bp PK-R promoter fragment is able to direct erythroid-specific transcription in a rat fetal liver cell-free transcription system and interacts both *in vitro* and *in vivo* in a erythroid-specific manner with GATA-1, CACC-binding proteins, and unidentified factors recognizing G/C-rich motifs.[28] Furthermore, Max-Audit *et al*. demonstrated the specific binding of GATA-1 and members of the CACC/Sp1 family to the proximal GATA binding site of a minimal promoter spanning nts –62 to +54.[10] GATA-1 and Sp1, two factors known to physically interact,[30] have also been shown to cooperatively activate transcription from a minimal (nts –62 to +43) rat PK-R promoter in *Drosophila* S2 cells.[29]

Because of the effect of the –83G>C mutation and its location in a well conserved region between rat and humans (Figure 1), we anticipated that nt –83G was an essential part of a *trans*-acting factor binding element. Systematic mutagenesis of the region disclosed the presence of a *cis*-element, whose core CTCTG extents from nts –87 to –83 (Figure 5). We designated this novel regulatory element in the PK-R promoter, PKR-RE1. Subsequent EMSA using K562 nuclear extract demonstrated *in vitro* DNA-protein interaction at the core of PKR-RE1 involving an as-yet-unidentified protein (Figure 6). In agreement with these data, Lacronique *et al*. previously showed by *in vitro* DNAse I footprinting that the corresponding conserved sequence in the rat PK-R promoter encompassing nts –64 to –58 was involved in DNA-protein interaction.[28] In gel shift assays, the labeled probe containing the CTCTG sequence and spanning nts –80 to –57 formed two retarded complexes which,

on the basis of mobility shift assays, were suggested to correspond to GATA-1-containing complexes, although GATA-1 later proved to bind more upstream.[28] Since the CTCTG motif is located just 10 bp upstream of the GATA-1 binding site, an appealing model would be that GATA-1 formed a complex with the *trans*-acting factor binding to PKR-RE1, similar to the previously described erythroid ternary complex between GATA-1, the helix-loop-helix factor TAL1, and the bridging LIM-only protein Lmo2.[31] Although, PKR-RE1 does not constitute an E-box, we tested whether GATA-1- and FOG-mediated *trans*-activation of the PK-R promotor was affected by the –83G>C mutation, but have so far not observed any difference between the wild-type sequence and the mutant (R.v.W., W.W.v.S., C.N., and F.C.N., unpublished results, November 2000). Based on the current knowledge, we therefore propose that the PKR-RE1 functions independently of GATA-1.

Since the CTCTG motif resembles no known transcription factor elements,[32] PKR-RE1 may be involved in a novel mechanism of erythroid-specific *trans*-activation. Future identification of the putative *trans*-acting factor(s) may provide important leads to our understanding of erythroid-specific transcriptional regulation involved in red cell differentiation and maturation.

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References

1. Zanella A, Bianchi P. Red cell pyruvate kinase deficiency: from genetics to clinical manifestations. Baillieres Best Pract Res Clin Haematol. 2000;13:57-81.
2. Bianchi P, Zanella A. Hematologically important mutations: red cell pyruvate kinase (third update). Blood Cells Mol Dis. 2000;26:47-53.
3. Satoh H, Tani K, Yoshida MC, Sasaki M, Miwa S, Fujii H. The human liver-type pyruvate kinase (PKL) gene is on chromosome 1 at band q21. Cytogenet Cell Genet. 1988;47:132-133.

4. Tani K, Fujii H, Nagata S, Miwa S. Human liver type pyruvate kinase: complete amino acid sequence and the expression in mammalian cells. Proc Natl Acad Sci U S A. 1988;85:1792-1795.
5. Kanno H, Fujii H, Hirono A, Miwa S. cDNA cloning of human R-type pyruvate kinase and identification of a single amino acid substitution ($Thr^{384}\rightarrow Met$) affecting enzymatic stability in a pyruvate kinase variant (PK Tokyo) associated with hereditary hemolytic anemia. Proc Natl Acad Sci U S A. 1991;88:8218-8221.
6. Lenzner C, Nürnberg P, Jacobasch G, Thiele B-J. Complete genomic sequence of the human PK-L/R-gene includes four intragenic polymorphisms defining different haplotype backgrounds of normal and mutant PK-genes. DNA Seq. 1997;8:45-53.
7. Marie J, Simon MP, Dreyfus JC, Kahn A. One gene, but two messenger RNAs encode liver L and red cell L' pyruvate kinase subunits. Nature. 1981;292:70-72.
8. Noguchi T, Yamada K, Inoue H, Matsuda T, Tanaka T. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. J Biol Chem. 1987;262:14366-14371.
9. Kanno H, Fujii H, Miwa S. Structural analysis of human pyruvate kinase L-gene and identification of the promoter activity in erythroid cells. Biochem Biophys Res Commun. 1992;188:516-523.
10. Max-Audit I, Eleouet JF, Roméo P-H. Transcriptional regulation of the pyruvate kinase erythroid-specific promoter. J Biol Chem. 1993;268:5431-5437.
11. Manco L, Ribeiro ML, Máximo V, et al. A new PKLR gene mutation in the R-type promoter region affects the gene transcription causing pyruvate kinase deficiency. Br J Haematol. 2000;110:993-997.
12. van Solinge WW, van Wijk HA, Kraaijenhagen RJ, Rijksen G, Nielsen FC. Novel mutations in the human red cell type pyruvate kinase gene: two promoter mutations in cis, a splice site mutation, a nonsense- and three missense mutations [abstract]. Blood. 1997;90(suppl 1):272a.
13. Rijksen G, Veerman AJ, Schipper-Kester GP, Staal GE. Diagnosis of pyruvate kinase deficiency in a transfusion-dependent patient with severe hemolytic anemia. Am J Hematol. 1990;35:187-193.
14. Recommended methods for the characterization of red cell pyruvate kinase variants. International Committee for Standardization in Haematology. Br J Haematol. 1979;43:275-286.
15. van Solinge WW, Kraaijenhagen RJ, Rijksen G, et al. Molecular modelling of human red blood cell pyruvate kinase: structural implications of a novel G_{1091} to a mutation causing severe nonspherocytic hemolytic anemia. Blood. 1997;90:4987-4995.

16. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene. 1989;77:51-59.
17. Nerlov C, Rørth P, Blasi F, Johnsen M. Essential AP-1 and PEA3 binding elements in the human urokinase enhancer display cell type-specific activity. Oncogene. 1991;6:1583-1592.
18. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 1983;11:1475-1489.
19. Kunkel TA. The mutational specificity of DNA polymerase-β during in vitro DNA synthesis. Production of frameshift, base substitution, and deletion mutations. J Biol Chem. 1985;260:5787-5796.
20. Tsai S-F, Martin DI, Zon LI, D'Andrea AD, Wong GG, Orkin SH. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature. 1989;339:446-451.
21. Beutler E, Gelbart T. Estimating the prevalence of pyruvate kinase deficiency from the gene frequency in the general white population. Blood. 2000;95:3585-3588.
22. Wang C, Chiarelli LR, Bianchi P, et al. Human erythrocyte pyruvate kinase: characterization of the recombinant enzyme and a mutant form (R510Q) causing nonspherocytic hemolytic anemia. Blood. 2001;98:3113-3120.
23. Beutler E, Gelbart T, Demina A. Racial variability in the UDP-glucuronosyltransferase 1 (*UGT1A1*) promoter: a balanced polymorphism for regulation of bilirubin metabolism? Proc Natl Acad Sci U S A. 1998;95:8170-8174.
24. Kaplan M, Renbaum P, Levy-Lahad E, Hammerman C, Lahad A, Beutler E. Gilbert syndrome and glucose-6-phosphate dehydrogenase deficiency: a dose-dependent genetic interaction crucial to neonatal hyperbilirubinemia. Proc Natl Acad Sci U S A. 1997;94:12128-12132.
25. Orkin SH. GATA-binding transcription factors in hematopoietic cells. Blood. 1992;80:575-581.
26. Simon MC. Gotta have GATA. Nat Genet. 1995;11:9-11.
27. Kugler W, Laspe P, Stahl M, Schröter W, Lakomek M. Identification of a novel promoter mutation in the human pyruvate kinase (*PK*) *LR* gene of a patient with severe haemolytic anaemia [erratum appears in Br J Haematol. 2002;119:289]. Br J Haematol. 1999;105:596-598.
28. Lacronique V, Boquet D, Lopez S, Kahn A, Raymondjean M. *In vitro* and *in vivo* protein - DNA interactions on the rat erythroid-specific L' pyruvate kinase gene promoter. Nucleic Acids Res. 1992;20:5669-5676.

29. Gregory RC, Taxman DJ, Seshasayee D, Kensinger MH, Bieker JJ, Wojchowski DM. Functional interaction of GATA1 with erythroid Kruppel-like factor and Sp1 at defined erythroid promoters. Blood. 1996;87:1793-1801.
30. Merika M, Orkin SH. Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. Mol Cell Biol. 1995;15:2437-2447.
31. Wadman IA, Osada H, Grutz GG, et al. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. EMBO J. 1997;16:3145-3157.
32. Wingender E, Chen X, Hehl R, et al. TRANSFAC: an integrated system for gene expression regulation. Nucleic Acids Res. 2000;28:316-319.

CHAPTER 3

Ex vivo analysis of aberrant splicing induced by two donor site mutations in *PKLR* of a patient with severe pyruvate kinase deficiency

Richard van Wijk[1], Annet C. W. van Wesel[1], Adri A. M. Thomas[2], Gert Rijksen[3], and Wouter W. van Solinge[1]

[1]Department of Clinical Chemistry, and [3]Department of Haematology, University Medical Centre Utrecht, Utrecht, The Netherlands; [2]Department of Developmental Biology, Utrecht University, Utrecht, The Netherlands.

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Abstract

Two single-nucleotide substitutions in *PKLR* constituted the molecular basis underlying pyruvate kinase (PK) deficiency in a patient with severe haemolytic anaemia. One novel mutation, IVS5+1G>A, abolished the intron 5 donor splice site. The other mutation, c.1436G>A, altered the intron 10 donor splice site consensus sequence and, moreover, encoded an R479H substitution. We studied the effects on *PKLR* pre-mRNA processing, using *ex vivo* produced nucleated erythroid cells from the patient. Abolition of the intron 5 splice site initiated two events in the majority of transcripts: skipping of exon 5 or, surprisingly, simultaneous skipping of exon 5 and 6 (Δ5,6). Subcellular localization of transcripts suggested that no functional protein was produced by the IVS5+1A allele. The unusual Δ5,6 transcript suggests that efficient inclusion of exon 6 in wild-type *PKLR* mRNA depends on the presence of splice enhancing elements in exon 5. The c.1436G>A mutation caused skipping of exon 10 but was mainly associated with a severe reduction in transcripts although these were, in general, normally processed. Accordingly, low amounts of PK were detected in nucleated erythroid cells of the patient, thus correlating with the patient's PK-deficient phenotype. Finally, several low-abundant transcripts were detected that represent the first examples of 'leaky-splicing' in *PKLR*.

Introduction

The enucleated mature red blood cell depends solely on anaerobic glycolysis for the constant generation of ATP in order to maintain cellular integrity and function. Disturbances in glycolysis shorten its cellular survival and frequently result in nonspherocytic haemolytic anaemia of variable severity. The most common cause of chronic nonspherocytic haemolytic anaemia is pyruvate kinase (PK) deficiency[1] (MIM 266200). PK catalyses the final step of glycolysis, converting phosphoenolpyruvate to pyruvate with the concomitant generation of ATP. PK deficiency is transmitted as an autosomal recessive disease, and molecular analysis has identified more than 130 mutations in the structural gene (*PKLR*) in association with PK deficiency.[2] The majority of these mutations constitute single-base substitutions, predicting amino acid changes of conserved residues in structurally and functionally important domains of PK. In contrast, mutations that affect *PKLR* transcription,[3,4] or processing of its pre-mRNA[5-11] are less frequently encountered.

Pre-mRNA processing[12-14] is a critical aspect of gene expression and comprises the precise removal of introns in such a way that the exons are joined to form mature mRNAs with intact

translational reading frames. The splicing reaction is carried out by the spliceosome which consists of five small ribonucleoprotein complexes U1, U2, U4, U5, and U6 snRNPs[15] and a large number of non-snRNP proteins.[16] The spliceosome is assembled *in vivo* onto the pre-mRNA as it is being synthesized, thus providing an intimate relationship between transcription and pre-mRNA processing.[17,18] Changes in splice site choice, *e.g.* as result of mutation, arise from changes in the assembly of the spliceosome and are thought to be regulated by altered binding of the initial factors to the pre-mRNA and the formation of early spliceosome complexes.[19] Fifteen percent of point mutations in human genes, associated with disease, affect the conserved splice site signals.[20] However, mutations can affect pre-mRNA processing in a multitude of ways.[21] Therefore, in order to fully comprehend the contribution of any specific mutation on disease, it is important to consider mRNA biogenesis, quality control, and metabolic fate.[22]

In this study we report on the identification of two single-base substitutions in *PKLR* of a patient with severe PK deficiency. A novel G>A substitution at the invariant GT dinucleotide of intron 5 and a similar substitution concerning the last nucleotide of exon 10, both affected the 5' splice site. Since pre-mRNA processing is a nuclear event and the reticulocyte is devoid of RNA synthesis,[23] we studied the effects of these mutations on *PKLR* pre-mRNA processing using *ex vivo* produced nucleated erythroid cells from the patient. Multiple aberrant transcripts were identified and characterized. Subsequent subcellular localization of mRNAs and Western Blot analysis established the relevance of each transcript with regard to the PK-deficient phenotype of the patient. In addition, the characterization of one unusual transcript which lacked both exon 5 and 6 as a result of the intron 5 donor site mutation, suggests that efficient inclusion of exon 6 in wild-type *PKLR* mRNA depends on the presence of splice enhancer elements in exon 5 in the primary transcript.

Subjects and methods

Patient

The patient is a 31 year old Dutch woman, diagnosed at birth with severe, transfusion-dependent haemolytic anaemia due to PK deficiency. Other causes of anaemia were ruled out. Splenectomy, performed at the age of four years, improved her condition only slightly. From age 15 years on, the transfusion dependency has gradually decreased and is, to date, limited to periods of infection or during pregnancy, in general when blood hemoglobin falls below 6.4 g/dL. Throughout her life she has displayed a remarkable heat-sensitivity which led to haemolytic crises not only during febrile episodes but also as a result of prolonged

exposure to sunlight. Her heterozygous parents were asymptomatic.
Appropriate informed consent was obtained from the patient and the normal control individual, a healthy female with no haemolysis and normal glycolytic enzyme activities.

Biochemical analysis
PK and hexokinase (HK) enzyme activities were measured according to standard methods.[24] Thermal stability of PK was determined by the PK heat stability test.[25]

Molecular analysis of PKLR
Genomic DNA was isolated by standard methods. The erythroid-specific promoter and individual exons of *PKLR*, including flanking intronic regions, were amplified by PCR as described[4] and sequenced with the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA). The IVS5+1G>A and c.1436G>A mutations were confirmed by *Hph*I and *Msc*I (New England Biolabs, Beverly, MA) digestion of independently amplified PCR products.

In vitro production of human nucleated erythroid cells
Nucleated erythroid cells from the patient and a normal control individual were produced from light-density cells as described.[26] Briefly, light-density cells were collected from 50 mL venous blood by Ficoll-Paque Plus (Amersham-Pharmacia Biotec, Uppsala, Sweden) density centrifugation. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM), containing 20% fetal bovine serum and supplemented with human stem cell factor (10 ng/mL) (a gift from dr. H. Lokhorst), erythropoietin (1 U/mL) (Janssen Cilag, Tilburg, The Netherlands), interleukin-3 (1 ng/mL) (Biosource Europe, Bruxelles, Belgium), dexamethasone (10^{-6} M), and β-estradiol (10^{-6} M) (both from Sigma, St Louis, MI). Cells were grown at 37°C at 5% CO_2 and maintained at $1–2\times10^6$ cells/mL. Proliferation and cellular morphology was monitored every other day by May-Grünwald-Giemsa stained cytocentrifuged smears. Cells were harvested after 10 days and at that moment the culture consisted of more than 95% (pro-)erythroblasts. Subsequently, RNA was isolated or cells were used for polysome profile analysis. The transcriptional and translational active state of the cells with regard to PK was verified by polysome analysis and Western Blot (see 'Discussion').

RT-PCR and estimation of allelic differences in transcript levels
Total RNA was isolated from nucleated erythroid cells using RNABee reagent (Campro Scientific, Veenendaal, The Netherlands), according to instructions of the manufacturer. RT-

PCR was performed using the GeneAmp RNA PCR Core Kit (Roche, Branchburg, NJ). 1.0 μg total RNA was reverse transcribed using random hexamers as primers. Because aberrant splicing events may not be confined to the immediate proximity of the location of both mutations, an extensive region was amplified from *PKLR* cDNA. In case of the IVS5+1G>A mutation, a region encompassing exons 4 to 7 was amplified with 30 pmol of primers PK-C4F: 5'-ACATTGCGCGACTCAACTTCTCC-3' (exon 4, nts 338–360) and PK-C7R: 5'-TGATCTTGATGCCGTGTCCTTCC-3' (exon 7, nts 931–909). The effects of the c.1436G>A mutation were studied by amplification of exons 9 to 12 with primers PK-C9F: 5'-GGGCAACTTCCCTGTGGAAGC-3' (exon 9, nts 1230–1250) and PK-C12R: 5'-GATGGGGTACAAGGGTAGGCTGG-3' (exon 12, nts +44 to +22, relative to the termination codon). In both cases, the reverse primer was 6-FAM labeled. Samples were heated for 5 minutes at 95°C and subjected to 35 cycles of amplification (denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 60 seconds), followed by an elongated extension step of 10 minutes after the last cycle. Controls without RNA as well as controls in which the reverse transcription step was omitted were included. Labeled fragments were separated on an ABI 310 Genetic Analyzer (Applied Biosystems). Sizing (bp) and peak areas of fragments were calculated using GeneScan Analysis Software (Applied Biosystems).

In order to estimate relative transcript levels from both alleles of the patient, RT-PCR product, encompassing exons 9 to 12, was digested by *Msc*I and subjected to agarose gel electrophoresis. The c.1436G>A mutation in exon 10 creates a unique recognition site for this enzyme and RT-PCR product from the 1436A allele renders fragments of 204 and 336 bp after digestion. The product from the other allele remains uncut (540 bp). Equal allelic amounts of exon 10 as amplified from the patient's genomic DNA served as a control, yielding digestion fragments of 212 and 50 bp in case of the 1436A allele whereas the DNA-PCR product from the other allele is uncut (262 bp).

Subcloning of RT-PCR products

RT-PCR products were cloned using the pGEM-T Easy Vector System Kit (Promega, Madison, WI). Plasmid DNA was isolated (Qiaprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and 50 to 65 inserts were sequenced with the vector-derived T7 primer.

Polysome profile analysis and Δ5 transcript instability

Ten minutes before harvesting the cultured nucleated erythroid cells, cycloheximide was added to a concentration of 100 μg/mL. Cells were washed three times with ice-cold PBS containing 1 μg of cycloheximide per mL and lysed in 200 μL of 20 mM Tris-HCl (pH 7.6),

15 mM KCl, 1.5 mM Mg-acetate, 0.1 mM EDTA, 0.1% Triton X-100, and 7 mM β-mercaptoethanol. Cells were kept on ice for 1 minute and Na-desoxycholate and Igepal (Sigma) were added to a final concentration of 0.5%. The suspension was incubated for 30 minutes on ice. Cell debris and nuclei were removed by a 5 minute centrifugation at 4°C at 10,000 rpm. The supernatant was immediately layered onto isokinetic 15-32% (w/w) sucrose gradients in 20 mM Tris-HCl pH 7.6, 100 mM K-acetate, 3 mM Mg-acetate, 0.1 mM EDTA, and 7 mM β-mercaptoethanol. Gradients were centrifuged for 45 minutes at 50,000 rpm in a SW55 Beckman rotor, and harvested by upward displacement through a spectrophotometer, set at 260 nm. Ten fractions of 0.25 mL were collected into tubes containing 0.125 mL phenol/chloroform. RNA was isolated and 0.15 μg RNA of each fraction was used for RT-PCR and subsequent fragment analysis.
To study the Δ5 transcript (*i.e.* the transcript lacking exon 5) instability, cells were isolated, lysed, and kept on ice. Aliquots of lysates were taken at 0, 10, 30, and 60 minutes and RNA was isolated for RT-PCR (primers PK-C4F and PK-C7R) and subsequent fragment analysis.

SDS-PAGE and Western Blot analysis

10^7 cultured nucleated erythroid cells of the patient and control were lysed in 100 μL buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM β-mercaptoethanol, 10 mM ε-aminocaproic acid, 10 mM EDTA, 1 mM PMSF. Samples were adjusted for protein content, subjected to 10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked for 1 hour at room temperature in 5% BSA, 0.1% Tween-20 in phosphate buffered saline (PBST) and incubated overnight with a polyclonal anti-PK antibody[27] in 1% BSA, PBST. The membrane was washed 3 times with PBST and incubated for 2 hours with 1:10,000 diluted anti-rabbit IgG-HRP-linked antibody (Cell Signaling Technology, Beverly, MA) in 1% BSA, PBST. Bands were visualized by Enhanced Chemiluminescence (PerkinElmer, Boston, MA), according to instructions of the manufacturer.

Results

Glycolytic enzyme activities

Typical haematological parameters of the patient are displayed in Table 1. Despite a reticulocytosis of 638‰, the patient was severely anaemic. The PK activity was in the normal range but was very low considering the massive reticulocytosis, illustrated by the strongly elevated HK activity. Furthermore, PK from the patient showed reduced thermal stability, indicated by the 90% decrease in PK activity in the heat stability test (Figure 1).

Table 1. Haematological parameters and glycolytic enzyme activities of the patient.

	patient	reference value
haemoglobin (g/dL)	7.1	11.8–14.4
RBC (10^{12}/L)	1.94	3.7–5.0
MCV (fL)	118	80–97
reticulocytes (‰)	638	5–25
PK (U/gHb)	9.0	6.9–14.5
HK (U/gHb)	8.90	1.02–1.58

***PKLR* sequence analysis**

DNA sequence analysis of the patient's *PKLR* gene revealed compound heterozygosity for two single base substitutions. The first mutation, on the paternal allele, concerned a guanine to adenine change at nt +1 of intron 5: IVS5+1G>A. This novel mutation abolishes the invariant GT dinucleotide of the intron 5 donor splice site. On the maternal allele we detected a previously reported guanine to adenine change at the last nt of exon 10 (c.1436G>A).[5,28] Apart from encoding an arginine to histidine change at residue 479, this mutation disrupts the consensus sequence of the intron 10 donor splice site. No other mutations were detected

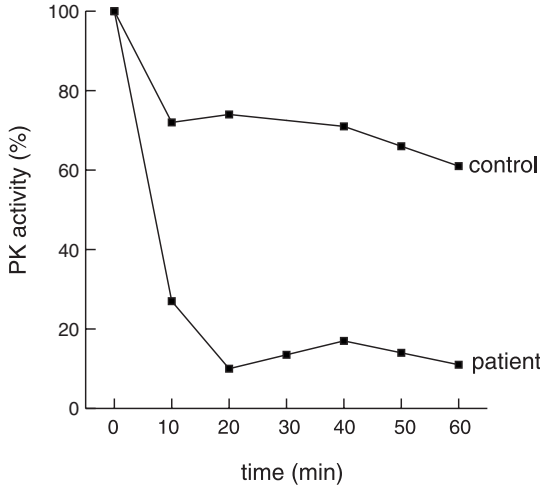

Figure 1. Thermal stability of PK. Thermal stability of PK in erythrocyte lysates from a normal control and the patient, was determined according to standard methods. Residual enzyme activity is expressed as percentage of the initial activity (set to 100%)

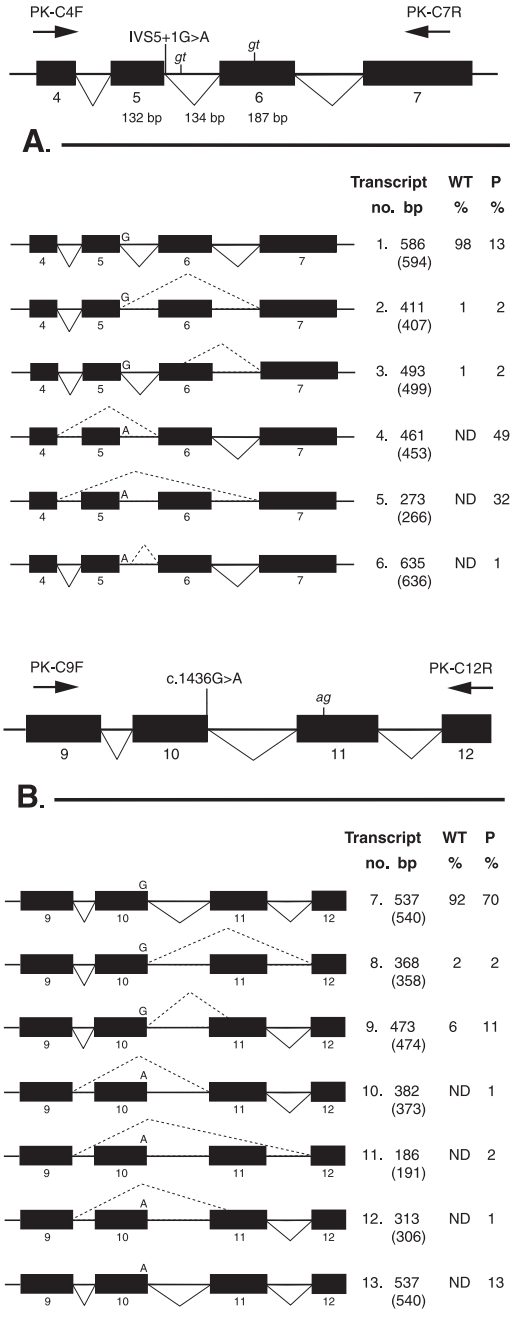
PK-C4F
IVS5+1G>A
gt
gt
PK-C7R
4
5
6
7
132 bp
134 bp
187 bp
A.
Transcript no. bp
WT %
P %
1. 586 (594) 98 13
2. 411 (407) 1 2
3. 493 (499) 1 2
4. 461 (453) ND 49
5. 273 (266) ND 32
6. 635 (636) ND 1
PK-C9F
c.1436G>A
ag
PK-C12R
9
10
11
12
B.
Transcript no. bp
WT %
P %
7. 537 (540) 92 70
8. 368 (358) 2 2
9. 473 (474) 6 11
10. 382 (373) ND 1
11. 186 (191) ND 2
12. 313 (306) ND 1
13. 537 (540) ND 13

Figure 2. Aberrant splicing induced by the IVS5+1G>A and c.1436G>A mutation. Schematic representation (not drawn to scale) of the region of *PKLR* flanking the IVS5+1G>A (panel A) and c.1436G>A (panel B) mutation. Location and direction of primers used for RT-PCR are indicated by arrows. Exons are numbered and depicted as solid black boxes connected by intronic sequences, relevant sizes (in bp) are indicated. The mutations and the location of cryptic splice donor (*gt*) and acceptor sites (*ag*) are depicted above the exons. Normal and aberrant processing of wild-type (WT) and patient (P) pre-mRNA is reflected by solid and dashed lines, respectively. The respective wild-type and mutant nucleotide is indicated for each transcript. Transcripts were characterized by DNA sequence analysis of cloned RT-PCR products and the fragment size (in bp) is indicated for each transcript. Note that software-calculated fragment sizes (in bp) were used throughout 'Results'. For completeness, the actual size is displayed in brackets, directly below the calculated size. Relative frequencies (%) of each transcript were derived from fragment analysis data. ND: Not Detected.

in coding region, flanking intronic sequences or the erythroid-specific promoter. Because both mutations were likely to affect correct *PKLR* pre-mRNA processing, we studied the effects of these mutations using erythroid cells, actively involved in RNA synthesis. For this, we cultured nucleated erythroid cells from the light-density fraction of venous blood of the patient and a control subject. After 10 days of culture, the initial 10^8 light-density cells of the patient rendered a culture consisting of 9.5×10^7 cells. More than 95% of these cells were morphologically identified as (pro-)erythroblasts. Total RNA was isolated from these cells.

The IVS5+1G>A mutation leads to skipping of exon 5 or simultaneous skipping of exon 5 and 6

For comprehensive analysis of aberrant processing induced by the IVS5+1G>A mutation, fluorescent RT-PCR products encompassing *PKLR* cDNA exons 4 to 7 were separated by capillary electrophoresis, cloned and evaluated. DNA sequence analysis of the cloned RT-PCR product of the control confirmed normal processing of wild-type *PKLR* transcripts as reflected by the majority of inserts (Figure 2A, transcript 1).

In agreement with this, one major 586 bp fragment was detected by fragment analysis (Figure 3A). In addition, clonal analysis revealed two rare inserts that reflected low level, alternative processing of wild-type *PKLR* pre-mRNA. One of these transcripts lacked exon 6 (Δ6) whereas the other transcript lacked 95 bp of the 3'-end of exon 6 due to processing at a cryptic donor site at nt 600 in exon 6. (Figure 2A, transcripts 2 and 3). These transcripts were, likely, represented by the 411 and 493 bp fragments, as detected by fragment analysis (Figure 3A).

DNA sequence analysis of cloned RT-PCR product of the patient revealed six distinct inserts. Three of these reflected the same transcripts as detected in the control, *i.e.* normally processed with regard to exon 5 (Figure 2A, transcripts 1 to 3; Figure 3B, 411, 493, and 586 bp fragments). We postulate that these transcripts were not associated with the IVS5+1G>A mutation because donor site mutations abolish normal splicing completely[20] and, moreover,

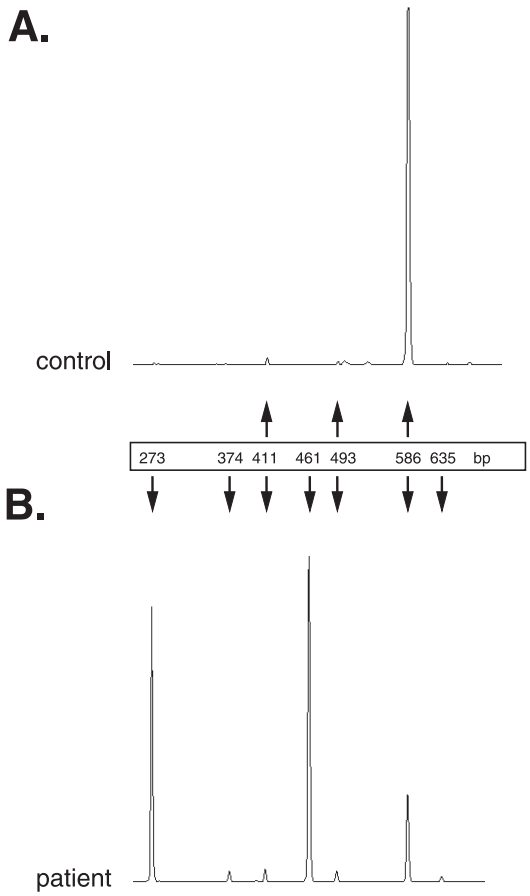

Figure 3. Detection of aberrant transcripts associated with the IVS5+1G>A mutation. Fluorescent-dye labeled RT-PCR products of total RNA from nucleated erythroid cells of a control subject (panel A) and the patient (panel B) were separated by capillary electrophoresis. Primers amplified a region of *PKLR* cDNA encompassing exons 4 to 7 and RT-PCR products are indicated by arrows. Peak heights reflect the fluorescence signal of the individual fragments.

the transcripts were detected in both the patient and the control. Consequently, they were derived from the other allele of the patient. The three other inserts encountered in the cloned RT-PCR product of the patient reflected transcripts aberrantly processed with regard to exon 5. They were not detected in the control (see above) and, thus, likely associated with the IVS5+1G>A mutation. Of these three transcripts, two were abundant. They lacked either exon 5 (Δ5) or both exon 5 and 6 (Δ5,6) (Figure 2A, transcripts 4 and 5), and corresponded to fragments of 461 and 273 bp, as detected by fragment analysis (Figure 3B). The third,

low-abundant, transcript contained the first 51 nt of intron 5 due to the use of a cryptic donor site at nt +52 in intron 5 (Figure 2A, transcript 6) and, likely, corresponded to the 635 bp fragment, as detected by fragment analysis (Figure 3B). No clones were encountered that could match the minor 374 bp fragment, detected by fragment analysis (Figure 3B). When reviewing the different transcripts identified in the patient and control, we speculate that the corresponding transcript lacks exon 5 and the 95 bp from the 3'-end of exon 6.

The c.1436G>A mutation causes skipping of exon 10 and is associated with strongly reduced transcript levels

Using primers spanning exons 9 to 12, we studied the effect of the c.1436G>A mutation on pre-mRNA processing. Fragment analysis (data not shown), and DNA sequence analysis of the cloned RT-PCR product of the control confirmed normal pre-mRNA processing in the majority of transcripts (Figure 2B, transcript 7). In addition, two minor transcripts were detected that lacked either exon 11 (Δ11) or 67 bp of the 5'-end of exon 11 due to alternative processing at a cryptic splice acceptor site at nt 1502 in exon 11 (Figure 2B, transcripts 8 and 9).

The cloned RT-PCR product of the patient revealed four distinct transcripts associated with the c.1436G>A mutation. Three of these were low-abundant transcripts that had the absence of exon 10 in common. They lacked exon 10 itself (Δ10), exon 10 and 11 together (Δ10,11), or exon 10 and 67 bp of the 5'-end of exon 11. (Figure 2B, transcripts 10 to 12). The majority of c.1436G>A associated transcripts was normally processed and, consequently, contained an adenine at nt 1436 (Figure 2B, transcript 13). Three transcripts were not associated with the c.1436G>A mutation and, thus, originated from the IVS5+1A allele. As in the control, the majority of these transcripts was normally processed whereas a minority resulted from alternative processing with regard to exon 11 (Figure 2B, transcripts 8 and 9).

Remarkably, when comparing the composition of clones from the patient, only 12 of the 63 clones represented transcripts derived from the 1436A allele. A similar asymmetrical clonal distribution was observed in the before-mentioned experiments regarding the IVS5+1G>A mutation: of the 56 selected clones, only six clones represented transcripts from the 1436A allele. This prompted us to estimate the relative amounts of transcripts from each allele of the patient. Therefore, we compared *Msc*I digestion fragments of exon 10-containing RT-PCR and DNA-PCR products. The c.1436G>A mutation in this exon introduces a unique recognition sequence for *Msc*I. Agarose gel electrophoresis of digested DNA-PCR product showed that half the amount of this 262 bp PCR product was cut into 212 and 50 bp fragments (Figure 4, the 50 bp fragment is not shown). In contrast, the two RT-PCR product digestion fragments (336 and 204 bp), representing the 1436A allele, were considerably less in amount than the undigested product (540 bp) which represented the 1436G allele (Figure 4).

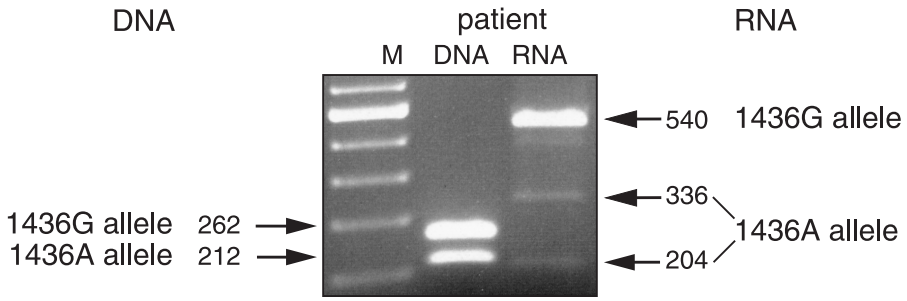

Figure 4. Association of the c.1436G>A mutation with strongly reduced transcript levels. Exon 10 was amplified from total RNA of the patient with primers spanning exons 9 to 12, and digested with *Msc*I. Equal allelic amounts of exon 10 as amplified from the patient's genomic DNA served as a control. One of the two fragments after *Msc*I digestion of genomic PCR product is not shown (50 bp fragment). M: molecular mass marker.

The Δ5,6 nonsense transcript is, in part, associated with polysomes whereas the Δ5 transcript is unstable

To examine whether the identified transcripts were translationally active, cell-extracts were fractionated on sucrose gradients. Absorbance profiles of samples of the control and patient indicated the presence of polysomes in fractions 4 to 10 (Figure 5A and 5B).

RNA obtained from each fraction was amplified by RT-PCR using primers PK-C4F and PK-C7R, and PCR products were analyzed by fragment analysis. As depicted in Figure 5C, the majority of wild-type mRNA was associated with polysomes. Low levels of the two other transcripts were present in all fractions, although we note that the mRNA alternatively spliced at the cryptic donor site in exon 6, appeared to accumulate in the lighter fractions, containing inactive ribosomes.

With regard to the patient, a substantial amount of Δ5,6 transcripts accumulated in the lighter fractions. Only part of this mRNA was polysome-associated (Figure 5D). The other transcripts were present in low amounts. Although there appeared to be a tendency for the normally processed 1436A mRNA to associate with polysomes (Figure 5D), levels were too low for accurate interpretation. The finding that the Δ5 mRNA was also present in low amounts was surprising because, thus far, this transcript had been the most abundant one. A possible explanation can be that the transcript was degraded during sample handling time (approximately one hour), prior to performing polysome analysis. Therefore, we performed a time-stability assay (see 'Methods'). The results confirmed that once the cells were lysed, the Δ5 transcript was rapidly degraded (Figure 6).

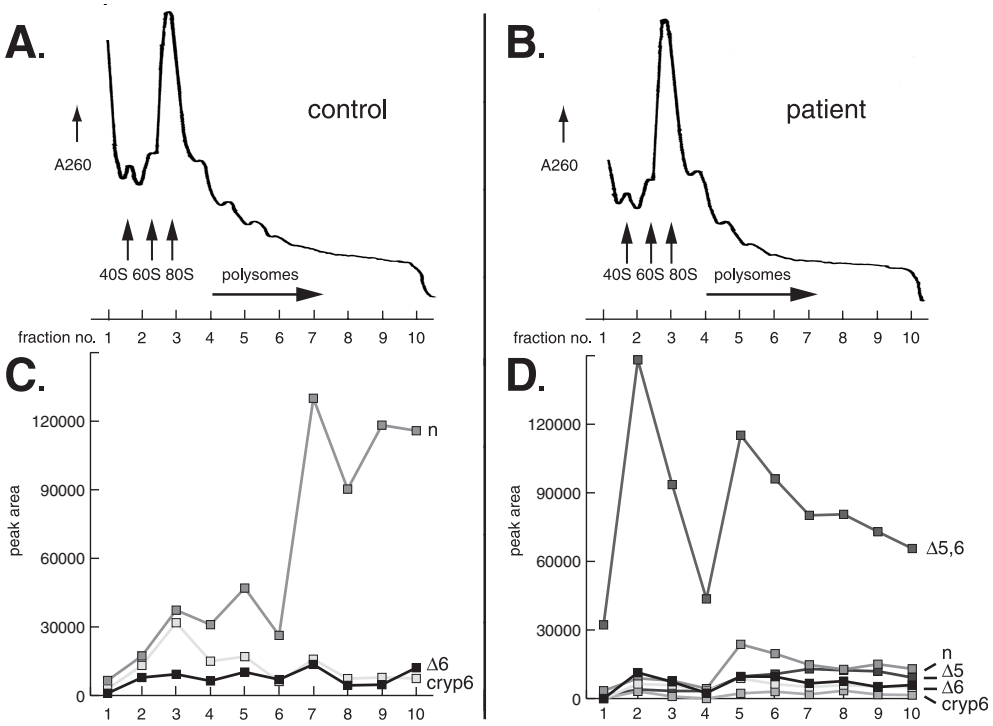

Figure 5. Subcellular localization of *PKLR* transcripts by RT-PCR analysis of polysomal fractions. (A, B) Polysome profile analysis of nucleated erythroid cellular extracts of the control and the patient. Polysomes were in gradient fractions 4 to 10. 40S, 60S, and 80S (monosomes) peaks are indicated by arrows. (C, D) Distribution of mRNAs from the control and the patient as determined by RT-PCR and subsequent fragment analysis. Primers amplified exons 4 to 7 from *PKLR* cDNA. For each transcript, the amount of RT-PCR product is plotted against the fraction numbers. Transcript composition is indicated as follows: n, normally processed; Δ6, deletion of exon 6; cryp6, spliced at the cryptic donor site in exon 6; Δ5,6, deletion of exon 5 and 6; Δ5, deletion of exon 5.

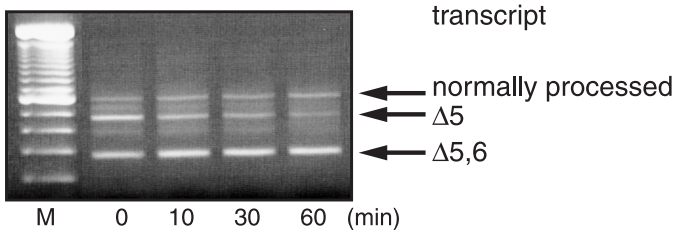

Figure 6. Instability of the exon 5 deleted transcript. Nucleated erythroid cell lysate from the patient was placed on ice and RNA was isolated at the indicated time intervals. RNA stability was analyzed by RT-PCR with primers spanning exons 4 to 7, and agarose gel electrophoresis. M: molecular mass marker.

Low amounts of PK monomers are synthesized by the cultured nucleated erythroid cells of the patient

Normally processed *PKLR* 1436A missense mRNA, albeit present at low levels, might direct active translation of R479H PK monomers. Using a polyclonal anti-PK antibody, a distinct band of the expected 63 kDa[29] was detected by Western Blot in the nucleated erythroid cells of the normal control, corresponding to (erythroid-specific) PK monomers (Figure 7). The smaller, faint band most likely corresponded to the partially proteolysed form of 57–58 kDa.[29,30] The same amount of total protein from nucleated erythroid cells of the patient displayed one faint, but distinct band of 63 kDa (Figure 7), indicative of low amounts of PK monomers.

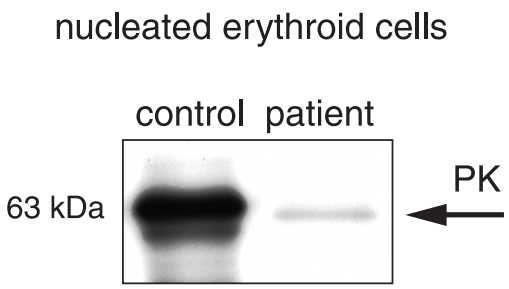

Figure 7. Detection of PK monomers in nucleated erythroid cells. Extracts of nucleated erythroid cells of the control and the patient were adjusted for protein content and separated by SDS-PAGE. Proteins were transferred to blot and PK monomers (63 kDa) were visualized by Western Blot, using a polyclonal anti-PK antibody.

Discussion

Severe pyruvate kinase deficiency in a Dutch patient with haemolytic anaemia was associated with two single nucleotide substitutions in the *PKLR* gene. One novel mutation, IVS5+1G>A, abolished the invariant dinucleotide at the donor splice site of intron 5. On the other allele, the previously reported c.1436G>A (R479H) missense mutation was detected which, additionally, altered the exonic consensus sequence of the intron 10 donor splice site. Both mutations were expected to exert their principal effect at the RNA level by interfering with correct processing of *PKLR* pre-mRNA. To study these effects, we used *ex vivo* produced nucleated erythroid cells from the patient. Both mutations induced aberrant processing of *PKLR* transcripts, and multiple mRNAs were identified. Subcellular localization of transcripts and Western Blot analysis established the relevance of each

transcript with regard to the PK-deficient phenotype of the patient. The transcript lacking exon 5 and 6 in particular was unusual which suggests the presence of a splice enhancer element in *PKLR* exon 5.

The recognition of the exon is, in general, invoked by the pairing between the 3' and 5' splice site across an exon in a process called exon definition.[31] Inactivation of the 5' splice site involves disruption of U1 snRNP binding to the donor site during early spliceosome assembly.[13] Consequently, the predominant effect of 5' splice site mutations is skipping of the upstream exon or the use of cryptic splice sites, when present in the vicinity of the wild-type site.[20,32] Indeed, the two major transcripts derived from the allele with the IVS5+1G>A mutation lacked exon 5: Δ5 and Δ5,6. In addition, we detected small amounts of a transcript with an in-frame insertion of 51 nt, resulting from processing at the cryptic donor site at nt +52 in intron 5. The fact that this cryptic site was used at such low frequency is likely the reflection of its markedly low 5' splice site score[33] of 63.0. The 132-bp deleted Δ5 transcript maintains the open reading frame, encoding a PK monomer (estimated mass 58 kDa) that lacks amino acids 126 to 169. In contrast, the 319 bp deleted Δ5,6 transcript contains a premature stopcodon at nt 710 in exon 7. Thus, if synthesized, this monomer (estimated mass 14 kDa) would be severely truncated, lacking approximately 75% of its peptide chain. Considering the abundance of Δ5 and Δ5,6 transcripts and their putative involvement in protein synthesis, we determined the subcellular localization of the respective mRNAs by polysome analysis. First, we established the transcriptional and translational activity of the cultured nucleated erythroid cells with regard to PK, using blood from a normal control individual. Wild-type *PKLR* mRNA was associated with polysomes, and PK monomers were detected by Western Blot, confirming the translationally active state of the cultured cells. With regard to the Δ5,6 transcript, a substantial amount accumulated in the lighter fractions and only part of the mRNA was polysome-associated. Thus, translation of Δ5,6 mRNA was inefficient. Moreover, it is most likely that such a severely truncated protein, if stable, would be non-functional.

The Δ5 transcript was unstable during polysome analysis. Western Blot analysis failed to identify any PK of the predicted size (Figure 7) which may be directly related to this instability or, alternatively, the mutant protein encoded by the Δ5 mRNA may be unstable. If translated, a nonfunctional protein is to be expected since the deletion of residues 126 to 169 includes a number of amino acids essential for the enzyme's function.[28,34-37]

For two reasons, the detection of the Δ5,6 mRNA was noteworthy. Firstly, this mRNA examplifies another nonsense transcript insensitive to nonsense mediated mRNA decay (NMD).[38-40] This surveillance mechanism rapidly degrades mRNAs with premature translation termination codons, thereby preventing the potentially deleterious effects of

truncated proteins.[41] The relative abundance of Δ5,6 mRNA suggests that this nonsense transcript bypasses NMD, similar to recently reported fibrinogen Aα[42] and β-globin[43] nonsense mRNAs. Secondly, simultaneous skipping of exon 5 and 6 is unusual by itself. Splice site mutations are rarely associated with multiple exon skipping[20,32] and only a few examples are available.[44,45] Our results suggest that the IVS5+1G>A mutation-induced skipping of exon 5 enhanced skipping of exon 6. Additional downstream (exon 7) or upstream (exon 4) skipping events were excluded (data not shown). We hypothesize that, in wild-type *PKLR* pre-mRNA, efficient inclusion of exon 6 depends on the presence in the primary transcript of, yet undefined, splice enhancing elements in exon 5. Likely candidates are exonic splice enhancers (ESEs).[46,47] ESEs are *cis*-acting exonic sequence motifs that promote exon definition by serving as RNA binding sites for specific serine/arginine-rich (SR) proteins.[48] The role of ESEs in constitutive splicing is now appreciated[49,50] and binding of SR proteins to constitutive exons plays an important role in the splicing reaction.[48] ESEs are very common in human genes and might be present in most, if not all, human exons.[51,52] Interference with the function of exonic cis-elements has been shown to be a common mechanism for inappropriate exon skipping.[53] The effects of ESE-disrupting mutations have, to our knowledge, thus far been limited to the ESE-containing exon. Recently, however, ESEs were found to be capable of activating not only weak splice sites within the same exon, but also weak splice sites of flanking adjacent exons, across a 430 bp intron.[54] These *in vitro* results, combined with the small size of *PKLR* intron 5 (134 bp), the weak splice sites flanking exon 6 (3' splice site score intron 5, 82.1; 5' splice site score intron 6, 72.4), and the fact that exon 6 is aberrantly processed in wild-type pre-mRNA (Figure 2A), strengthen our hypothesis regarding the requirement of upstream exonic splice enhancing elements for efficient inclusion of *PKLR* exon 6. Such a regulatory mechanism may also explain why point mutations in an upstream exon can cause skipping of only the downstream exon.[55]

It is noteworthy to realize that the observation of splice site mutations leading to multiple exon skipping can easily been obscured by the frequently applied investigative approaches to study such mutations that involve the use of primers located only in the immediate upstream and downstream exons. Consequently, multiple exon skipping events will go by undetected.

Although not invariant, the guanine at the –1 nt of the 5' splice site is very common in primates (78%).[33] The predominant feature of mutating the –1 nt is skipping of the upstream exon. Normal processing of pre-mRNA may be observed, albeit usually at low levels. Previous studies regarding the c.1436G>A mutation were unable to demonstrate aberrant splicing induced by this mutation.[5,28] Instead, low levels of normally processed transcript were detected.[28] Our results confirmed the association of the c.1436G>A mutation with a

strong reduction in transcript levels. Furthermore, we demonstrate here that this mutation does effect pre-mRNA processing by the characterization of three aberrant transcripts that lacked exon 10. The majority of 1436A transcripts, however, was normally processed which may be a reflection of the modest decrease in 5' splice site score of the mutated donor site (71.5), compared to the wild type donor site (83.9). Of interest was the presence of the Δ10,11 transcript. The equal amounts of transcripts lacking exon 11 detected in control and patient's RNA suggests, however, that simultaneous skipping of exon 10 and 11 in this case more likely reflects 'leaky splicing' (see below) that led to skipping of exon 11 on transcripts that had already skipped exon 10 due to the c.1436G>A mutation.

During this study we identified low-abundant alternative transcripts, not associated with mutation. They likely resulted from inefficient processing or 'leaky splicing' events as has been reported for other genes.[56,57] We report here for the first time alternative processing of wild-type *PKLR* pre-mRNA. Correct processing of exon 6 and 11 appeared to be inefficient and in both cases the exons were either skipped or alternatively processed at exonic cryptic splice sites. Although such low-abundant transcripts are not likely to result in clinical expression, their characterization may render employable knowledge. For example, the identification of a *PKLR* splice site mutation (IVS6–2A>T),[10] prompted the investigators to analyze the patient's cDNA. This revealed no abnormalities other than a 67-bp deletion of the 5' end of exon 11 which, in turn, led the authors to conclude that this aberrant transcript was related to the intron 6 acceptor site mutation.[10] Figure 2B clearly shows that the 67-bp deletion from exon 11 was a result of inefficient processing of this exon in wild-type *PKLR* pre-mRNA. We propose that the actual effect of the IVS6–2A>T mutation has escaped detection and remains to be established.

No functional PK monomers were produced from the patient's IVS5+1A allele. However, we detected low amounts of PK monomers in nucleated erythroid cells of the patient which is in agreement with the low levels of normally processed 1436A missense mRNA. Hence, the patient can be regarded as pseudo-homozygous for R479H PK. Recently, Valentini *et al*. characterized recombinant wild type and R479H mutant PK, expressed in *Escherichia coli*.[58] Structural data concerning this mutant showed that the His479 side chain was fully solvent-exposed and that the crystal structure of R479H PK was identical to that of the wild-type (recombinant) protein.[58] Thus, structural perturbations induced by the R479H substitution are likely to be limited. Furthermore, kinetic parameters of the catalytically active tetrameric mutant enzyme appeared to be essentially unaffected.[58] Altogether, we postulate that the PK-deficient phenotype of the patient is directly related to strongly reduced amounts of R479H PK tetramers. The only discrepancy comes from the patient's heat-sensitivity (see 'Subjects') which suggests decreased stability of the R479H PK tetramer during febrile episodes or even

prolonged exposure to sunlight. This is in agreement with reduced thermal stability of the patient's PK *in vitro* (Figure 1) but contrasts with the nearly unaffected thermal stability of recombinant R479H PK,[58] thereby underscoring the differences in fate and function of mutant enzymes *in vivo* and *in vitro*.[59]

Insight into the basic mechanisms of *PKLR* pre-mRNA processing and the factors that influence splice site selection may eventually result in therapeutic possibilities designed to target the splicing pathway. Such approaches have been shown to be feasible by the successful treatment of nucleated erythroid cells from thalassemic patients using antisense oligonucleotides that restored correct splicing[60,61] and may well be applied to other human diseases like PK deficiency.

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References

1. Hirono A, Kanno H, Miwa S, Beutler E. Pyruvate kinase deficiency and other enzymopathies of the erythrocyte. The Metabolic & Molecular Bases of Inherited Disease. New York: McGraw-Hill; 2001:4637-4664.
2. Zanella A, Bianchi P. Red cell pyruvate kinase deficiency: from genetics to clinical manifestations. Baillieres Best Pract Res Clin Haematol. 2000;13:57-81.
3. Manco L, Ribeiro ML, Máximo V, et al. A new PKLR gene mutation in the R-type promoter region affects the gene transcription causing pyruvate kinase deficiency. Br J Haematol. 2000;110:993-997.
4. van Wijk R, van Solinge WW, Nerlov C, et al. Disruption of a novel regulatory element in the erythroid-specific promoter of the human *PKLR* gene causes severe pyruvate kinase deficiency. Blood. 2003;101:1596-1602.
5. Kanno H, Wei DC, Chan LC, et al. Hereditary hemolytic anemia caused by diverse point mutations of pyruvate kinase gene found in Japan and Hong Kong. Blood. 1994;84:3505-3509.

6. Lenzner C, Nürnberg P, Thiele BJ, et al. Mutations in the pyruvate kinase L gene in patients with hereditary hemolytic anemia. Blood. 1994;83:2817-2822.
7. Kanno H, Fujii H, Wei DC, et al. Frame shift mutation, exon skipping, and a two-codon deletion caused by splice site mutations account for pyruvate kinase deficiency. Blood. 1997;89:4213-4218.
8. Zanella A, Bianchi P, Baronciani L, et al. Molecular characterization of *PK-LR* gene in pyruvate kinase-deficient Italian patients. Blood. 1997;89:3847-3852.
9. Fujii H, Kanno H, Akaba K, Hayasaka K, Miwa S. Exonic point mutation 1552 CGT→AGT is not a missense mutation but causes an aberrant splicing of human L-type pyruvate kinase gene. Blood. 1997;90:5b.
10. Zanella A, Bianchi P, Fermo E, et al. Molecular characterization of the *PK-LR* gene in sixteen pyruvate kinase-deficient patients. Br J Haematol. 2001;113:43-48.
11. Manco L, Bento C, Ribeiro ML, Tamagnini G. Consequences at mRNA level of the PKLR gene splicing mutations IVS10(+1)G→C and IVS8(+2)T→G causing pyruvate kinase deficiency. Br J Haematol. 2002;118:927-928.
12. Krämer A. The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu Rev Biochem. 1996;65:367-409.
13. Burge CB, Tuschl T, Sharp PA. Splicing of precursors to mRNAs by the spliceosomes. In: Gesteland RF, Cech TR, Atkins JF, eds. The RNA World. New York: Cold Spring Harbor Laboratory Press; 1999:525-560.
14. Hastings ML, Krainer AR. Pre-mRNA splicing in the new millennium. Curr Opin Cell Biol. 2001;13:302-309.
15. Yu Y-T, Scharl EC, Smith CM, Steitz JA. The growing world of small nuclear ribonucleoproteins. In: Gesteland RF, Cech TR, Atkins JF, eds. The RNA World. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1999:487-524.
16. Zhou Z, Licklider LJ, Gygi SP, Reed R. Comprehensive proteomic analysis of the human spliceosome. Nature. 2002;419:182-185.
17. Proudfoot NJ, Furger A, Dye MJ. Integrating mRNA processing with transcription. Cell. 2002;108:501-512.
18. Maniatis T, Reed R. An extensive network of coupling among gene expression machines. Nature. 2002;416:499-506.
19. Black DL. Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem. 2003;27:291-336.
20. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair

substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet. 1992;90:41-54.

21. Faustino NA, Cooper TA. Pre-mRNA splicing and human disease. Genes Dev. 2003;17:419-437.
22. Mendell JT, Dietz HC. When the message goes awry: disease-producing mutations that influence mRNA content and performance. Cell. 2001;107:411-414.
23. Benöhr HC, Waller HD. Metabolism in haemolytic states. Clin Haematol. 1975;4:45-62.
24. Beutler E. Red Cell Metabolism: A Manual of Biochemical Methods. Orlando: Grune & Stratton; 1984
25. Miwa S, Boivin P, Blume KG, et al. Recommended methods for the characterization of red cell pyruvate kinase variants. Br J Haematol. 1979;43:275-286.
26. Migliaccio G, Di Pietro R, di Giacomo V, et al. In vitro mass production of human erythroid cells from the blood of normal donors and of thalassemic patients. Blood Cells Mol Dis. 2002;28:169-180.
27. Rijksen G, Veerman AJ, Schipper-Kester GP, Staal GE. Diagnosis of pyruvate kinase deficiency in a transfusion-dependent patient with severe hemolytic anemia. Am J Hematol. 1990;35:187-193.
28. Kugler W, Willaschek C, Holtz C, et al. Eight novel mutations and consequences on mRNA and protein level in pyruvate kinase-deficient patients with nonspherocytic hemolytic anemia. Hum Mutat. 2000;15:261-272.
29. Kahn A, Marie J. Pyruvate kinases from human erythrocytes and liver. Methods Enzymol. 1982;90:131-140.
30. Marie J, Kahn A. Proteolytic processing of human erythrocyte pyruvate kinase: study of normal and deficient enzymes. Biochem Biophys Res Commun. 1979;91:123-129.
31. Berget SM. Exon recognition in vertebrate splicing. J Biol Chem. 1995;270:2411-2414.
32. Nakai K, Sakamoto H. Construction of a novel database containing aberrant splicing mutations of mammalian genes. Gene. 1994;141:171-177.
33. Shapiro MB, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. 1987;15:7155-7174.
34. Cohen-Solal M, Préhu C, Wajcman H, et al. A new sickle cell disease phenotype associating Hb S trait, severe pyruvate kinase deficiency (PK Conakry), and an $\alpha 2$

globin gene variant (Hb Conakry). Br J Haematol. 1998;103:950-956.
35. Baronciani L, Beutler E. Analysis of pyruvate kinase-deficiency mutations that produce nonspherocytic hemolytic anemia. Proc Natl Acad Sci U S A. 1993;90:4324-4327.
36. Demina A, Varughese KI, Barbot J, Forman L, Beutler E. Six previously undescribed pyruvate kinase mutations causing enzyme deficiency. Blood. 1998;92:647-652.
37. Neubauer B, Lakomek M, Winkler H, Parke M, Hofferbert S, Schröter W. Point mutations in the L-type pyruvate kinase gene of two children with hemolytic anemia caused by pyruvate kinase deficiency. Blood. 1991;77:1871-1875.
38. Hilleren P, Parker R. Mechanisms of mRNA surveillance in eukaryotes. Annu Rev Genet. 1999;33:229-260.
39. Maquat LE, Carmichael GG. Quality control of mRNA function. Cell. 2001;104:173-176.
40. Wilusz CJ, Wang W, Peltz SW. Curbing the nonsense: the activation and regulation of mRNA surveillance. Genes Dev. 2001;15:2781-2785.
41. Frischmeyer PA, Dietz HC. Nonsense-mediated mRNA decay in health and disease. Hum Mol Genet. 1999;8:1893-1900.
42. Asselta R, Duga S, Spena S, et al. Congenital afibrinogenemia: mutations leading to premature termination codons in fibrinogen Aα-chain gene are not associated with the decay of the mutant mRNAs. Blood. 2001;98:3685-3692.
43. Romão L, Inácio A, Santos S, et al. Nonsense mutations in the human β-globin gene lead to unexpected levels of cytoplasmic mRNA accumulation. Blood. 2000;96:2895-2901.
44. Steingrimsdottir H, Rowley G, Dorado G, Cole J, Lehmann AR. Mutations which alter splicing in the human hypoxanthine-guanine phosphoribosyltransferase gene. Nucleic Acids Res. 1992;20:1201-1208.
45. Teraoka SN, Telatar M, Becker-Catania S, et al. Splicing defects in the ataxia-telangiectasia gene, *ATM*: underlying mutations and consequences. Am J Hum Genet. 1999;64:1617-1631.
46. Blencowe BJ. Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. Trends Biochem Sci. 2000;25:106-110.
47. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet. 2002;3:285-298.
48. Graveley BR. Sorting out the complexity of SR protein functions. RNA. 2000;6:1197-1211.

49. Schaal TD, Maniatis T. Multiple distinct splicing enhancers in the protein-coding sequences of a constitutively spliced pre-mRNA. Mol Cell Biol. 1999;19:261-273.
50. Mayeda A, Screaton GR, Chandler SD, Fu X-D, Krainer AR. Substrate specificities of SR proteins in constitutive splicing are determined by their RNA recognition motifs and composite pre-mRNA exonic elements. Mol Cell Biol. 1999;19:1853-1863.
51. Fairbrother WG, Yeh R-F, Sharp PA, Burge CB. Predictive identification of exonic splicing enhancers in human genes. Science. 2002;297:1007-1013.
52. Liu H-X, Zhang M, Krainer AR. Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. Genes Dev. 1998;12:1998-2012.
53. Liu H-X, Cartegni L, Zhang MQ, Krainer AR. A mechanism for exon skipping caused by nonsense or missense mutations in *BRCA1* and other genes. Nat Genet. 2001;27:55-58.
54. Lam BJ, Hertel KJ. A general role for splicing enhancers in exon definition. RNA. 2002;8:1233-1241.
55. Lo Ten Foe JR, Kruyt FAE, Zweekhorst MBM, et al. Exon 6 skipping in the Fanconi anemia C gene associated with a nonsense/missense mutation (775C→T) in exon 5: the first example of a nonsense mutation in one exon causing skipping of another downstream. Hum Mutat. 1998;suppl 1:S25-27.
56. Slomski R, Schloesser M, Berg LP, et al. Omission of exon 12 in cystic fibrosis transmembrane conductance regulator (*CFTR*) gene transcripts. Hum Genet. 1992;89:615-619.
57. O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA, Goodship JA. A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nat Genet. 2003;33:497-501.
58. Valentini G, Chiarelli LR, Fortin R, et al. Structure and function of human erythrocyte pyruvate kinase. Molecular basis of nonspherocytic hemolytic anemia. J Biol Chem. 2002;17:23807-23814.
59. van Wijk R, Rijksen G, Huizinga EG, Nieuwenhuis HK, van Solinge WW. HK Utrecht: missense mutation in the active site of human hexokinase associated with hexokinase deficiency and severe nonspherocytic hemolytic anemia. Blood. 2003;101:345-347.
60. Lacerra G, Sierakowska H, Carestia C, et al. Restoration of hemoglobin A synthesis in erythroid cells from peripheral blood of thalassemic patients. Proc Natl Acad Sci U S A. 2000;97:9591-9596.

61. Suwanmanee T, Sierakowska H, Fucharoen S, Kole R. Repair of a splicing defect in erythroid cells from patients with β-thalassemia/HbE disorder. Mol Ther. 2002;6:718-726.

CHAPTER 4

Fourteen novel mutations in *PKLR* associated with pyruvate kinase deficiency

Richard van Wijk[1], Eric G. Huizinga[2], Gert Rijksen[3], Joannes J. M. Marx[4], and Wouter W. van Solinge[1]

[1]Department of Clinical Chemistry, [3]Department of Hematology, and [4]Eijkman-Winkler Institute for Microbiology, Infectious Diseases, and Inflammation, University Medical Center Utrecht, Utrecht, The Netherlands, [2]Department of Crystal & Structural Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands.

Abstract

Erythrocyte pyruvate kinase (PK-R) deficiency is the most frequent cause of hereditary hemolytic anemia due to defective glycolysis. We report the molecular basis underlying PK-R deficiency in 28 patients from 26 unrelated families. Twenty-four different point mutations were detected in *PKLR*, 14 of which are reported here for the first time. Of these novel mutations, 12 encoded an amino acid substitution: Ile90Asn, Gly95Arg, Gly111Arg, Ala154Thr, Gly165Val, Leu272Val, Ile310Asn, Arg337Trp, Gly358Glu, Leu374Pro, Arg385Lys, and Arg569Gln. Two novel mutations are postulated to either affect correct splicing (IVS4–2A>C) or to result in a truncated PK-R monomer (Arg488Stop). The c.331G>A mutation (Gly111Arg, PK Utrecht) was one of the most frequently detected mutant *PKLR* alleles and is restricted to the Dutch population. To better understand the mechanisms by which the novel amino acid substitutions could impair enzymatic function and lead to PK-R deficiency, we evaluated their position and the nature of substitution in the three-dimensional model of the recently elucidated crystal structure of human erythrocyte PK. The predicted arginine side chain at residue 111 in PK Utrecht causes severe sterical hindrance and unfavorable charge interactions. Residues Arg337, Gly358, Arg385, and Arg569 are located at the subunit interfaces, and allosteric interactions are likely to be disrupted upon mutation. In particular, mutations Arg337Trp and Arg385Lys disrupt salt bridges crucial for the T- and R-state transition. Gly95Arg and Gly165Val disrupt the functionally important ATP binding site and B domain. Amino acid changes Leu374Pro, Ile90Asn, Ala154Thr, and Ile310Asn perturb the hydrophobic core. Leu272Val, identified in an asymptomatic individual with normal enzymatic activity, causes minimal structural perturbation and may be a nonfunctional amino acid substitution.

Introduction

Defective glycolysis is an important cause of hereditary non-spherocytic hemolytic anemia and can frequently be attributed to a deficiency of pyruvate kinase (PK). PK is a key regulatory enzyme of glycolysis and catalyzes the irreversible phosphoryl group transfer from phosphoenolpyruvate (PEP) to ADP, yielding pyruvate and ATP. The enzyme is allosterically activated by PEP and fructose-1,6-diphosphate (FDP) and has an absolute requirement for cations, normally Mg^{2+} and K^{+}. Of the four PK isozymes in mammals, red-blood cell PK (PK-R) is exclusively expressed in erythroid cells. Together with the liver-specific isozyme PK-L, PK-R is transcribed from the *PKLR* gene by means of tissue-specific

expression.[1,2] The PK-M1 (expressed in skeletal muscle) and PK-M2 (expressed in fetal and most other adult tissues) isozymes are also produced from one gene (*PKM2*) but from distinct mRNAs, generated by alternative splicing.[3] Because the mature enucleated erythrocyte is totally dependent on glycolysis, a deficiency of PK-R results in a continuous lack of ATP. This shortens the life span of the red blood cell, leading to non-spherocytic hemolytic anemia.[4] PK deficiency is inherited in an autosomal recessive manner with an estimated prevalence of 51 cases (*i.e.* homozygous or compound heterozygous patients) per million in the white population.[5] The clinical symptoms are variable, ranging from pronounced neonatal jaundice requiring multiple transfusions to a fully compensated hemolytic anemia.[6] To date, more than 150 mutations in *PKLR* have been associated with PK deficiency (reference 7, and this study). The vast majority of these mutations are missense mutations that affect residues that are important for the enzyme's stability and function. Parameters of PK are so finely tuned that even moderate molecular alterations may significantly perturb cell metabolism.[8]

Human red blood cell PK (PK-R) is a homotetramer and each subunit can be divided into four domains: the N domain (residues 1–84), A domain (residues 85–159 and 263–431), B domain (residues 160–262), and C domain (residues 432–574) (Figure 1).[8] Domain A is the most highly conserved whereas the B and C domain are more variable.[9] The active site lies in a cleft between the A domain and the flexible B domain. The B domain is capable of rotating away from the A domain generating either the 'open' or 'closed' conformation. The C domain contains the binding site for FDP. The allosteric transition from the inactive T-state to the active R-state results in a catalytically active PK tetramer, consisting of an assembly of domains and subunits in which allosteric and catalytic sites are able to communicate with each other across the relatively long distance that separates the FDP binding site from the catalytic center. Tetrameric interactions at the interfaces between the A domains (A/A' subunit interface) and the C domains (C/C' subunit interface), as well as A/B and A/C interdomain interactions are considered to be key determinants of the allosteric response.[8,10-15] However, a comprehensive analysis of the allosteric transition awaits the direct comparison of crystal structures from the T- and R-state enzymes from the same species.

We established the molecular defect underlying PK deficiency in a group of 28 different patients from 26 unrelated families. Twenty-four different mutations were detected in *PKLR*, associated with PK deficiency. Twenty of these mutations, including 14 novel ones, encoded a single amino acid substitution. The structural perturbation predicted by the novel missense mutations may lead to severe impairment of enzymatic function. The mechanism by which this occurs can be quite diverse, affecting, for instance, allosteric properties, substrate binding, effector binding, or overall protein stability. The recently elucidated crystal

Table 1. Clinical classification of PK deficient patients investigated in this study

Patient M/F	origin	PKLR mutation cDNA nt	effect	age of diagnosis	PK activity V_{max} in IU/gHb	HK activity V_{max} in IU/gHb	splenectomy	regular transfusions	Hb g/dL	reticulo ‰	phenotype
DP87 (F)	Dutch	**283G>A**/401T>A	**G95R**/V134D	birth	3.5	6.7	yes	yes	9.1	193	severe
ME72 (F)	Dutch	**269T>A/331G>A**	**I90N/G111R**	birth	<1.0	6.9	yes	yes	6.4	700	severe
ME74[a] (F)	Dutch	**269T>A/331G>A**	**I90N/G111R**	birth	<1.0	7.6	yes	yes	6.9	498	severe
AC76 (F)	American Black	320T>C/1529G>A	M107T/R510Q	2 years	NA	NA	yes	yes	8.6	270	severe
SS95 (F)	Dutch	**331G>A/929T>A**	**G111R/I310N**	birth	6.4	1.10	no	yes	7.2	33	severe
AB66 (M)	Dutch	**331G>A**/1492C>T	**G111R**/R498C	18 years	1.3	4.67	yes	no	9.6	187	severe
SB02[b] (M)	Dutch	**IVS4–2A>C/494G>T**	**splicing/G165V**	birth	1.5	NA	-	-	NA	NA	severe
SN99 (F)	Danish	401T>A/1529G>A	V134D/R510Q	2 months	4.7	1.48	no	yes	9.3	14	severe
CM99 (F)	Finnish	401T>A/1529G>A	V134D/R510Q	3 months	4.7	2.11	no	yes	7.5	126	severe
CB93 (M)	Dutch	721G>T/1529G>A	E241X/R510Q	birth	<1.0	3.80	no	yes	7.5	70	severe
BF98 (M)	Danish	**1009C>T**/1456C>T	**R337W**/R486W	birth	3.8	7.10	no	yes	8.6	46	severe
US02 (M)	Danish	**1009C>T**/1456C>T	**R337W**/R486W	5 months	6.4	4.24	no	yes	6.7	13	severe
YI90 (M)	Syrian	**1073G>A/1073G>A**	**G358E/G358E**	10 years	1.4	8.24	yes	yes	7.8	NA	severe
JW65 (M)	Dutch	1178A>G/1456C>T	N393S/R486W	32 years	17%[d]	NA	yes	no	9.6	556	severe
DH95 (M)	Dutch	1269G>A/1654G>A	splicing/V552M	6 months	5.1	NA	no	yes	5.6	44	severe
DM96 (M)	Albanian	1373G>A/1373G>A	G458D/G458D	1 year	100%[d]	500%[d]	no	yes	5.9	450	severe
GH92 (F)	Dutch	**1462C>T**/1529G>A	**R488X**/R510Q	birth	1.5	8.10	no	yes	5.8	147	severe
EV70 (F)	Dutch	**1462C>T**/1529G>A	**R488X**/R510Q	3 years	0.8	NA	yes	no	6.1	851	severe
JR63 (M)	Dutch	**331G>A**/1456C>T	**G111R**/R486W	33 years	4.2	2.64	no	no	10.7	43	mild
SH68 (F)	Dutch	**331G>A**/1456C>T	**G111R**/R486W	30 years	5.0	NA	no	no	11.7	31	mild
CC78 (F)	Dutch	**1121T>C/1706G>A**	**L374P/R569Q**	21 years	1.9	1.94	no	no	10.9	71	mild
RC73[c] (M)	Dutch	**1121T>C/1706G>A**	**L374P/R569Q**	26 years	1.4	1.45	no	no	12.2	61	mild
KL70 (M)	Dutch	**1154G>A**/1529G>A	**R385K**/R510Q	7 years	0.9	1.95	no	no	12.2	48	mild
MN03 (F)	Dutch	1269G>A/1456C>T	splicing/R486W	birth	1.3	NA	no	no	8.7	109	mild
MD00 (M)	Dutch	1456C>T/1529G>A	R486W/R510Q	3 months	1.7	2.54	no	no	9.3	42	mild
MB86 (M)	Dutch	1529G>A/1529G>A	R510Q/R510Q	1 year	NA	NA	no	no	9.6	70	mild
IM60 (F)	Chech	**460G>A/N**	**A154T**/N	43 years	4.8	1.31	no	no	10.6	12	asymptomatic
KT93 (M)	Ghanese	**814C>G/N**	**L272V**/N	5 years	8.4	1.51	no	no	12.0	NA	asymptomatic
Reference values	-	-	-	-	6.9–14.5	1.02–1.58	-	-	13.8–17.1 (M) 11.8–15.4 (F)	5-25	-

Patients are categorized according to the severity of the phenotype,[16] mutations reported here for the first time are in bold-face typing. [a] Sister of ME72, [b] this patient died one day after birth due to severe hemolytic anemia, [c] brother of CC78, [d] relative to normal. NA, Not available; N, Normal.

structure of human erythrocyte PK[8] has enabled the evaluation of amino acid substitutions at the molecular level and this in turn may provide insight with respect to a genotype-phenotype correlation. To gain insight into the mode of action of the here reported novel amino acid substitutions, we evaluated the concerned residues and the predicted molecular perturbation in the three-dimensional model of human erythrocyte PK.

Patients, materials and methods

Patients

Twenty-eight patients were investigated. These were either newly identified patients or previously diagnosed patients who were referred to our center for confirmation of the diagnosis and to establish the molecular basis of PK deficiency. Other causes of (hemolytic) anemia were ruled out. Table 1 lists the clinical data of these patients who belong to 26 unrelated families, mostly from Dutch origin. The age at which the diagnosis PK deficiency was made, and PK and hexokinase (HK) enzyme activity measurements at the time of diagnosis are listed. HK activity measurements were included to estimate the contribution of the relatively young cell fraction (displaying much higher enzymatic activities of PK and HK) to the total PK activity as measured. Furthermore, clinical data regarding splenectomy and the need for regular transfusions are indicated for each patient as well as their most recent Hb levels and reticulocyte counts. The patient's phenotypes were classified according to Zanella *et al.* (*i.e.* severe phenotype: pre-splenectomy Hb levels lower than 8 g/dL and/or more than 50 transfusions and/or splenectomized; mild phenotype: Hb levels greater than 9 g/dL, fewer than five transfusions, non-splenectomized).[16]

Appropriate informed consent was obtained from all patients or their parents.

Biochemical and molecular analysis

Hematological parameters were measured on a Cell-Dyn 4000 hematology analyzer (Abbott Diagnostics, Santa Clara, CA). PK and HK enzyme activities were measured according to standard methods.[17]

Genomic DNA was isolated by standard methods. The erythroid-specific promoter and individual exons of *PKLR*, including flanking intronic regions, were amplified by PCR as described previously[18] and sequenced with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 310 Genetic Analyzer (Applied Biosystems). *PKLR* cDNA nucleotide and amino acid numbering starts at the initiator methionine of reference sequence P30613 (GenBank) and mutations are described according

to the mutation nomenclature system.[19] Novel mutations were confirmed by restriction enzyme digestion of independently amplified PCR products, except for the c.283G>A mutation, which was confirmed by independent PCR and DNA sequence analysis. The following enzymes were used ((+) or (–) indicates the presence or absence of a recognition sequence in case of presence of the mutation): c.269T>A, *Sfa*NI (–); c.331G>A, *Msp*I (–); IVS4–2A>C, *Bsr*I (–); c.460G>A, HPY-CH4III (+); c.494G>T, *Bsr*I (–); c.814C>G, *Bsp*MI (–); c.929T>A, *Dpn*II (–); c.1009C>T, *Nla*III (+); c.1073G>A, *Afe*I (+); c.1121T>C, *Msp*I (+); c.1154G>A, *Mnl*I (–); c.1462C>T, *Bsl*I (–); c.1706G>A, *Bsp*MI (+) (all enzymes obtained from New England Biolabs, Beverly, MA). For detection of the c.460G>A mutation a single mismatch was introduced in the reverse primer to create a recognition sequence for HPY-CH4III. Detection of the c.1706G>A mutation by *Bsp*MI digestion requires the *in cis* presence of cytosine at the polymorphic nt 1705.
None of the novel mutations were detected in DNA of a normal control population (n = 100), screened for by the appropriate method.

Structural analysis and molecular modeling
Amino acid substitutions were evaluated using the three-dimensional structure of recombinant human erythrocyte R-state tetrameric PK, cystallized in complex with FDP and phosphoglycolate, a substrate analog (Protein Data Bank Entry 1LIU).[8] Mutated residue Arg111 was modeled using the program O[20] and coordinates from 1LIU. Figures were generated using computer programs Molscript[21] and Raster3D.[22]

Results and Discussion

Based on the clinical picture, 26 of 28 investigated patients were expected to be either compound heterozygous or homozygous for a mutation in *PKLR*. One additional patient with anemia but without hemolysis (IM60) was expected to be heterozygous whereas another asymptomatic individual (KT93) was detected by chance. Table 1 shows that the molecular diagnosis was complete in all patients. In total, 24 different single-base changes were identified in *PKLR* of which 14 mutations have not been reported before. Twenty mutations, including 12 novel ones, encoded an amino acid change whereas four mutations predicted either a truncated peptide (mutations c.721G>T and c.1462C>T) or aberrant *PKLR* mRNA (IVS4–2A>C and c.1269G>A).
The structural effects of the 12 novel amino acid substitutions were evaluated in the three-dimensional model of human erythrocyte PK. The location of the residues concerned is shown in Figure 1. The results from structural analysis are summarized in Table 2.

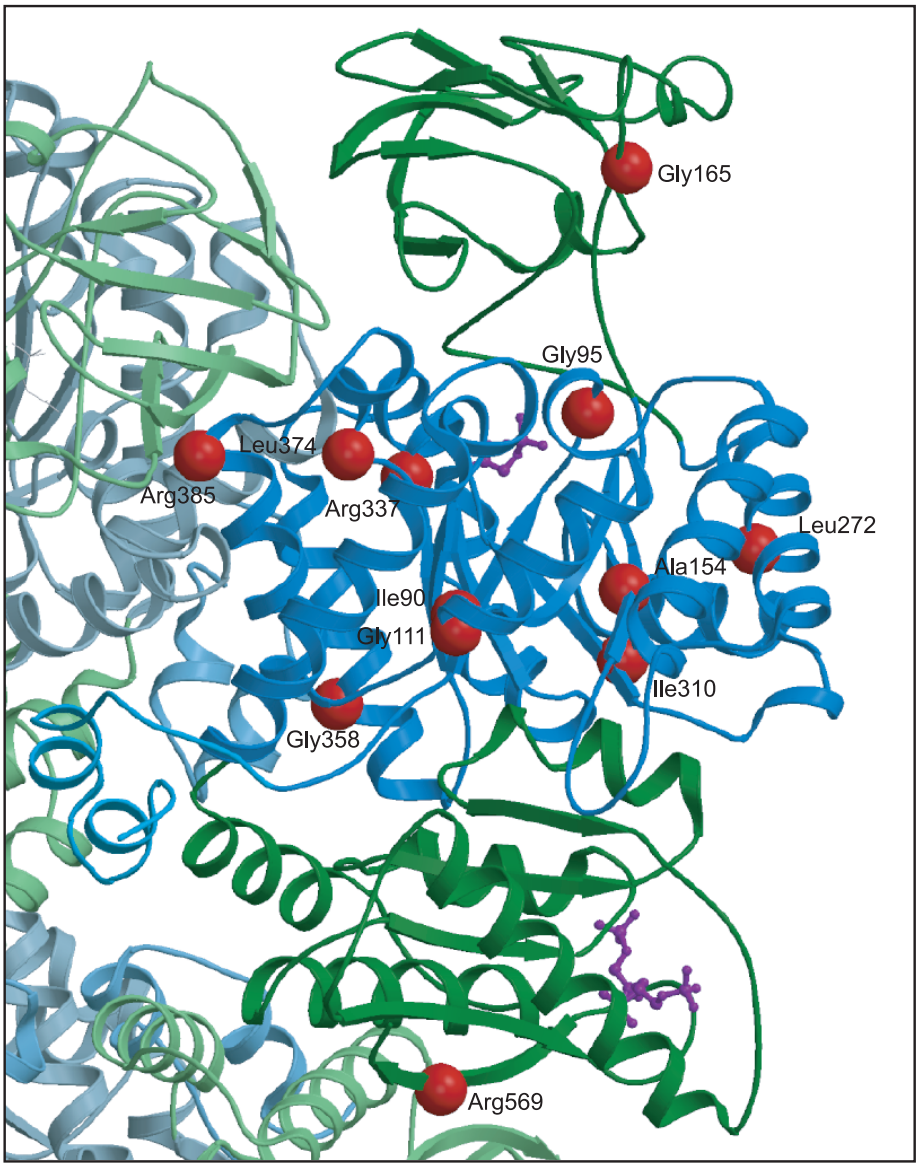

Figure 1. **PK monomer with depicted positions of the substituted amino acids described in this study (color version, addendum page 164).** The figure highlights one PK subunit in the tetrameric structure. The residues affected by the novel missense mutations are shown as red spheres. The A domain is colored blue. The B (top) and C domains (bottom) are colored green. The N-terminal domain is colored light-blue. The substrate analog phosphoglycolate and the allosteric effector fructose-1,6-diphosphate are colored purple and drawn in ball-and-stick mode in their binding pockets in the A and C domains, respectively.

Table 2. Structural implications of novel missense mutations in *PKLR*

***PKLR* allele**	**amino acid change**	**secondary structure**	**role in structure / likely effect of mutation**
c.269T>A	Ile90Asn	Aβ1	BH, SH
c.283G>A	Gly95Arg	Aα1	BH, NA, SH
c.331G>A	Gly111Arg	Aα1	SH, UC
c.460G>A	Ala154Thr	Aβ3	BH, SH
c.494G>T	Gly165Val	Bβ1	SH
c.814C>G	Leu272Val	Aα3	BH
c.929T>A	Ile310Asn	Aβ5	BH
c.1009C>T	Arg337Trp	Aα6a	AS, SH, SI(A), LC
c.1073G>A	Gly358Glu	Aα6	BH, SI(A), SH
c.1121T>C	Leu374Pro	Aα7a	DS, BH, SH
c.1154G>A	Arg385Lys	Aα7	SI(A), HB
c.1706G>A	Arg569Gln	Cβ5	SI(C)

BH: buried hydrophobic, the original residue is buried in the hydrophobic core
SH: sterical hindrance, the new side chain needs more room than is available
LH: leaves a hole, a buried side chain is replaced by a smaller side chain – the resulting hole must be filled up with water or a structural rearrangement must occur
AS: residue is located in the active site, including PEP binding site and metal ion binding sites
NA: residue is located near or at the ATP binding site
SI: subunit interface, residue is located in either the A/A', SI(A) or C/C', SI(C) interface of two subunits – mutation may disturb allosteric interactions
LC: loss of charged contact
UC: unfavorable charge interaction
DS: disrupts secondary structure (*e.g.* introduction of a proline residue in the middle of a strand or helix)

The c.331G>A (Gly111Arg) mutation is a frequent mutation in the Dutch population

The c.331G>A mutation predicts the substitution of a highly conserved glycine by arginine at residue 111 located at the end of helix Aα1. The introduction of an arginine side chain causes severe sterical hindrance and unfavorable charge interactions with side chains of Arg86, Ile90, Val418, and His422, thereby destabilizing helix Aα1 and its neighborhood (Figure 2).

The c.331G>A mutation was detected in five of the 27 unrelated families (Table 1). Because all five families were of Dutch origin, the Gly111Arg PK-R variant represents an important

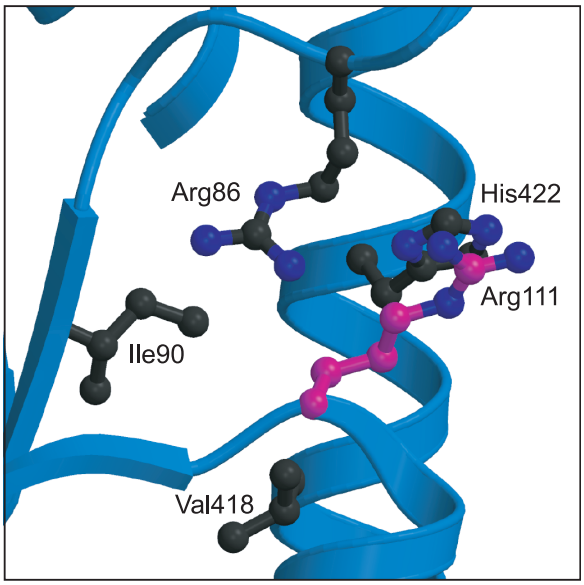

Figure 2. Modeling of the Gly111Arg substitution (color version, addendum page 165). Ribbon drawing of the neighborhood of the Gly111Arg substitution with selected residues shown in ball-and-stick. The modeled conformation of Arg111 is depicted with magenta carbon atoms. Arginine side chains can adopt several orientations, but only one is shown. In all possible conformations of Arg111, sterical clashes or unfavorable charge interactions would occur between its side chain and one or more of the neighboring residues depicted.

cause of PK deficiency in The Netherlands. In fact, together with the common c.1529G>A (12 out of 50 mutant *PKLR* alleles) and c.1456C>T (7 out of 50 alleles) mutations, the c.331G>A allele (5 out of 50 alleles) underlies almost 50% of all cases of PK deficiency reported in this study (Table 1). We designated this variant PK Utrecht. Haplotype analysis using the recently reported erythroid-specific promoter polymorphism -248delT,[18] and the known polymorphisms in intron 5 (IVS5+51C/T), intron 11 (variable number of ATT-repeats), and exon 12 (c.1705A/C, c.1738C/T, and +267T/C), showed an identical haplotype in all families: T-T-16-C-T-C. This strongly suggests a single origin of PK Utrecht in the Dutch population.

All patients in which the c.331G>A mutation was encountered were compound heterozygous, and four different mutations were detected *in trans* to the 331A allele: c.269T>A (Ile90Asn) in the related patients ME72 and ME74, c.929T>A (Ile310Asn) in patient SS95, c.1492C>T (Arg498Cys) in patient AB66, and c.1456C>T (Arg486Trp) in the unrelated patients JR63 and SH68 (Table 1). The absence of homozygous patients limits the evaluation of the Gly111Arg substitution with regard to its deleterious effect and the PK-deficient phenotype. A comparison of the phenotypes of the six compound heterozygous patients shows that four patients are severely affected whereas two patients display a mild phenotype (Table 1). These latter two, unrelated, patients have co-inherited the c.1456C>T mutation which likely accounts for their mild phenotype. The c.1456C>T mutation encodes the substitution of Arg486 with tryptophan.[23] Despite this drastic amino acid replacement at

the A/C domain interface, the introduction of the tryptophan side chain causes little structural perturbation and limited changes in biochemical parameters.[8] Accordingly, its inheritance is usually associated with mild clinical symptoms[24] and even homozygous patients exhibit, in general, a mild anemia.[25]

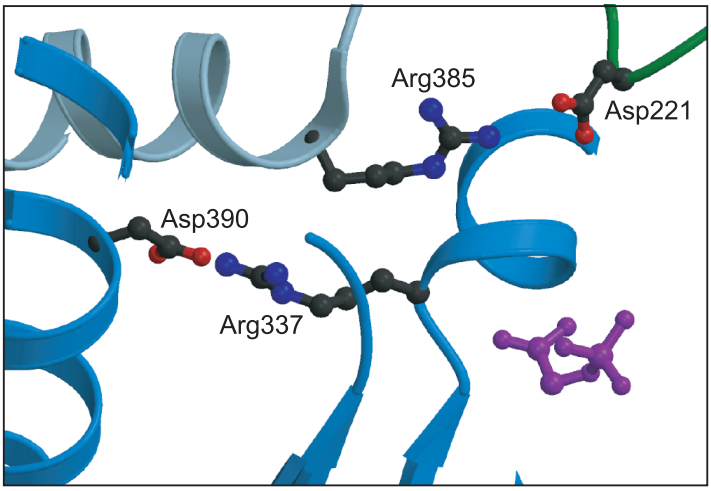

Figure 3. **Structural role of Arg337 and Arg385 (color version, addendum page 165).** Arg337 is located close to the substrate-binding site in the A domain (blue) and forms a salt bridge with Asp390 of the same subunit. Arg385, located in the A domain of an opposing subunit (gray), forms a salt bridge with Asp221 of the B domain (green). In the crystal structure, the substrate binding site is occupied by the substrate analog phosphoglycolate drawn in purple ball-and-stick representation. Mutation Arg337Trp would cause severe sterical hindrance and abolish charge interactions. The more conservative mutation of Arg385Lys would not cause sterical hindrance and would retain in part the potential for hydrogen bonding and charge interactions. Due to their position close in domain and subunit interfaces close to the active site these mutations will likely affect catalysis and allosteric regulation.

Amino acid substitutions in the A/A' and C/C' subunit interfaces: Arg337Trp, Gly358Glu, Arg385Lys, and Arg569Gln

Arg337 and Arg385 are highly conserved residues located in the A/A' subunit interface close to the active site. In fact, Arg337 is one of three consecutive amino acids (^{337}RGD^{339}) that are absolutely conserved among 50 PK sequences.[9] A comparison between the T-state structures of *Leishmania mexicana*[12] and *Escherichia coli*,[10] and the R-state structures of PK-M1,[26,27] yeast,[11] and human PK-R,[8] shows that Arg337 and Arg385 are involved in salt bridge formation to the same residue in the R- and T-state, respectively. In the T-state structures, Arg385 (human PK-R numbering) forms a salt bridge with Asp390 of the opposing monomer. In the R-state structures, this inter-subunit salt bridge is lost and Asp390

now forms a salt bridge with Arg337 of the same subunit, while Arg385 now establishes an inter-subunit salt bridge near the active site with Asp221 of the B domain of the opposing monomer (Figure 3).

The Arg385–Asp221 salt bridge anchors the B domain in the 'closed conformation', competent for PEP binding.[10] In agreement with this, Wooll *et al.* have shown that the loss of this salt bridge, brought about by a single amino acid substitution (Ser402Pro, *i.e.* Ser446 in PK-R) at the A/A' subunit interface of the (R-like) PK-M1 isozyme, is accompanied by increased rotational flexibility of the B domain.[14] Furthermore, substitution of the residues equivalent to Arg385 and Asp390 in *Escherichia coli* (*i.e.* Arg292 and Asp297) by aspartate and arginine, respectively, rendered the enzyme totally inactive.[13] Therefore, mutations Arg337Trp and Arg385Lys appear to disturb interactions crucial to the T- to R-state allosteric transition and, in the case of Arg385Lys, may affect the closure of the active site by the B domain.

Patients BF98 and US02 were both compound heterozygous for the c.1009C>T (Arg337Trp) and c.1456C>T (Arg486Trp) mutation (Table 1). Despite the co-inheritance of the mild 1456T allele (see above), they were both severely affected. Thus, the severe clinical phenotype of patients BF98 and US02 may result from the detrimental effect of the Arg337Trp substitution. Interestingly in this respect are the clinical features observed in a heterozygous PK-deficient patient. In this patient heterozygosity for the c.1010G>A missense mutation, which predicts the substitution of Arg337 by glutamine, was associated with the existence of hemolysis.[28] Similarly, DNA analysis of another, moderately affected, patient displayed no other abnormality than heterozygosity for the c.1010G>C missense mutation which encodes the substitution of Arg337 by proline.[24] This suggests that *PKLR* alleles encoding a substitution of residue 337 display a dominant mode of inheritance, as has been suggested for certain other PK variants.[29,30]

Considering the important structural role for Arg385 in both the T- and R-state structures of PK it was surprising that patient KL70, compound heterozygous for c.1154G>A (Arg385Lys) and c.1529G>A (Arg510Gln) was only mildly affected (Table 1). In contrast, another missense mutation affecting Arg385 (Arg385Trp) caused severe hemolysis in a 1-year-old transfusion-dependent boy in spite of the *in trans* presence of the mild 1456T allele.[5] This apparent discrepancy may be explained by the fact that substitution of arginine by lysine, which are both positively charged, is a more conservative change than substitution of Arg385 with tryptophan and does not destroy the potential to form salt bridges.

Gly358 is completely buried in the A/A' subunit interface. Introduction of a negatively charged glutamate at position 358 perturbs this interface and causes severe PK deficiency as illustrated by patient YI90 (Table 1). This patient was designated as homozygous for the

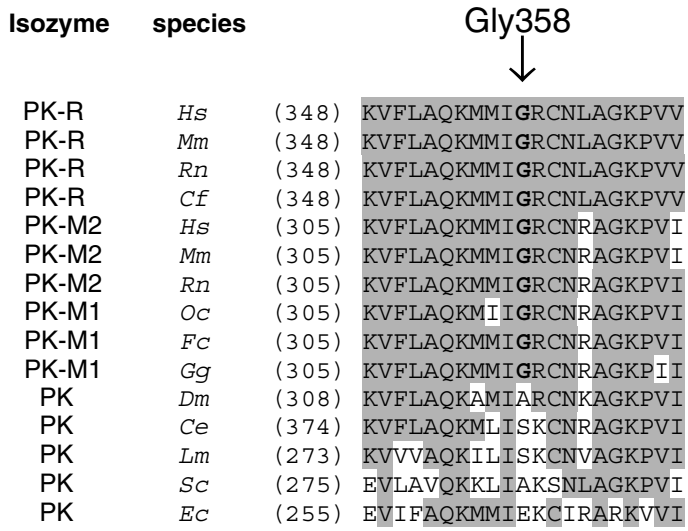

Figure 4. **Amino acid conservation of Gly358.** Neighbouring amino acids of PK-R residue Gly358 of different PKs among selected species shows that the conservation of Gly358 is limited to mammals. Species are: *Hs* (*Homo sapiens*, human); *Mm* (*Mus musculus*, house mouse); *Rn* (*Rattus norvegicus*, Norway rat); *Cf* (*Canis familiaris*, dog); *Oc* (*Oryctolagus cuniculus*, rabbit); *Fc* (*Felis catus*, cat); *Gg* (*Gallus gallus*, chicken); *Dm* (*Drosophila melanogaster*, fruit fly); *Ce* (*Caenorhabditis elegans*, worm); *Lm* (*Leishmania mexicana*, protozoan); Sc (*Saccharomyces cerevisiae*, yeast); Ec (*Escherichia coli*, bacterium).

causative c.1073G>A missense mutation that encodes Gly358Glu. He is a 10-year-old boy from Syrian ancestry who suffered from hemolytic anemia since birth. He presented with hyperbilirubinemia shortly after birth for which he received an exchange transfusion. Thereafter, he required a blood transfusion every two to three weeks because of persistent hemolysis. He underwent splenectomy at age four years after which transfusion dependency decreased to once every four to six months. To date, he is a pale and icteric boy who is otherwise doing well. Because his parents were not available for study, we cannot rule out the possibility that the patient is in fact compound heterozygous for c.1073G>A and a deletion spanning (part of) exon 8. Interestingly, Gly358 is the least conserved residue of all amino acids described in this study (Figure 4 and reference 9) and the residue at the equivalent position 265 in *Escherichia coli* is glutamate, like it is in our patient. This illustrates that disease-causing mutations in humans may occur in other species (*e.g.* the mouse[31]) without leading to disease[32] because of the presence of other compensating sequence variations.[33]

Arg569 is a solvent exposed residue, located five residues from the C terminal end of PK-R

in the C/C' subunit interface. It is the most C-terminal mutation reported to date (reference 7, and this study). The arginine side chain is directed away from the interface and does not make specific hydrogen bonds or charge interactions. At first sight introduction of a glutamine side chain at this position would not seem to have any drastic effect. However, because Arg569 residues from different subunits are located close together across the subunit interface, substitution of Arg569 with glutamine involves the loss of two positive charges in the C/C' interface. This additive effect on the local electrostatic potential might be the basis for adverse effects of this mutation.

The two related patients (CC78 and RC73) carrying the Arg569Gln PK-R variant both showed a very mild phenotype (Table 1). The *in trans* inherited allele in both patients was c.1121T>C which predicts the substitution of Leu374 by proline (see below). Patient CC78 was first diagnosed with PK deficiency at 21 years-of-age because of a life-long history of mild anemia. Subsequent testing of her brother (RC73) revealed the same *PKLR* genotype. Both patients have never received any blood transfusion nor suffered from hemolytic crises.

Amino acid substitutions in the active site and B domain: Gly95Arg and Gly165Val

Gly95 is a highly conserved residue in the ATP binding site and the introduction of valine is likely to affect catalysis. On the DNA level, the c.283G>A mutation that encodes the Gly95Arg substitution alters the last nucleotide of exon 3. Thereby, it disrupts the exonic consensus sequence that constitutes the 5' splice site of intron 3 and may affect correct processing of *PKLR* pre-mRNA.[34] A dual effect of this mutation is thus expected, similar to a previously described case of severe PK deficiency.[35]

Amino acid substitutions in the B domain are relatively rare (reference 7, and this study). The only B domain alteration identified in this study concerns the substitution of glycine by valine at residue 165. The introduction of the valine side chain causes severe sterical hindrance, in particular with Trp201. Gly165 resides in a critical region of PK, illustrated by the importance of active site residue Arg163[11,12,26,27,36] which links domains A and B, and is involved in 'closure' of the B domain upon substrate binding. Substitution of Arg163 by Cys (PK Linz) in a homozygous patient was associated with severe, transfusion-dependent hemolytic anemia.[37]

Mutations c.283G>A (Gly95Arg) and c.494G>T (Gly165Val) were identified in two severely affected patients (Table 1). The clinical picture of patient DP87 has been previously reported.[38] She is one of the most severely affected patients described in this study. To date, at 16 years-of-age, this pale girl displays retarded growth and requires blood transfusions every four to six weeks. The patient with the predicted Gly165Val PK-R variant (SB02) died one day after birth due to severe hemolytic anemia (Table 1). The *in-trans PKLR* allele of

this patient carried the IVS4–2A>C splice site mutation which, most likely, abolishes normal splicing completely (see below). We postulate that because of the lethal combination of these two mutations, the Gly165Val substitution leads to severely impaired enzyme function.

Amino acid substitutions in the hydrophobic core: Ile90Asn, Ala154Thr, Ile310Asn, and Leu374Pro

Residues Ile90, Ala154, Ile310, and Leu374 are completely buried inside the densely packed hydrophobic core. Consequently, the introduction of proline instead of leucine in helix Aα7a, and polar residues at positions 90 and 310 (asparagine) and position 154 (threonine), perturb the hydrophobic interior of the protein, forcing the surrounding side chains to rearrange (Table 2).

Although the Ala154Thr substitution represents the least dramatic amino acid replacement, Ala154 is a highly conserved residue and close to active site residues Arg116 (substrate binding[8] and ATP/ADP binding[12,26,27]) and Asp156 (K^+ binding[11,12,14,27]). The heterozygous individual in which this mutation was identified (IM60) showed PK activity in the heterozygous range (Table 1), thereby confirming the association of this mutation with PK deficiency.

Leu272Val causes little structural perturbation and may represent a nonfunctional substitution

The substitution of leucine at residue 272 by valine is a conservative amino acid replacement of which the causative mutation (c.814C>G) was identified by chance in an asymptomatic individual who was heterozygous for this mutation. This individual (KT93) displayed normal PK activity in the absence of reticulocytosis (Table 1). It is of course possible that altered enzymatic properties of the Leu272Val PK-R hetero- or homotetramer obscure the interpretation of *in vitro* enzymatic activity of PK, as measured under optimized experimental conditions. It is also conceivable, however, that the subtle alterations induced by valine at residue 272 are without functional consequences. The c.814C>G mutation was not detected in the normal control population but these results hold limited value because the control individuals are of Caucasian origin whereas KT93 originates from Ghana. Interestingly in this respect is the fact that PK deficiency is very rare in Africa. Hence, screening of a representative African control population may resolve the question whether the African *PKLR* c.814C>G allele is either very rare, or a polymorphic variant that has remained undetected because of only subtle effects on enzymatic activity. More conclusive evidence regarding the effects on PK enzymatic function awaits the biochemical characterization of the Leu272Val PK-R homotetramer.

Other mutations in PKLR identified in this study

Two other novel single-base substitutions identified in this study were associated with PK deficiency. These constituted a nonsense mutation (c.1462C>T) and a splice-site mutation in intron 4 (IVS4–2A>C). The novel c.1462C>T mutation predicts a truncated PK monomer, which, if stable, would lack 86 amino acids from its C-terminal end, thereby disrupting the FDP binding site. It is highly likely that such a drastic alteration causes severe impairment of enzymatic function. The IVS4–2A>C mutation abolishes the invariant AG dinucleotide[39] of the IVS4 splice acceptor site and, most likely, abolishes normal processing of *PKLR* pre-mRNA completely.

The twelve novel missense mutations reported here concerned residues that are fully conserved among mammals. In addition, most residues are also highly conserved among PK from 50 different species[9] and, apart from Gly165Val and Arg569Gln, are confined to the highly conserved A domain (Figure 1). The three-dimensional model of human erythrocyte PK enabled a close evaluation of these newly identified missense mutations. The predicted induced molecular perturbation was shown to affect structurally and functionally important residues in PK in such a way that its enzymatic function is likely to be severely reduced. In all cases a causal relationship could be substantiated between the amino acid substitution and PK deficiency. The clinical pictures associated with PK deficiency in this study ranged from very mild (*e.g.* patient RC73) to very severe (*e.g.* DP87), and even lethal (SB02). Most patients are compound heterozygous. Hence, if stable, five different PK-R tetramers may be assembled from two different PK monomers. This limits the genotype-phenotype correlation to homozygous patients. However, even homozygous PK-deficient patients may display considerable variation in their clinical picture as has been demonstrated for c.1529G>A homozygotes.[40] In spite of these difficulties it remains of great value to study PK-deficient patients. Ultimately, a more general picture may emerge regarding the effects of specific mutations. A good example with regard to the latter is the acceptance of the 1456T allele as a ‘mild’ PK-deficient allele. Also in this study this common *PKLR* allele was more frequently associated with a mild phenotype, allelic frequency 0.50 (n = 16), than with a severe phenotype, allelic frequency 0.06 (n = 32) (Table 1).

In order to be able to establish a genotype-phenotype correlation in PK deficiency, which may be of importance in counseling PK-deficient patients, a first prerequisite is the identification of the molecular mechanism by which amino acid substitutions lead to impaired enzymatic activity. Although the sophisticated mode of action of PK is finely tuned we have reason to believe that nonfunctional amino acid substitutions, like Leu272Val, may be tolerable in PK. The structural information as employed in this study has provided insight regarding the nature of

mutation and its associated effects on protein structure and, hence, function. Thereby, it contributes to a better understanding of disease-causing amino acid changes in PK and, *vice versa*, provides knowledge about the enzyme's structure and function.

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References

1. Noguchi T, Yamada K, Inoue H, Matsuda T, Tanaka T. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. J Biol Chem. 1987;262:14366-14371.
2. Tani K, Fujii H, Nagata S, Miwa S. Human liver type pyruvate kinase: complete amino acid sequence and the expression in mammalian cells. Proc Natl Acad Sci U S A. 1988;85:1792-1795.
3. Noguchi T, Inoue H, Tanaka T. The M_1- and M_2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. J Biol Chem. 1986;261:13807-13812.
4. Valentine WN, Paglia DE. The primary cause of hemolysis in enzymopathies of anaerobic glycolysis: a viewpoint. Blood Cells. 1980;6:819-829.
5. Beutler E, Gelbart T. Estimating the prevalence of pyruvate kinase deficiency from the gene frequency in the general white population. Blood. 2000;95:3585-3588.
6. Hirono A, Kanno H, Miwa S, Beutler E. Pyruvate kinase deficiency and other enzymopathies of the erythrocyte. The Metabolic & Molecular Bases of Inherited Disease. New York: McGraw-Hill; 2001:4637-4664.

7. Bianchi P, Zanella A. Hematologically important mutations: red cell pyruvate kinase (third update). Blood Cells Mol Dis. 2000;26:47-53.
8. Valentini G, Chiarelli LR, Fortin R, et al. Structure and function of human erythrocyte pyruvate kinase. Molecular basis of nonspherocytic hemolytic anemia. J Biol Chem. 2002;17:23807-23814.
9. Enriqueta Muñoz M, Ponce E. Pyruvate kinase: current status of regulatory and functional properties. Comp Biochem Physiol B Biochem Mol Biol. 2003;135:197-218.
10. Mattevi A, Valentini G, Rizzi M, Speranza ML, Bolognesi M, Coda A. Crystal structure of *Escherichia coli* pyruvate kinase type I: molecular basis of the allosteric transition. Structure. 1995;3:729-741.
11. Jurica MS, Mesecar A, Heath PJ, Shi W, Nowak T, Stoddard BL. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. Structure. 1998;6:195-210.
12. Rigden DJ, Phillips SE, Michels PA, Fothergill-Gilmore LA. The structure of pyruvate kinase from *Leishmania mexicana* reveals details of the allosteric transition and unusual effector specificity. J Mol Biol. 1999;291:615-635.
13. Valentini G, Chiarelli L, Fortin R, Speranza ML, Galizzi A, Mattevi A. The allosteric regulation of pyruvate kinase. J Biol Chem. 2000;275:18145-18152.
14. Wooll JO, Friesen RHE, White MA, et al. Structural and functional linkages between subunit interfaces in mammalian pyruvate kinase. J Mol Biol. 2001;312:525-540.
15. Fenton AW, Blair JB. Kinetic and allosteric consequences of mutations in the subunit and domain interfaces and the allosteric site of yeast pyruvate kinase. Arch Biochem Biophys. 2002;397:28-39.
16. Zanella A, Bianchi P, Fermo E, et al. Molecular characterization of the *PK-LR* gene in sixteen pyruvate kinase-deficient patients. Br J Haematol. 2001;113:43-48.
17. Beutler E. Red Cell Metabolism: A Manual of Biochemical Methods. Orlando: Grune & Stratton; 1984.
18. van Wijk R, van Solinge WW, Nerlov C, et al. Disruption of a novel regulatory element in the erythroid-specific promoter of the human *PKLR* gene causes severe pyruvate kinase deficiency. Blood. 2003;101:1596-1602.
19. den Dunnen JT, Antonarakis SE. Nomenclature for the description of human sequence variations. Hum Genet. 2001;109:121-124.
20. Jones TA, Kjeldgaard M. Electron-density map interpretation. Methods Enzymol. 1997;277:173-208.
21. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Cryst. 1991;24:946-950.

22. Merritt EA, Bacon DJ. Raster3D: photorealistic molecular graphics. Methods Enzymol. 1997;277:505-524.
23. Baronciani L, Beutler E. Analysis of pyruvate kinase-deficiency mutations that produce nonspherocytic hemolytic anemia. Proc Natl Acad Sci U S A. 1993;90:4324-4327
24. Pastore L, Della Morte R, Frisso G, et al. Novel mutations and structural implications in R-type pyruvate kinase-deficient patients from Southern Italy. Hum Mutat. 1998;11:127-134.
25. Zanella A, Bianchi P, Baronciani L, et al. Molecular characterization of *PK-LR* gene in pyruvate kinase-deficient Italian patients. Blood. 1997;89:3847-3852.
26. Muirhead H, Clayden DA, Barford D, et al. The structure of cat muscle pyruvate kinase. EMBO J. 1986;5:475-481.
27. Larsen TM, Laughlin LT, Holden HM, Rayment I, Reed GH. Structure of rabbit muscle pyruvate kinase complexed with Mn^{2+}, K^{+}, and pyruvate. Biochemistry. 1994;33:6301-6309.
28. Zarza R, Alvarez R, Pujades A, et al. Molecular characterization of the PK-LR gene in pyruvate kinase deficient Spanish patients. Red Cell Pathology Group of the Spanish Society of Haematology (AEHH). Br J Haematol. 1998;103:377-382.
29. Kahn A, Marie J, Galand C, Boivin P. Chronic haemolytic anaemia in two patients heterozygous for erythrocyte pyruvate kinase deficiency. Electrofocusing and immunological studies of erythrocyte and liver pyruvate kinase. Scand J Haematol. 1976;16:250-257.
30. Etiemble J, Picat C, Dhermy D, Buc HA, Morin M, Boivin P. Erythrocytic pyruvate kinase deficiency and hemolytic anemia inherited as a dominant trait. Am J Hematol. 1984;17:251-260.
31. Waterston RH, Lindblad-Toh K, Birney E, et al. Initial sequencing and comparative analysis of the mouse genome. Nature. 2002;420:520-562.
32. Ferrer-Costa C, Orozco M, de la Cruz X. Characterization of disease-associated single amino acid polymorphisms in terms of sequence and structure properties. J Mol Biol. 2002;315:771-786.
33. Steward RE, MacArthur MW, Laskowski RA, Thornton JM. Molecular basis of inherited diseases: a structural perspective. Trends Genet. 2003;102:505-513.
34. Shapiro MB, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. 1987;15:7155-7174.
35. van Wijk R, van Wesel ACW, Thomas AAM, Rijksen G, van Solinge WW. *Ex vivo*

analysis of aberrant splicing induced by two donor site mutations in *PKLR* of a patient with severe pyruvate kinase deficiency. Br J Haematol, *accepted for publication*.

36. Cheng X, Friesen RH, Lee JC. Effects of conserved residues on the regulation of rabbit muscle pyruvate kinase. J Biol Chem. 1996;271:6313-6321.
37. Neubauer B, Lakomek M, Winkler H, Parke M, Hofferbert S, Schröter W. Point mutations in the L-type pyruvate kinase gene of two children with hemolytic anemia caused by pyruvate kinase deficiency. Blood. 1991;77:1871-1875
38. Rijksen G, Veerman AJ, Schipper-Kester GP, Staal GE. Diagnosis of pyruvate kinase deficiency in a transfusion-dependent patient with severe hemolytic anemia. Am J Hematol. 1990;35:187-193.
39. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet. 1992;90:41-54.
40. Lenzner C, Nurnberg P, Jacobasch G, Gerth C, Thiele BJ. Molecular analysis of 29 pyruvate kinase-deficient patients from central Europe with hereditary hemolytic anemia. Blood. 1997;89:1793-1799.

CHAPTER 5

Molecular modelling of human red blood cell pyruvate kinase: structural implications of a novel G_{1091} to A mutation causing severe nonspherocytic hemolytic anemia

Wouter W. van Solinge[1]*, Rob J. Kraaijenhagen[1], Gert Rijksen[2], Richard van Wijk[1]*, Bjarne B. Stoffer[3], Michael Gajhede[3], and Finn C. Nielsen[4]

[1]Clinical Laboratory, Eemland Hospital, Amersfoort, The Netherlands; [2]Department of Hematology, University Hospital, Utrecht, The Netherlands; [3]Institute of Chemistry, University of Copenhagen, Copenhagen, Denmark; [4]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark. *present address: Department of Clinical Chemistry, University Medical Center Utrecht, Utrecht, The Netherlands.

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Abstract

We present a novel G_{1091} to A mutation in the human liver and red blood cell (RBC) pyruvate kinase (PK) gene causing severe hemolytic anemia. In two families, three children were severely PK-deficient compound heterozygotes exhibiting the G_{1091} to A mutation and a common G_{1529} to A mutation on the other allele. In one family, the mother, a G_{1091} to A heterozygote, later had a second baby with a new husband, also a G_{1091} to A carrier. The baby was homozygous for the G_{1091} to A mutation and died 6 weeks after birth from severe hemolysis. Both mutant alleles were expressed at the RNA level. The G_{1091} to A mutation results in the substitution of a conserved glycine by an aspartate in domain A of RBC PK, whereas the G_{1529} to A mutation leads to the substitution of a conserved arginine residue with glutamine in the C-domain. Molecular modelling of human RBC PK, based on the crystal structure of cat muscle PK, shows that both mutations are located outside the catalytic site at the interface of domains A and C. The mutations are likely to disrupt the critical conformation of the interface by introducing alternative salt bridges. In this way the Gly364 to Asp and Arg510 to Gln substitutions may cause PK deficiency by influencing the allosteric properties of the enzyme.

Introduction

Pyruvate kinase (PK) catalyses the conversion of phosphoenol pyruvate to pyruvate and is an important regulator of glycolysis. Two different genes have been characterized in mammals. One encodes the liver and red blood cell (RBC) isoenzymes (*PKLR* gene), whereas the other generates the muscle isoenzymes M_1 and M_2.[1-3] Mutations in the *PKLR* gene are associated with PK deficiency, an important cause of nonspherocytic hemolytic anemia. The clinical symptoms are variable, ranging from a mild, compensated hemolytic anemia to death in early childhood.[4]

In the last few years a variety of different mutations in the *PKLR* gene have been identified, facilitating the molecular diagnosis of PK deficiency.[5,6] Because of the lack of structural and functional information, it has been difficult to predict the phenotypic consequences of specific mutations. PK forms tetramers and its catalytic activity is allosterically regulated by phosphorylation and allosteric effectors. The tertiary structure of human R-type PK (HRPK) has not been determined, but the primary sequence is homologous to cat and rabbit muscle PK isoform M_1 (M_1PK),[7-9] whose structures have been solved at 2.6 Å and 2.9 Å resolution, respectively (Larsen *et al.*[9]; Brookhaven Data Bank, accession code 1PKM [Allen and

Muirhead]). M_1PK is composed of four domains: first, the N-terminal domain N, which is involved in subunit contacts in the tetrameric molecule; second, domain A, a classic α/β-barrel, that comprises the catalytic site; third, domain B, which loops out from the end of the third β-strand in domain A (the functional role of domain B is unclear); and, finally, the C-terminal domain C, which forms an open twisted αβ-type domain that may play an important role for tetramerization. However, the M_1 type PK is the only PK enzyme which is not allosterically regulated and hence functionally different from the RBC type PK. Reflections on the effect of mutations in the human RBC PK gene have so far been based on the three-dimensional model of cat muscle, assuming that these conclusions were valid for the human RBC gene.

In this study, we report the finding of a novel PK mutation in two Danish families that causes severe nonspherocytic hemolytic anemia. We have examined the expression of the mutant alleles at the RNA and protein level. Simulation of the effect of the mutations in a human RBC PK model, based on the crystal structure of muscle PK, shows that none of the mutations involves the catalytic site, but both are likely to disrupt the structure of the critical interface between domains A and C. We postulate that the G_{1091} to A and common G_{1529} to A mutation cause PK deficiency by disrupting the allosteric properties of PK.

Materials and methods

Nomenclature

Nucleotides and amino acids are numbered according to Kanno et al.[7] (GenBank D13232-D13243).

Patients

The proband was an 11-year-old Danish boy. At birth he suffered from a severe hemolytic crisis, with a hemoglobin concentration of 10.3 g/dL, reticulocytosis, and hepatosplenomegaly. PK activity was undetectable, and other possible causes of hemolysis were excluded. At present, he requires blood transfusions every three months. His parents do not suffer from any clinically noticeable hemolysis. After the boy's mother was divorced and remarried, she and her new husband had a baby girl. At birth, she was severely anemic and died after six weeks, despite blood transfusions. The PK-activity of the baby was undetectable.

During the molecular investigations a second Danish family was referred to our laboratories

for molecular analysis of the *PKLR* genes. Both parents were healthy adults of Danish origin with no known relation to the first family. The clinical course of their first child (girl) at birth was identical as described above for the proband. At present, she is three years old and requires blood transfusions every three months. The second child in this family, a boy, was also born with a hemoglobin concentration of 11.3 g/dL and reticulocytosis. His liver and spleen were normal. PK-activity was low. Presently, he is one year old and receives transfusions every three months. Informed consent was obtained from all subjects.

DNA and RNA isolation

DNA from EDTA-stabilized blood and parrafin-embedded liver coupes was isolated as described.[10,11] Total RNA was isolated from isolated blood reticulocytes as described.[12]

Polymerase chain reaction (PCR) procedures

To have high specificity and yield for sequencing purposes, the human *PKLR* gene was first amplified with primers PKr-1 (exon 1: 5'-CCC AGG CCC ACA CTG AAA GC-3') and Pkr-6 (exon 12: 5'- GTG TGG GCT GGA GAA CGT AGA-3') using the XL-PCR kit from Perkin-Elmer (Norwalk, CT) according to the manufacturer's instructions, with modifications.[13] Briefly, components were mixed using 0.2 mmol/L of each dNTP, 50 pmol of each primer, and 1.1 mmol/L Mg $(OAc)_2$. After 4 minutes of denaturation at 95°C, 4 U of

Table 1. Oligonucleotide primers used for sequencing

Exon 1:	PK-1F:	5'-cct ttt ctc ttc tct gtc tcc c-3'
	PK-1R:	5'-ggc tcc tag ttt tca ccc tca t-3'
Exon 2:	PK-2F:	5'-ctg cag aac tga tcc cca gcc-3'
	PK-2R:	5'-ccc tgt agc ttg acc cat ccc-3'
Exon 3:	PK-3F:	5'-cat ggg gag gaa ggg cag gt-3'
	PK-3R:	5'-acc aat agg ccc tgt gtg gct-3'
Exon 4:	PK-4F:	5'-ttg cct ctc atg ttc tgg ggg-3'
	PK-4R:	5'-tgg cca gga cct cga ggc at-3'
Exon 5:	PK-5F:	5'-ggc cac ctt ccc ctg aaa cc-3'
	PK-5R:	5'-ttt ccg gcc ctg gcc cag c-3'
Exon 6:	PK-6F:	5'-tcc ggg gct cag aac tca cat-3'
	PK-6R:	5'-agg aga agg gaa tgt gcc cag-3'
Exon 7:	PK-7F:	5'-gca act gtg ccc cgt cct ca-3'
	PK-7R:	5'-gtg atg ggg aat agc gac agg-3'
Exon 8:	PK-8F:	5'-cca tca cct ttc ttc tcc tgc-3'
	PK-8R:	5'-ttc acc cac agg tgt ccc taa-3'
Exon 9:	PK-9F:	5'-agt cac agt gtg agt cct ac-3'
	PK-9R:	5'-tat gga agg gat ttg gtt cc-3'
Exon 10:	PK-10F:	5'-ttc cat acc cca gtg ccc ctt-3'
	PK-10R:	5'-acc cct gac cca aag ctc cat-3'
Exon 11:	PK-11F:	5'-cac agc ttg tta gtg aca cct g-3'
	PK-11R:	5'-gct cct gat aca aat ggt agg a g-3'
Exon 12:	PK-12F:	5'-gtg tga gcc acc aca cct gtc-3'
	PKr-6:	5'-GTG TGG GCT GGA GAA CGT AGA-3'

Nucleotides in lower case are located in intron. Nucleotides in uppercase are located in exon.

r*Tth* DNA polymerase XL was added. Cycles were as follows: 1 minute at 94°C, 10 minutes at 64°C, and 10 minutes at 72°C. After cycle no. 19, the extension time was increased with 15 seconds per cycle for a total of 30 cycles.

The amplified XL-PCR product was used as template in nested PCRs in which each exon and introns 1, 5, and 8, including the intron-exon bounderies, were amplified. Exon 1 was amplified directly from genomic DNA using primers PK-1F and PK-1R (Table 1). The reaction mixture in 100 µL volume contained 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.01% (wt/vol) gelatine, 0.2 mmol/L of each dNTP, 1 mmol/L of each primer, 1 µL XL-PCR product, and 2.5 U AmpliTaq DNA polymerase. All reagents were obtained from Perkin-Elmer. The samples were subjected to 30 cycles of amplification with denaturation at 94°C for 45 seconds (5 minutes at 95°C in the first cycle), annealing at 64°C for 45 seconds, and extension at 72°C for 45 seconds, followed by an elongated extension time of 7 minutes after the last cycle. Negative controls, without added DNA, were included in each run to exclude amplification of contaminating DNA.

Reverse transcription-PCR (RT-PCR) was performed as described.[12] cDNA synthesis was initiated with random hexamers. cDNA was amplified with primers PKr-5 (exon 8: 5'-GGT GAG CGA CGG CAT CAT GG-3') and CDPK-9 (exon 9: 5'-CGC ATG CTG CAT CTT CAC CGC-3') for detection of the nt 1091 mutation. For the detection of the nt 1529 mutation, primers CDPK-11 (exon 11: 5'-CTC AGC CCA GCT TCT GTC TCG-3') and PKr-6 were used. PCR conditions were as described under nested-PCR. Normal human liver RNA was included as positive control.[12] These products were used for direct DNA sequencing. For all RNA samples amplified in a RT-PCR we routinely included a control where the reverse transcription step was omitted.

Sequencing of PCR products

Automated sequencing was performed with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer/Applied Biosystems, Foster City, CA), according to the instructions, and sequence reactions were analyzed on a Perkin-Elmer/Applied Biosystems ABI 310 Genetic Analyzer.

Restriction endonuclease digestion

All mutations in DNA and RNA and the polymorphisms in DNA were confirmed and characterized using restriction endonuclease digestion when applicable. To confirm the 1091 mutation detected by sequencing, we amplified a 264-bp fragment containing exon 8 from genomic DNA using primers PK-8F and PK-8R (Table1). The fragment contains three restriction sites for *Cac*8I (GCN/NGC), yielding fragments of 4, 22, 83, and 155 bps. The

1091 G/A mutation removes two sites simultaneously (fragments of 22 bp and 242 bp). For confirmation of the nt 1529 mutation, we amplified exon 11, with primers PK-11F and PK-11R (fragment of 330 bp). The mutation creates a restriction site for *Sty*I (C/CWWGG), producing fragments of 190 and 140 bp. The same enzymes were used for confirmation of the mutations in RNA. Primers used for RT-PCR were as mentioned above in "Polymerase chain reaction (PCR) procedures."

To detect the polymorphism at nt 1705 (A/C),[14] we amplified a fragment using primers PK-12F and PKr-6 (235 bp). *Bsp*HI (T/CATGA) will only cleave this product, when an adenine is present (132 bp and 103 bp).

The nt 1738 (C/T) (this study, Van Solinge *et al*.,[15] and Bianchi *et al*.[16]) polymorphism was detected using *Bse*RI [GAGGAG$(N)_{10}$ / and $(N)_8$CTCCTC], only cleaving a fragment amplified with primers PK-12F and PKr-6 if a thymine is present at this position (182 bp and 53 bp). The polymorphism at nt +51 in intron 5 (C/T) (this study, Fujii *et al*.,[17] and Baronciani *et al*.[18]) was detected by sequencing intron 5, because no useful restriction enzyme recognition sites are present.

The trinucleotide ATT repeat in intron 11[19] was amplified with 4,7,2',7'-tetrachloro-6-carboxy-fluorescein (TET)-labeled primers JHATT[19] and PK-TNR-R[4] and examined with the GeneScan short denatured module on the ABI 310 Genetic Analyzer and by sequencing the fragments.

Protein studies

Enzyme assays and the heat stability assay of PK were performed according to the methods of Beutler.[20] The antibody consumption assay was performed essentially as described earlier.[21] Oligomerization in the presence and absence of 10 mmol/L fructose-1,6-diphosphate (FDP) was studied by gel filtration experiments on a Sephacryl S-200 superfine column (Pharmacia, Uppsala, Sweden) using lactate dehydrogenase (130 kDa) as an internal standard.[22]

Molecular modelling

Coordinates of the x-ray crystal structure of the M_1 muscle type PK from *Felis domesticus*, obtained from the Brookhaven Data Bank as 1PKM (submitted by Allen and Muirhead), were used as a scaffold for the construction of a model of HRPK. Initially, the corrected cDNA sequence of the human *PKLR*-gene[7,23] was aligned with the sequences derived from both 1PKM (Allen and Muirhead) and 1PKN[9] structures, using the Alignment of Multiple Protein Sequences (AMPS) program, as described.[24] This resulted in the alignment as shown in Figure 1.

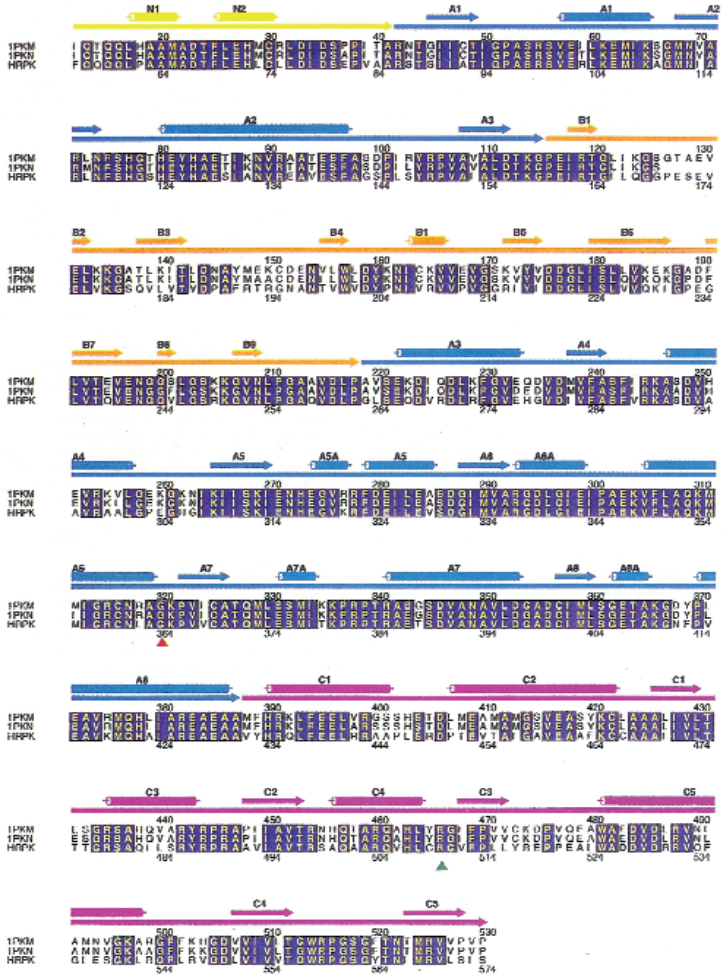

Figure 1 (color version, addendum page 166). Alignment of muscle-type pyruvate kinase from *Felis domesticus* (1PKM) and *Oryctolagus cuniculus* (1PKN) with R-type PK from human (HRPK). The alignment was generated with AMPS, as described in the Materials and Methods and drawn with the program ALSCRIPT.[35] Amino acids that are completely conserved among the three PKs are shown with yellow letters in boxes shaded with magenta. Only residues of human R-type PK (56-574) corresponding to 1PKM (12-530) are shown. The secondary structure elements, α-helixes and β-strands, assigned to 1PKM are shown above the sequences as cylinders and filled arrows, respectively. The domain organization of PK is represented by filled arrows right under the secondary structure elements. Color code: the N-terminal domain N is yellow; the catalytic domain A is blue, domain B is orange, and the C-terminal twisted αβ-type domain C is pink. The numbers above the sequences are in accordance with the numbering in 1PKM, whereas the numbers beneath the sequences refer to the codon numbering of HRPK. The red-filled triangle indicates the position of residue 364 (glycine to aspartic acid mutation), and the green filled triangle indicates the position of residue 510 (arginine to glutamine mutation).

After alignment of the sequences, the HRPK was constructed from 1PKM with the program package from Biosym Technologies (San Diego, CA) in the following way: (1) the coordinates of 1PKM were read into the program INSIGHTII, (2) followed by alignment of the sequence of 1PKM with the HRPK sequence according to Figure 1 using the program HOMOLOGY and (3) transferring of 1PKM coordinates to HRPK. (4) The raw model of HRPK was modified to eliminate close Van der Waals contacts. (5) The adjusted model of HRPK was then subjected for minimization in the program DISCOVER for 6,000 iterations with the steepest descendants algorithm assuming a pH of 7.0, essentially as described.[25] The minimized model of HRPK was used for inspection of the mutations described in this study.

Results

Pyruvate kinase mutations

The proband was a compound heterozygote with a novel G_{1091} to A mutation in exon 8 and a second mutation (G_{1529} to A) in exon 11 on the other allele of the *PKLR* gene (Figure 2A, only G_{1091} to A shown). The G_{1091} to A mutation results in the substitution of a conserved

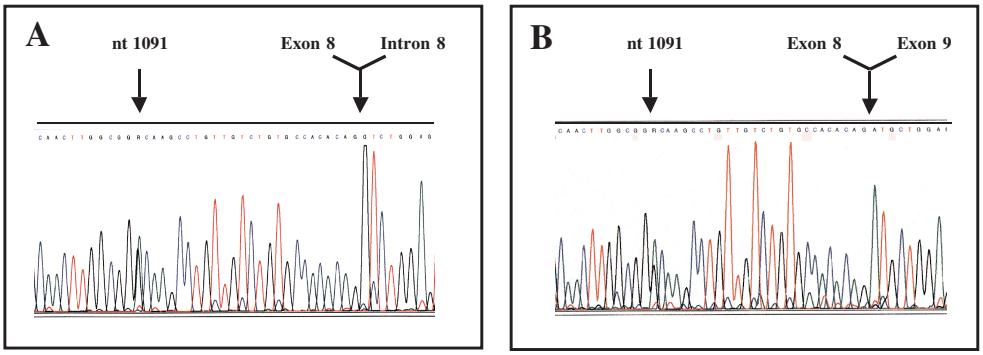

Figure 2. Detection of the nucleotide G_{1091} to A mutation in exon 8 of the human *PKLR* gene and RNA of the proband by sequencing. (A) DNA; (B) RNA.

glycine by an aspartate at codon 364, whereas the G_{1529} to A mutation leads to the substitution of a conserved arginine residue with glutamine at codon 510. The latter is a well-known and common mutation among non-gypsy whites.[4,26] The G_{1091} to A mutation was also present in the mother, whereas the G_{1529} to A mutation was detected in his father. Analysis of the *PKLR* gene of the second husband showed that he was a heterozygote carrier

of the G_{1091} to A mutation. Analysis of DNA obtained from liver coupes showed that the deceased baby was a G_{1091} to A homozygote (data not shown). The pedigree and mutation analysis of the family are shown in Figure 3. The second Danish family also exhibited the

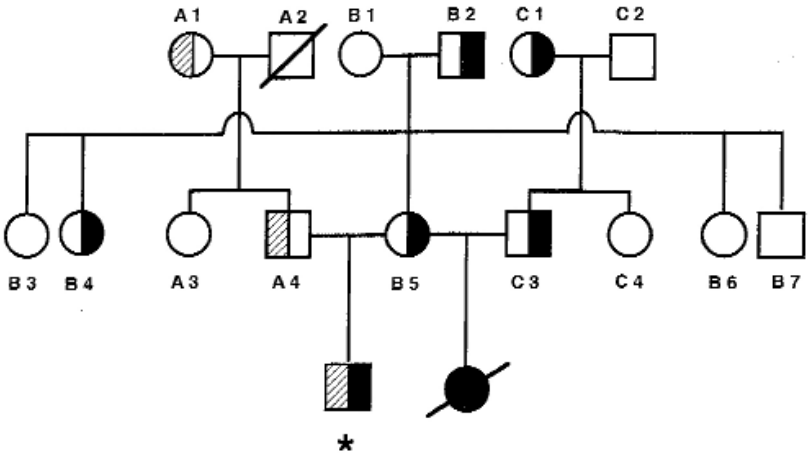

Figure 3. Pedigree of family 1. Filled areas denote the nt 1091 mutation. Shaded areas denote the nt 1529 mutation. *Proband.

G_{1091} to A and G_{1529} to A mutations. The daughter and son were both compound heterozygotes and the 1091 mutation was inherited from the father, whereas the mother was carrier of the 1529 mutation. RT-PCR analysis of RNA followed by sequencing and restriction enzyme analysis indicated that both mutated alleles were expressed (Figure 2B, only G_{1091} to A shown). The prevalence of the two mutations was determined by screening 100 normal controls for the G_{1091} to A and G_{1529} to A mutations. None was detected, indicating that the G_{1091} to A and G_{1529} to A allele frequency is less than 1:200.

Polymorphisms

A C/T polymorphic site was identified in exon 12, at position 1738, 13 nucleotides downstream of the translation termination codon.[15,16] Estimated from 100 chromosomes of unrelated individuals, the allelic frequency was as follows: C-alleles, 0.26; T-alleles, 0.74. Observed homozygosity for C/C was 0.04, for C/T was 0.43, and for T/T was 0.53. In addition, the polymorphism in intron 5 at nt +51[17,18] was used to determine the haplotypes of our patients.

Using four polymorphisms, we performed DNA haplotype analysis. The 1529 mutation was linked to IVS-5-T, 14 ATT repeats in intron 11, nt 1705-C, and nt 1738-T. The 1091 mutation

Table 2. Characteristics of two Danish families with PK deficiency

					Polymorphisms			
	PK (U/gHb)	HK (U/gHb)	PK mutation	amino acid	IVS-5	repeats	nt 1705	nt 1738
Family 1								
Proband	1.3	5.9	1091G→A/1529G→A	364Gly→Asp/510Arg→Gln	C/T	12/14	A/C	C/T
Mother (B5)	6.0	1.15	1091G→A	364Gly→Asp	C/T	12/14	A/C	C/T
Father 1 (A4)	5.0	0.97	1529G→A	510Arg→Gln	C/T	14/15	C/C	T/T
Father 2 (C3)	6.4	1.2	1091G→A	364Gly→Asp	C/T	12/15	A/C	C/T
Daughter	—*	—	1091G→A/1091G→A	364Gly→Asp/364Gly→Asp	C/C	12/12	A/A	C/C
Family 2								
Father	5.4	2.9	1091G→A	364Gly→Asp	C/T	12/15	A/C	C/T
Mother	8.1	1.7	1529G→A	510Arg→Gln	T/T	14/14	C/C	T/T
Daughter	2.8	4.2	1091G→A/1529G→A	364Gly→Asp/510Arg→Gln	C/T	12/14	A/C	C/T
Son	—	—	1091G→A/1529G→A	364Gly→Asp/510Arg→Gln	C/T	12/14	A/C	C/T
Reference	8.4-14.4	1.1-1.8						

Abbreviations: PK, pyruvate kinase; HK, hexokinase; IVS-5, polymorphism at nt 51 in intervening sequence-5.

* No sample available

was linked to IVS-5-C, 12 ATT repeats in intron 11, nt 1705-A, and nt 1738-C, in all patients (Table 2).

Enzyme activities

RBC PK activity of the proband in family I as well as the affected children in family II was low, but detectable (Table 2). However, although blood samples were taken shortly before blood transfusion, it cannot be excluded that the activity in the patients was, at least partly, originating from the presence of residual donor cells. To evaluate the mean cell age of the RBC population, the cell age-dependent enzymes hexokinase and glucose-6-phosphate dehydrogenase were measured too. The largely increased activities of these enzymes indicate a very young mean cell population and emphasize the extent of the PK deficiency. Unfortunately, no blood samples were available from the deceased daughter in family I nor from the affected son in family II. As expected, the proband's heterozygous parents have low PK activities. The second husband as well as the father in family II are heterozygous too. Altough the mother in family II is a carrier of the G_{1529} to A mutation, her PK activity was not significantly lowered.

Protein studies

Biochemical studies at the protein level were hampered by the low PK activity and the potential presence of donor RBCs in the patients. It could not be excluded that the residual enzyme activity in the proband was originating mainly from donor cells. Yet, we decided to characterize the enzyme from patient and heterozygotes. However, we were unable to detect any significant aberrations. First, the expression of inactive enzyme protein was assayed by performing an antibody comsumption assay. The amount of immuno-reactive material consumed by a fixed enzyme activity appeared to be normal in all cases. However, in the patient, a minor part of the activity could not be precipitated with the antibody for R-type PK, suggesting the presence of PK isoforms that are not recognized by the antibody (data not shown). In severe PK deficiency, the compensatory presence of M_2-type PK has been shown in some cases.[27] However, no enzyme activity could be precipitated with antibody against M_2-type PK. In another set of experiments, the heat stability of the enzyme was investigated, but no labile enzyme could be detected. The allosteric properties of the enzyme were investigated by studying the effects of FDP on the activity at low phosphoenolpyruvate concentrations. Again, the enzyme from patient and heterozygotes behaved completely normal. Also, the extent of tetramerization in presence and absence of FDP was studied by gel filtration experiments, but no signs of altered subunit interactions were observed (results not shown).

Because of the limitations as described above, these results did not allow any conclusions on the expression of mutant enzyme at the protein level.

Molecular modelling

Alignment of cat M_1PK, rabbit M_1PK, and human RBC PK (HRPK) shows that HRPK is approximately 70% similar to the two enzymes (Figure 1). Like cat and rabbit M_1PK, HRPK may therefore be divided into four domains. Residues 42-85 form the N-terminal domain. Residues 86-159 and 263-431 form domain A, a classic α/β-barrel, that comprises the catalytic site. The residues that comprise domain B span from codons 160 to 262 and loop out from the end of the third β-strand in domain A. Finally, the C-terminal C-domain from residues 432 to 574 forms an open twisted αβ-type domain. The mutated Gly364 is located in the A-domain, whereas Arg510 is situated in the C-domain. Both amino acids are conserved in cat and rabbit M_1PK (Figure 1), yeast, rat LPK, chicken MPK,[8] mouse RPK,[28]

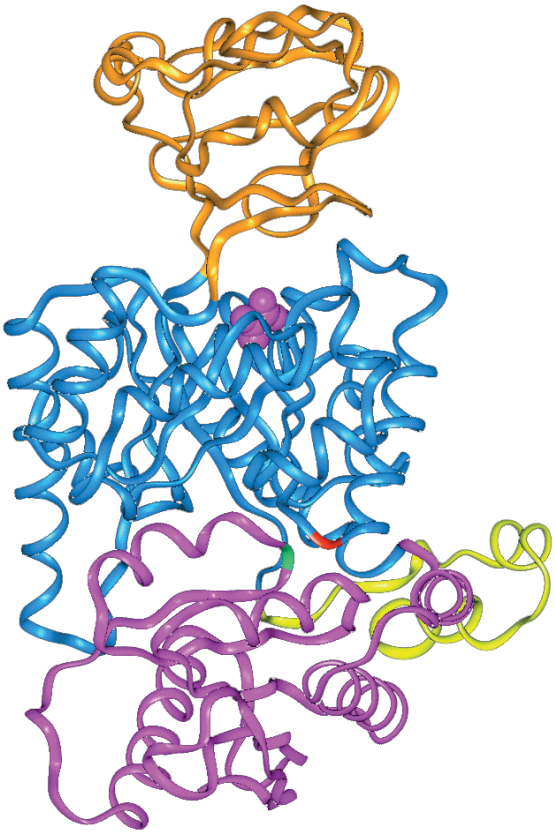

Figure 4 (color version, addendum page 167). Cα-ribbon trace of the model of human R-type PK monomer. The color code is as for Figure 1: the N-terminal domain N is yellow; catalytic domain A is blue, domain B is orange, and the C-terminal twisted αβ-type domain C is pink. The positions of the amino acids 364 (G_{1091} to A mutation) and 510 (G_{1529} to A mutation) are indicated by red and green color, respectively. The active site bound pyruvate molecule taken from 1PKN[9] is represented by pink space filling.

and dog RPK.[29] In *Bacillus stearothermophilus* the glycine 364 is conserved, whereas arginine 510 is not.[30]

Figure 4 shows the Cα-ribbon trace of the model of human RBC PK. Placement of the mutations in this model shows that the mutations are both located at the interface of domains A and C. Glycine 364 is situated in the loop connecting Aα6 and Aβ7, whereas Arg510 connects Cα4 and Cβ3. Please note that the substituted amino acids in the HRPK are not located *in cis in vivo* because mutations are present on separate alleles. Therefore, Figure 4

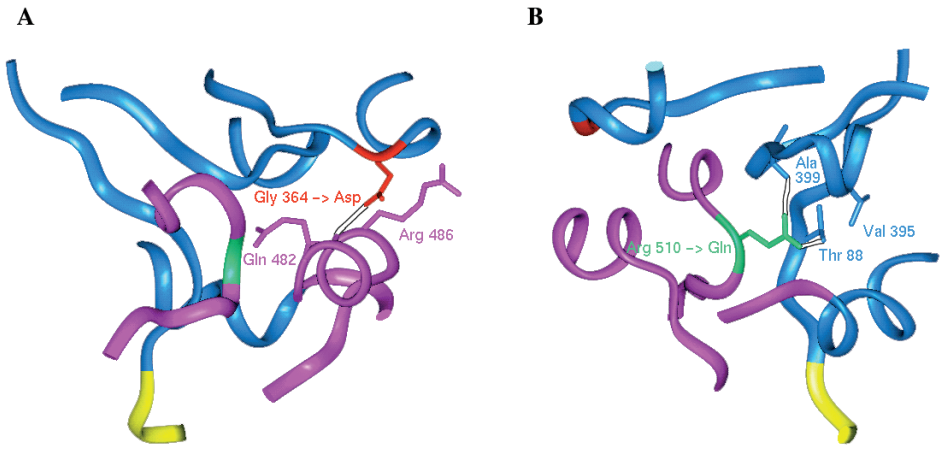

Figure 5 (color version, addendum page 168). Close-up view of areas around the amino acids 364 (A) and 510 (B). Residues in close contact with the mutated residues, translated from codons 364 and 510, are shown with side chains, whereas the residues further apart are shown in Cα-ribbon trace representation as in Figure 4. The color code is as for Figure 1. (A) and (B) are shown with the mutated aspartic acid and glutamine replacing the wild-type glycine and arginine, respectively. Hydrogen bonds less than 3.0 Å are shown as white lines.

only indicates the position of the mutated codons. We then configured the mutated codons into the model and made a close-up view of areas around the respective parts of the PK monomer, as shown in Figure 5. The Gly364 to Asp mutation in domain A is situated close to glutamine at position 482 and to Arg486 in domain C of the monomer. Asp364 is not involved in any salt bridge formation to Arg486, but it forms a hydrogen bond to the backbone carbonyl of Gln482. The glutamine for arginine substitution at position 510 leads to the formation of two hydrogen bonds (Thr88 and Ala399), whereas the wild-type has only one hydrogen bond to Ala399.

Discussion

Patients with clinically apparent PK deficiency are rare in the Western-European population. Although calculating the probability of the events as they happened to the first Danish family will be difficult, we think this is a very rare and unlucky event indeed. Not only did the son from the first marriage inherit both affected alleles, but in addition the mother remarried with a carrier of PK-deficiency and the daughter born from this marriage again inherited two affected alleles, which proved fatal. The appearance of a second unrelated Danish family at the same time, again with two affected siblings and identical mutations is coincidental, but raises questions concerning the prevalence of these mutations in Denmark. Although the nt 1529 mutation represents 45% of the diseased alleles in non-gypsy whites[4,26] and probably is the most prevalent mutation in Western-Europe,[31,32] both mutations were not detected in a control group of 100 healthy individuals.

Within the two families, the G_{1091} to A mutation was present in the heterozygous, homozygous, and compound heterozygous form. No other sequence variants were found in the patients heterozygous for the G_{1091} to A mutation other than the polymorphisms (*vide infra*). Patients in the two families who are heterozygous for the G_{1091} to A mutation all have decreased PK activity. When the nt 1091 mutation is inherited with the nt 1529 mutation on the other allele, severe PK deficiency and clinical symptoms occur (proband and two siblings in family 2). The patient with the nt 1091 mutation present on both alleles was clinically severely affected, PK-deficient, and died. Thus, the G-to-A change at position nt 1091 correlates with the disease. In addition, the substituted amino acids are phylogenetically conserved, indicating that they are important for the function of the enzyme. Taking all these findings into consideration, we conclude that the G-to-A change at position nt 1091 causes PK deficiency.

We used four polymorphisms to study the haplotype of the described mutations.[15-18] In accordance with previous studies, the nt 1529 mutation is linked to nt 1705-C and 14 ATT repeats in intron 11, supporting the theory of a common ancestor gene for this mutation. We find that the nt 1529 is linked to nt 1738-T and a thymine at nt 51 in intron 5, substantiating this hypothesis. The nt 1091 mutation was linked in all affected individuals to nt 1705-A, nt 1738-C, 12 ATT repeats, and a cytosine at nt 51 in intron 5.

Enzymatic studies and gelfiltration experiments of PK from our patients did not demonstrate any abnormality other than the reduced activity. There may be several reasons for this. First, it is possible that the mutant enzymes do not differ in their kinetic properties. Many mutations present in compound heterozygous or homozygous form have reduced activity, but normal responses in kinetic studies.[4] Interestingly, even identical mutations can exhibit different kinetic profiles. Moreover, the tetrameric structure of PK makes interpretation of gel filtration data

difficult, because several hybrid proteins can be present. Secondly, interference of donor cells in the compound heterozygous patients, differences in enzymatic behaviour *in vivo* and *in vitro*, unstability of mutant enzymes, and overexpression of M_2PK might influence results.[27,33]
To obtain more insight in the pathogenesis of the nt 1091 and nt 1529 mutations, we constructed the three-dimensional model of human RBC PK, using the known crystal structure of cat PKM as scaffold. Previous studies in other species have indicated that the precise conformation of the area of the molecule that is mutated in this study may be critical, not only in respect to proper alignment of residues in the active site, but also for allosteric transitions and binding of ADP and FDP.[8,30] Walker *et al*.[30] have proposed the existense of two pockets at the interface of domains A and C: the Arg42-pocket (in humans Arg86) that is important for ADP binding and the residue 466-pocket (in humans Arg510), which is possibly important for binding of a second allosteric activator. These pockets are divided by the fourth α helix of domain C (Cα4), which is bound by hydrogen bonds and salt bridges to domain A by connection from Ala399 (in domain A) to Arg510 (in domain C), Asp400 (domain A) to Arg488 (domain C), and Asn113 (domain A) to Val506 in domain C (human numbering). In this way, a critical conformation of the interface between domains A and C is specified. The area around Gly364 is spacious and the insertion of the aspartate is not likely to disrupt the structure of PK by a simple sterical effect. However, aspartate comes in close contact with glutamine 482, and a salt bridge that would disrupt the crucial interaction between Arg488 and Asp400 is likely to be established (Figure 5A). In a similar manner, substitution of Arg510 with glutamine reduces the conformational freedom between domains A and C, because an extra connection between these domains is created by formation of a salt bridge to Thr88. Probably the salt bridge from Gln510 to Ala399 will be intact, but structural changes caused by the hydrogen bond to Thr88 will affect the energy balance in this area and influence allosteric transitions (Figure 5B). Moreover, Thr88 is close to Arg86, which is the proposed ADP binding residue.
We propose that the Gly364 to Asp and Arg510 to Gln mutations affect the interdomain structure between domains A and C and that the function of both the Arg86 and Arg510 pockets might be affected. Since transition from the T-state to the high-affinity R-state of PK requires rotation of all subunits in the tetramer by flexible hinges,[34] the formation or breakage of intersubunit contacts could restrict the conformation of the active site as well.

Acknowledgement

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References

1. Miwa S, Kanno H, Fujii H: Pyruvate kinase deficiency. Historical perspective and recent progress of molecular genetics. Am J Hematol. 1993;42:31-35.
2. Noguchi T, Inoue H, Tanaka T. The M1 and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. J Biol Chem. 1986;261:13807-13812.
3. Noguchi T, Yamada K, Inoue H, Matsuda T, Tanaka T. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. J Biol Chem. 1987;262:14366-14371.
4. Baronciani L, Beutler E. Molecular study of pyruvate kinase deficient patients with hereditary nonspherocytic hemolytic anemia. J Clin Invest. 1995;95:1702-1709.
5. Miwa S, Fujii H. Molecular basis of erythroenzymopathies associated with hereditary hemolytic anemia: tabulation of mutant enzymes. Am J Hematol. 1996;51:122-132.
6. Baronciani L, Bianchi P, Zanella A. Hematologically important mutations: red cell pyruvate kinase. Blood Cells Mol Dis. 1996;22:259-264.
7. Kanno H, Fujii H, Hirono A, Miwa S. cDNA cloning of human R-type pyruvate kinase and identification of a single amino acid substitution (Thr384→Met) affecting enzymatic stability in a pyruvate kinase variant (PK Tokyo) associated with hereditary hemolytic anemia. Proc Natl Acad Sci USA. 1991;88:8218-8221.
8. Muirhead H, Clayden DA, Barford D, Lorimer CG, Forthergill-Gilmore LA, Schiltz E, Schmitt W. The structure of cat muscle pyruvate kinase. EMBO J. 1986;5:475-481.
9. Larsen TM, Laughlin LT, Holden HM, Rayment I, Reed GH. Structure of rabbit muscle pyruvate kinase complexed with Mn^{2+}, K^{+}, and pyruvate. J Biochem. 1994;33:6301-6309.
10. Mies C. A simple and rapid method for isolating RNA from paraffin embedded tissues for reverse transcription-polymerase chain reaction (RT-PCR). J Histochem Cytochem. 1994;42:811-813.
11. Van Solinge WW, Lind B, Van Wijk R, Hart HCH, Kraaijenhagen RJ. Clinical expression of a rare β-globin gene mutation co-inherited with hemoglobin E-disease. Eur J Clin Chem Clin Biochem. 1996;34:949-954.
12. Lind B, Van Solinge WW, Schwartz M, Thorsen S. Splice site mutation in the human protein C gene associated with venous thrombosis: Demonstration of exon skipping by ectopic transcript analysis. Blood. 1993;82:2423-2432.
13. Cheng S, Fockler C, Barnes WM, Higuchi R. Effective amplification of long targets

from cloned inserts and human genomic DNA. Proc Natl Acad Sci USA. 1994;91:5695-5699.

14. Kanno H, Fujii H, Hirono A, Omine M, Miwa S. Identical point mutations of the R-type pyruvate kinase (PK) cDNA found in unrelated PK variants associated with hereditary hemolytic anemia. Blood. 1992;79:1347-1350.
15. Van Solinge WW, Van Wijk R, Kraaijenhagen RJ, Rijksen G, Nielsen FC. Identification of a novel mutation (PK-Dordrecht) and a novel polymorphism in the human red cell type pyruvate kinase gene. Br J Haematol. 1996;93:198.
16. Bianchi P, Zanella A, Zappa M, Vercellati C, Terragna C, Baronciani L, Sirchia G. A new point mutation G/A 1168 (Asp390-Asn) in an Italian patient with erythrocyte pyruvate kinase deficiency. Blood. 1995;86:133a.
17. Fujii H, Kanno H, Miwa S. Cumulative information of genotype-phenotype relationship of the homozygous pyruvate kinase deficiency. Blood. 1995;86:133a.
18. Baronciani L, Westwood B, Beutler E. Study of the molecular defects in pyruvate kinase (PK) deficient patients affected by hereditary nonspherocytic hemolytic anemia (HNHA). Clin Res. 1995;43:341a.
19. Lenzner C, Jacobasch G, Reis A, Thiole B, Nürnberg P. Trinulcleotide repeat polymorphism at the PKLR locus. Human Mol Genet. 1994;3:523.
20. Beutler E. Red Cell Metabolism. A Manual of Biochemical Methods. Orlando: Grune and Stratton; 1984.
21. Staal GEJ, Rijksen G, Vlug AMC, Vromen-van den Bos B, Akkerman JWN, Gorter G, Dierick J, Petermans M. Extreme deficiency of L-type pyruvate kinase with moderate clinical expression. Clin Chim Acta. 1982;118:241-253.
22. Oude Weernink PA, Rijksen G, Mascini EM, Staal GEJ. Phosphorylation of pyruvate kinase type K is restricted to the dimeric form. Biochim Biophys Acta. 1992;1121:61-68.
23. Baronciani L, Beutler E: Analysis of pyruvate kinase-deficiency mutations that produce nonspherocytic hemolytic anemia. Proc Natl Acad Sci USA. 1993;90:4324-4327.
24. Barton GJ. Protein multiple sequence alignment and flexible pattern matching. Methods Enzymol. 1990;183:403-428.
25. Christensen U, Olsen K, Stoffer BB, Svensson B. Substrate binding mechanism of Glu180→Gln, Asp176→Asn, and wild-type glucoamylase from *Aspergillus niger*. Biochemistry. 1996;35:15009-15018.
26. Baronciani L, Beutler E. Prenatal diagnosis of pyruvate kinase deficiency. Blood. 1994;84:2354-2356.

27. Kanno H, Wei DC, Chan LC, Mizoguchi H, Ando M, Nakahata T, Narisawa K, Fujii H, Miwa S. Hereditary hemolytic anemia caused by diverse point mutations of pyruvate kinase gene found in Japan and Hong Kong. Blood. 1994;84;3505-3509.
28. Kanno H, Morimoto M, Fujii H, Tsujimura T, Asai H, Noguchi T, Kitamura Y, Miwa S. Primary structure of murine red blood cell-type pyruvate kinase (PK) and molecular characterization of PK deficiency identified in the CBA strain. Blood. 1995;86:3205-3210.
29. Whitney KM, Goodman SA, Bailey EM, Lothrop CD, Jr. The molecular basis of canine pyruvate kinase deficiency. Exp Hematol. 1994;22:866-874.
30. Walker D, Chia WN, Muirhead H. Key residues in the allosteric transition of *Bacillus stearothermophilus* pyruvate kinase identified by site-directed mutagenesis. J Mol Biol. 1992;228:265-276.
31. Lakomek M, Huppke P, Neubauer B, Pekrun A, Winkler H, Schröter W. Mutations in the R-type pyruvate kinase gene and altered enzyme kinetic properties in patients with hemolytic anemia due to pyruvate kinase deficiency. Ann Hemat. 1994;69:253-260.
32. Lenzner C, Nürnberg P, Jacobasch G, Gerth C, Thiele BJ. Molecular analysis of 29 pyruvate kinase-deficient patients from central europe with hereditary hemolytic anemia. Blood. 1997;89:1793-1799.
33. Max-Audit I, Rosa R, Marie J. Pyruvate kinase hyperactivity genetically determined: metabolic consequences and molecular characterization. Blood. 1980;56:902-909.
34. Mattevi A, Bolognesi M, Valentini G. The allosteric regulation of pyruvate kinase. FEBS Lett. 1996;389:15-19.
35. Barton GJ. ALSCRIPT: a tool to format multiple sequence alignments. Protein Eng. 1993;6:37-40.

CHAPTER 6

Distinct phenotypic expression of two *de novo* missense mutations affecting the dimer interface of glucose-6-phosphate dehydrogenase

Richard van Wijk[1], Eric G. Huizinga[2], Ingrid Prins[3], Arjenne Kors[3], Gert Rijksen[4], Marc Bierings[3], and Wouter W. van Solinge[1]

[1]Department of Clinical Chemistry, [3]Department of Paediatrics, Wilhelmina Kinderziekenhuis, and [4]Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands; [2]Department of Crystal & Structural Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands.

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Abstract

Mutations encoding class I glucose-6-phosphate dehydrogenase (G6PD) variants are associated with chronic nonspherocytic hemolytic anemia (CNSHA), the most severe phenotypic expression of G6PD deficiency. These mutations frequently affect the G6PD dimer interface that is essential for enzymatic activity. We detected two *de novo* missense mutations concerning residues located close together in the dimer interface in two patients with severe G6PD deficiency. A novel c.1225C>T missense mutation was identified in a male neonate who presented with hemolysis and severe hyperbilirubinemia and the predicted Pro409Ser substitution constituted a novel class I variant, designated G6PD Utrecht. G6PD deficiency in the second patient was due to the once previously reported class I variant G6PD Sumaré (Val431Gly). Structural analysis revealed that the mutated residues Pro409 and Val431, located on different subunits, interact directly across the subunit interface and perturb formation of the G6PD dimer upon mutation. Favism and mild chronic hemolysis characterized the phenotype of the patient with G6PD Sumaré which contrasts with the more severe clinical picture of the patient with G6PD Utrecht and, in addition, that of the patient orginally described with G6PD Sumaré. We postulate that this G6PD variant is at the crossing between class I and class II G6PD deficiency and its ultimate phenotypic expression is either aggravated or ameliorated by other (extra)genetic factors.

Introduction

Chronic nonspherocytic hemolytic anemia (CNSHA) is the most severe phenotypic expression of glucose-6-phosphate dehydrogenase (G6PD) deficiency[1] (OMIM#305900). Because G6PD's principal role in the red blood cell is to provide reductive potential in the form of NADPH to protect the cell against oxidative stress,[2] the CNSHA phenotype results from a continuous, severe lack of reducing power. This feature distinguishes class I G6PD variants from other deficient G6PD variants (classes II to IV)[3] which are associated with episodes of oxidative stress evoked by infections or drugs, resulting in acute hemolytic anemia. The residual G6PD activity of class I variants is variable, ranging from activities as high as 35% of normal, to severely reduced or even undetectable enzyme activity.[4] Some residual enzyme activity is believed to be present, however, because total lack of G6PD has been shown to be a lethal condition at an early stage in embryonic development.[5] The deficiency is more pronounced in red blood cells, when compared to other cells, because of the long life span of the erythrocyte after the loss of capacity of protein synthesis.[6]

Most mutations underlying class I variants are located in and around exon 10 of the G6PD gene (*G6PD* on chromosome Xq28). The homology model of human G6PD based on the crystal structure of *Leuconostoc mesenteroides*[7] and, more recently, the crystal structure of human G6PD[8] itself shows that the corresponding amino acids are located close to the dimer interface and the structural NADP, indicating that integrity of these regions is important for enzyme stability and *in vivo* activity.
We studied two patients with severe G6PD deficiency and identified two G6PD variants that both resulted from a *de novo* mutation. One of the mutations was novel whereas the other is reported here for the second time. The affected amino acids, located and interacting with one another at the dimer interface, were, in contrast to the literature, associated with distinct phenotypic expression upon mutation.

Materials and methods

Informed consent was obtained from all subjects.

Case report: patient 1
A male baby was born at 37 weeks of gestation with a birth weight of 2330 g. He was the second baby of Dutch Caucasian nonconsanguineous parents. The family history was negative for anemia. The mother has blood group B Rhesus-negative. Therefore, she received anti-D gammaglobulins at 30 weeks of gestation. On the first day of life, total serum bilirubin rose to 167 µmol/L (reference value 0–1 day: <100 µmol/L) and intense phototherapy commenced. Initially, hemoglobin was 16.3 g/dL on day 1 (reference value 1–3 days: 13.4–21.6 g/dL) but decreased to 12.0 g/dL on the second day of life with a reticulocytosis of 235‰ (reference value <2 days: 30–60‰). Because the baby's blood group was O Rhesus-positive, the working diagnosis at that moment was hemolysis due to anti-D antibodies despite a negative Coombs' test. On day 5 he received a blood transfusion. The highest bilirubin level was 212 µmol/L on day 3 (reference value 3–5 days: <200 µmol/L). At discharge (day 9) his hemoglobin was 14.9 g/dL (reference value 0–3 months: 9.4–13.4 g/dL).
On follow up (age: 7 weeks) he was pale but not jaundiced. He was breast-fed and received 25 µg of vitamin K once daily. Hemoglobin was 8.0 g/dL, reticulocytes 140‰ (reference value: 0–20‰) and serum iron 13 µmol/L (reference value: 10–32 µmol/L). As the anti-D antibodies should have disappeared after 7 weeks, he was started on iron supplements with

the working diagnosis of iron deficiency anemia due to hemolysis. At age 9 weeks, the blood hemoglobin was 6.7 g/dL, reticulocytes were 218‰ (Table 1) and total serum bilirubin was 43 μmol/L (reference value: 3–21 μmol/L). The iron supplements were withdrawn. At this point he was diagnosed with hemolytic anemia due to severe G6PD deficiency (see Results and discussion). To date at age three years, the boy has experienced numerous hemolytic episodes, mostly triggered by infections, complicating his chronic hemolysis, frequently requiring blood transfusion.

Case report: patient 2

This boy is the second son of Caucasian nonconsanguineous Dutch parents born at 41 weeks of gestation with a normal hemoglobin (17.7 g/dL) and without signs of neonatal jaundice (bilirubin: 134 mmol/L on the fourth day). From the age of 1 month he was treated with ferrousfumarate for anemia (hemoglobin 8.5 g/dL) for a period of 2 months. The further history is uneventful.

At the age of 29 months, he presented with an episode of severe hemolysis 3 days after eating fava beans for the first time: hemoglobin 6,7 g/dL (reference value: 11.8–14.4 g/dL); lactate dehydrogenase (LD), 4142 U/L (reference value: 470–900 U/L); reticulocytes, 140‰. Further analysis led to the diagnosis of G6PD deficiency (see Results and discussion). During the following year, he was mildly jaundiced one more time during an episode of gastroenteritis, for which his parents sought no medical attention. Hemoglobin was between 9.3 and 9.9 g/dL with 47–65‰ reticulocytes and LD 748–802 U/L, all suggestive of mild ongoing hemolysis.

Biochemical analysis

G6PD activity and activity measurements of the red blood cell age-related enzymes pyruvate kinase (PK) and hexokinase (HK) were determined according to standardized procedures.[9]

Molecular analysis

DNA was isolated from peripheral white blood cells and exons 2–13 of *G6PD* of the patients were amplified by PCR according to previously described methods.[10] DNA sequence analysis was performed as described[11] with the appropriate primers. Screening of a normal control population (n = 50) for the novel c.1225C>T mutation was performed by *Msp*I digestion, a recognition site of which is abolished by the mutation. cDNA nucleotide and amino acid numbering starts at the initiator methionine and sequence variations are described according to the mutation nomenclature system.[12]

Genotyping the dinucleotide (TA) insertion polymorphism in the UDP-

glucuronosyltransferase 1 (*UGT1)* promoter was modified from the method described by Monaghan *et al.*[13] 6-FAM labeled sense primers were used for amplification and fluorescent PCR products were analyzed on the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using GeneScan 3.1 software.

Molecular modeling

The mutated residues Ser409 and Gly431 were modeled using the program O[14] and coordinates from the crystal structure of human G6PD (protein data bank entry 1QKI).

Results and Discussion

Severe G6PD deficiency was diagnosed in both patients based on low to undetectable G6PD activity. To rule out possible contamination with donor erythrocytes (patient 1) and to establish glycolytic enzyme activities after recovery from hemolytic crisis (patient 2), repeated measurements of G6PD, HK, and PK activities were performed at age 7 and 41 months, respectively. These measurements showed undetectable G6PD activity, despite a high reticulocyte count, and elevated activity of HK and PK, consistent with a young red cell population (Table 1). The undetectable residual enzymatic activity hampered further enzymatic characterization. Glycolytic enzyme activities of the patient's relatives were all within normal range (Table 1).

Table 1. Glycolytic enzyme activities of patients 1 and 2

Patient 1			**Father**	**Mother**	**Brother**	**Reference**
Age at diagnosis	9 weeks[a]					
Follow-up		7 months				
PK (U/gHb)	26.9	22.4	8.4	16.7	14.2	6.9–14.5
HK (U/gHb)	7.70	5.91	1.42	1.66	1.46	1.02–1.58
G6PD (U/gHb)	2.3	<1.0	10.6	10.6	11.0	7.1–11.5
hemoglobin (g/dL)	6.7	8.5				9.4–13.4
reticulocytes (‰)	218	135				0–20

Patient 2			**Father**	**Mother**	**Brother**	**Reference**
Age at diagnosis	29 months					
Follow-up (age)		41 months				
PK (U/gHb)	27.1	28.4	NA	12.2	13.0	6.9–14.5
HK (U/gHb)	5.63	3.77		ND	1.29	1.02–1.58
G6PD (U/gHb)	<1.0	<1.0		9.6	11.6	7.1–11.5
hemoglobin (g/dL)	6.7	9.3				9.4–13.4
reticulocytes (‰)	140	62				0–20

[a] Eight weeks following transfusion. NA, Not available; ND, Not determined.

We established the *G6PD* genotypes by DNA sequence analysis of the complete coding region. Regarding patient 1, we detected a novel hemizygous cytosine to thymine substitution at nt 1225 (c.1225C>T) in exon 10 (Figure 1A) coding for serine instead of the highly conserved proline at residue 409.[15] No other mutations were detected in *G6PD*.

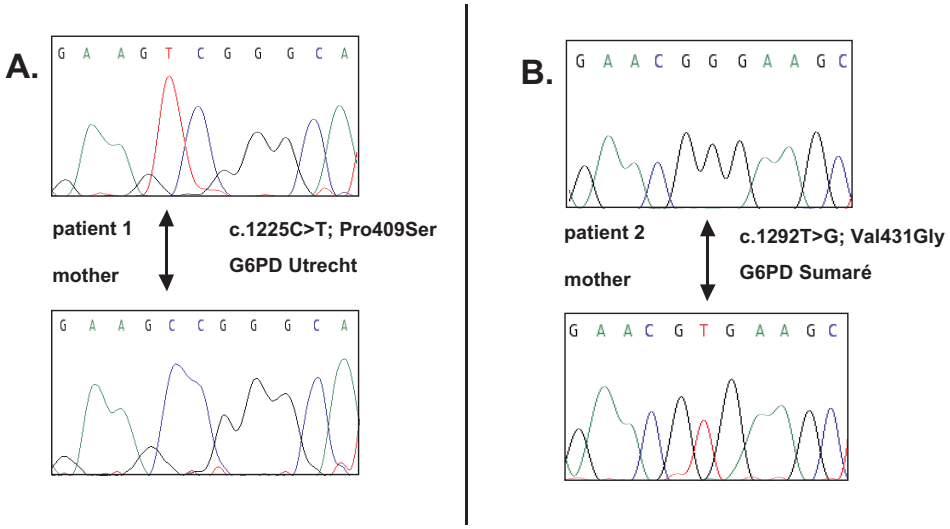

Figure 1. (A) DNA sequence analysis of *G6PD* of patient 1 (top) shows the novel hemizygous cytosine to thymine substitution (arrow) at nt 1225 in exon 10 of *G6PD*. This substitution is absent in *G6PD* of the mother (bottom), indicating a *de novo* missense mutation. The mutation predicts a proline to serine substitution at residue 409 in G6PD (G6PD Utrecht). (B) DNA sequence analysis of *G6PD* of patient 2 (top) revealed a hemizygous thymine to guanine substitution at nt 1292 in exon 11 (Val431Gly, G6PD Sumaré). This substitution is absent in the mother (bottom), indicating a *de novo* mutation.

Screening of a control population by *Msp*I digestion, of which a recognition sequence is abolished by this mutation, failed to detect the 1225T allele. The severe clinical phenotype of chronic hemolysis is consistent with class I G6PD deficiency[3] and we designated this novel variant G6PD Utrecht. Regarding patient 2, DNA analysis revealed a hemizygous thymine to guanine substitution at nt 1292 in exon 11 (c.1292T>G) (Figure 1B). This mutation encodes the once previously reported class I G6PD variant Sumaré (Val431Gly).[16] Neither mutation was encountered during subsequent DNA analysis of exons 10 and 11 in any of the, respective, relatives, notably the mothers (Figures 1A and B). Because we could confirm the parent–child relationship by parental DNA testing (data not shown), we inferred the occurence of a *de novo* mutational event in both patients. Moreover, this most likely occurred in early embryogenesis or in the maternal germline because buccal-cell DNA

samples from both patients also demonstrated hemizygosity for the respective mutations (data not shown). To our knowledge, this adds two G6PD variants that have arisen *de novo* to the five previously reported in class I G6PD deficiency.[17-21]

The severe clinical phenotype in patient 1, in particular with regard to the hyperbilirubinemia, in the immediate perinatal period was unusual because excessively increased hemolysis, especially in the first few days of life, does not constitute the cardinal factor in the pathogenesis of hyperbilirubinemia in G6PD-deficient neonates.[22] The presence of a dinucleotide (TA) insertion polymorphism[23] in the promoter of the gene that codes for UDP glucuronosyltransferase 1 (*UGT1*), the enzyme that catalyzes bilirubin conjugation by the liver, however, is known to be actively involved in the pathogenesis of G6PD deficiency associated neonatal hyperbilirubinemia.[24] One dinucleotide insertion increases the incidence of hyperbilirubinemia from 9.7% in G6PD-deficient neonates homozygous for the normal promoter (TA_6/TA_6), to 31.6% in those heterozygous (TA_6/TA_7), and 50% in those homozygous (TA_7/TA_7) for this polymorphism.[24,25] Indeed, we detected this polymorphism in the heterozygous state in our patient (Figure 2A).

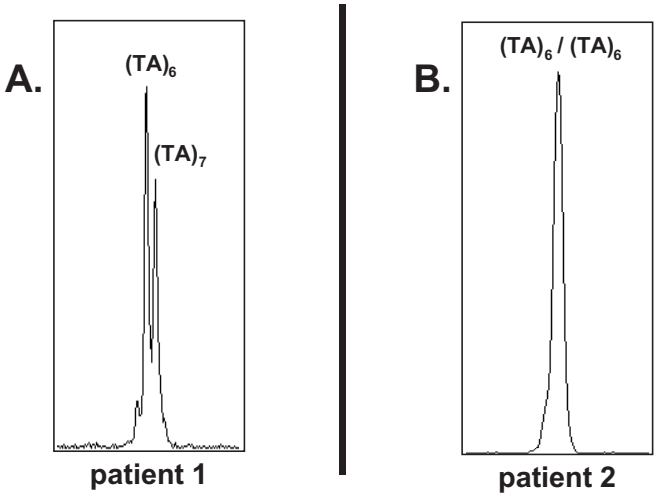

Figure 2. Dinucleotide (TA) insertion polymorphism in the UDP-glucuronosyltransferase 1 (*UGT1)* promoter was determined by detection of fluorescent PCR products. Patient 1 was heterozygous for one dinucleotide insertion (TA_6/TA_7) (panel A) whereas patient 2 was homozygous for the wild-type promoter (TA_6/TA_6) (panel B).

Nearly half of the mutations encoding class I G6PD variants directly affect the subunit interface of the G6PD dimer.[8] Structural analysis of the crystal structure of human G6PD[8] reveals that residues Pro409 (mutated in patient 1) and Val431 (mutated in patient 2) are also

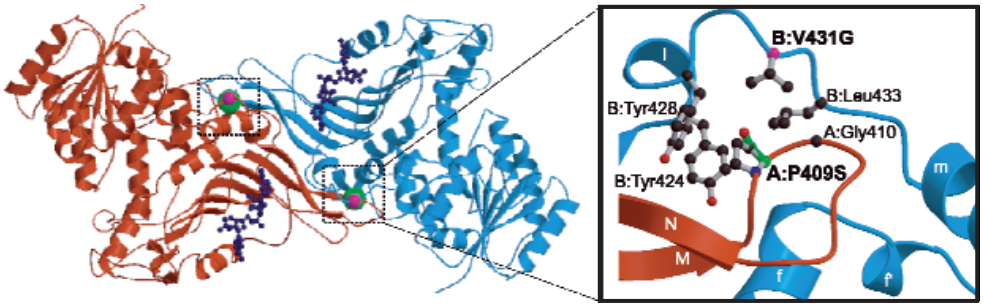

Figure 3 (color version, addendum page 168). Ribbon representation of a G6PD dimer with green spheres indicating the position of Pro409 and magenta spheres indicating the position of Val431. Subunits A and B are colored red and blue, respectively. The structural NADP molecules are shown in dark blue ball-and-stick representation. Dashed rectangles depict the two regions in the G6PD dimer interface disrupted by the Pro409Ser and Val431Gly substitutions. Note that although residues Pro409 and Val431 are located close together, they in fact reside on different subunits. (Inset) Close-up view of the vicinity of Pro409 (here shown on subunit A) and Val431 (subunit B) with selected residues shown in ball-and-stick (oxygen, red; nitrogen, blue; carbon, black). The carbon atoms of the mutated residues are shown in green (Ser409) and magenta (Gly431). The mutations were modeled using the program O.[14] The figure was generated from the atomic coordinates of protein data bank entry 1QKI[8] using computer programs Molscript[35] and Raster3D.[36]

involved in interactions among the subunits of the G6PD dimer (Figure 3). Pro409 is located in a loop that connects β strands M and N that act as a scaffold for the interface and NADP-binding residues.[8,26] The Pro409-containing loop is involved in numerous interactions with the other subunit, specifically with residues from helices f, f', l, and m, and residues from a loop that connects helices l and m. This latter loop contains the mutated residue Val431. Thus, Pro409 and Val431, located on different subunits, interact directly in the subunit interface. Pro409 makes inter-subunit contacts with Tyr424, Tyr428, and Val431 and mutation to serine would disrupt some of these contacts. Likewise, inter-subunit contacts of Val431 with Pro409 are likely to be disrupted upon mutation of Val431 to glycine. Moreover, the Pro409Ser and Val431Gly mutations both introduce a residue that allows for greater main chain flexibility, which could destabilize subunit interactions. The critical nature of the conformation of the Pro409-containing loop is further illustrated by the effect of mutations of the adjacent residue Gly410. Mutations of Gly410 to alanine,[27] cysteine,[28] or aspartate[29] all lead to class I deficiency. In conclusion, mutations Pro409Ser and Val431Gly could weaken dimer interactions, compromising the stability and enzymatic activity of G6PD. The subsequent lack of NADPH leads to CNSHA, although the different phenotypes in both patients point out the importance of both the position as well as the nature of amino acid replacement with regard to the extent of chronic hemolysis.

The mild phenotype of G6PD Sumaré, as expressed in patient 2, also differed from the

clinical picture of the only other patient carrying this variant to date.[16] Saad *et al.*[16] originally identified G6PD Sumaré in a 22-year-old white male of unknown origin from southeastern Brazil who displayed persistent chronic jaundice since birth, splenomegaly, mild chronic anemia (Hb 13.0 g/dL), and reticulocytosis (140‰).[16] This patient's daughter, compound heterozygous for G6PD Sumaré and the common G6PD A– variant, had jaundice at birth and mild chronic hemolysis whereas several respiratory infections did not evoke hemolytic crises.[30] In contrast, neonatal jaundice was absent in our patient and, in fact, he had been clinically normal until he presented with favism at the age of 29 months. Only then was he diagnosed with mild chronic hemolysis due to severe G6PD deficiency.

The vast majority of cases of favism occur in individuals carrying the severely deficient, non-CNSHA, class II G6PD variants. However, favism as the first clinical symptom of class I G6PD deficiency has been described before, namely, in association with G6PD Tokyo (Glu416Lys).[31] G6PD Tokyo was exclusively associated with CNSHA in three unrelated Polish G6PD-deficient families, whereas in a fourth, unrelated, family three members presented with favism during childhood and only further observation confirmed CNSHA in these patients.[31] From a structural point of view, only a few mutations causing class II G6PD deficiency affect residues in the G6PD dimer interface. Strikingly, however, one of these concerns another, recently described substitution of residue 431: Val431Met, G6PD Surabaya.[32] This variant was discovered in an 18-year-old male following an episode of acute hemolytic attack but without chronic hemolysis.[32] This is intriguing because alternate substitutions of the same amino acid at the G6PD dimer interface are seldomly associated with distinct phenotypic expression.[1,8] In fact, the only example, to our knowledge, involves the substitution of Glu398 by Lys in class I variant G6PD Puerto Limon[33] whereas substitution of Glu398 with Gly characterizes class II variant G6PD Anadia.[27]

In conclusion, Val431 is located in a region of the G6PD dimer interface crucial for enzymatic activity as illustrated by the severe CNSHA phenotype of G6PD Utrecht. Substitution of Val431 by Gly, however, places G6PD Sumaré at the crossing between class I and class II G6PD deficiency. Consequently, phenotypic expression in the two reported patients with G6PD Sumaré is either aggravated or ameliorated by other factors. These factors may be environmental or genetic, either linked or unlinked to the G6PD gene.[34] With regard to the difference in neonatal jaundice associated with the inheritance of G6PD Sumaré, as discussed above, the co-inheritance of the *UGT1* promoter polymorphism represents one such factor. Our patient was homozygous for the normal promoter (TA_6/TA_6, Figure 2B) whereas, unfortunately, the *UGT1* promoter genotype of the original patient is unknown.

Understanding genotype to phenotype interactions is important in counseling patients with

G6PD deficiency. As illustrated by our study, these interactions are complex and dependent on many factors. Describing these mutations and the associated phenotypes is important in order to widen our knowledge of G6PD deficiency.

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References

1. Vulliamy TJ, Luzzatto L. Glucose-6-phosphate dehydrogenase deficiency and related disorders. In: Handin RI, Lux SE, IV, P. ST, eds. Blood. Principles and Practice of Hematology. Philadelphia: Lippincott Williams & Wilkins; 2003:1921-1950
2. Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L. Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. EMBO J. 1995;14:5209-5215.
3. Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group. Bull World Health Organ. 1989;67:601-611.
4. Beutler E. G6PD deficiency. Blood. 1994;84:3613-3636.
5. Longo L, Vanegas OC, Patel M, et al. Maternally transmitted severe glucose 6-phosphate dehydrogenase deficiency is an embryonic lethal. EMBO J. 2002;21:4229-4239.
6. Mason PJ. New insights into G6PD deficiency. Br J Haematol. 1996;94:585-591.
7. Rowland P, Basak AK, Gover S, Levy HR, Adams MJ. The three-dimensional structure of glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* refined at 2.0 Å resolution. Structure. 1994;2:1073-1087.
8. Au SWN, Gover S, Lam VMS, Adams MJ. Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural $NADP^+$ molecule and provides insights into enzyme deficiency. Structure Fold Des. 2000;8:293-303.
9. Beutler E, Blume KG, Kaplan JC, Lohr GW, Ramot B, Valentine WN. International Committee for Standardization in Haematology: recommended methods for red-cell enzyme analysis. Br J Haematol. 1977;35:331-340.
10. Beutler E, Kuhl W, Gelbart T, Forman L. DNA sequence abnormalities of human

glucose-6-phosphate dehydrogenase variants. J Biol Chem. 1991;266:4145-4150.
11. van Wijk R, van Solinge WW, Nerlov C, et al. Disruption of a novel regulatory element in the erythroid-specific promoter of the human *PKLR* gene causes severe pyruvate kinase deficiency. Blood. 2003;101:1596-1602.
12. den Dunnen JT, Antonarakis SE. Nomenclature for the description of human sequence variations. Hum Genet. 2001;109:121-124.
13. Monaghan G, Ryan M, Seddon R, Hume R, Burchell B. Genetic variation in bilirubin UPD-glucuronosyltransferase gene promoter and Gilbert's syndrome. Lancet. 1996;347:578-581.
14. Jones TA, Kjeldgaard M. Electron-density map interpretation. Methods Enzymol. 1997;277:173-208.
15. Notaro R, Afolayan A, Luzzatto L. Human mutations in glucose 6-phosphate dehydrogenase reflect evolutionary history. FASEB J. 2000;14:485-494.
16. Saad STO, Salles TSI, Arruda VR, Sonati MF, Costa FF. G6PD Sumaré: a novel mutation in the G6PD gene (1292 T→G) associated with chronic nonspherocytic anemia. Hum Mutat. 1997;10:245-247.
17. Vulliamy TJ, D'Urso M, Battistuzzi G, et al. Diverse point mutations in the human glucose-6-phosphate dehydrogenase gene cause enzyme deficiency and mild or severe hemolytic anemia. Proc Natl Acad Sci USA. 1988;85:5171-5175.
18. Zimmerman SA, Ware RE, Forman L, Westwood B, Beutler E. Glucose-6-phosphate dehydrogenase Durham: a de novo mutation associated with chronic hemolytic anemia. J Pediatr. 1997;131:284-287.
19. Vulliamy TJ, Kaeda JS, Ait-Chafa D, et al. Clinical and haematological consequences of recurrent G6PD mutations and a single new mutation causing chronic nonspherocytic haemolytic anaemia. Br J Haematol. 1998;101:670-675.
20. Costa E, Cabeda JM, Vieira E, et al. Glucose-6-phosphate dehydrogenase aveiro: a de novo mutation associated with chronic nonspherocytic hemolytic anemia. Blood. 2000;95:1499-1501.
21. Roos D, van Zwieten R, Wijnen JT, et al. Molecular basis and enzymatic properties of glucose 6-phosphate dehydrogenase Volendam, leading to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. Blood. 1999;94:2955-2962.
22. Kaplan M, Hammerman C, Renbaum P, Levy-Lahad E, Vreman HJ, Stevenson DK. Differing pathogenesis of perinatal bilirubinemia in glucose-6-phosphate dehydrogenase-deficient *versus* -normal neonates. Pediatr Res. 2001;50:532-537.
23. Bosma PJ, Chowdhury JR, Bakker C, et al. The genetic basis of the reduced

expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. N Engl J Med. 1995;333:1171-1175.

24. Kaplan M, Renbaum P, Levy-Lahad E, Hammerman C, Lahad A, Beutler E. Gilbert syndrome and glucose-6-phosphate dehydrogenase deficiency: a dose-dependent genetic interaction crucial to neonatal hyperbilirubinemia. Proc Natl Acad Sci USA. 1997;94:12128-12132.
25. Kaplan M, Hammerman C, Beutler E. Heterozygosity for a polymorphism in the promoter region of the UGT1A1 gene. J Hepatol. 2001;35:148-150.
26. Naylor CE, Rowland P, Basak AK, et al. Glucose 6-phosphate dehydrogenase mutations causing enzyme deficiency in a model of the tertiary structure of the human enzyme. Blood. 1996;87:2974-2982.
27. Beutler E, Vulliamy TJ. Hematologically important mutations: glucose-6-phosphate dehydrogenase. Blood Cells Mol Dis. 2002;28:93-103.
28. Hirono A, Kuhl W, Gelbart T, Forman L, Fairbanks VF, Beutler E. Identification of the binding domain for $NADP^+$ of human glucose-6-phosphate dehydrogenase by sequence analysis of mutants. Proc Natl Acad Sci USA. 1989;86:10015-10017.
29. Beutler E, Westwood B, Prchal JT, Vaca G, Bartsocas CS, Baronciani L. New glucose-6-phosphate dehydrogenase mutations from various ethnic groups. Blood. 1992;80:255-256.
30. Saad STO, Costa FF. Mild hemolysis in a girl with G6PD Sumaré (class I variant) associated with G6PD A–. Blood Cells Mol Dis. 2003;30:238-240.
31. Jablonska-Skwiecinska E, Lewandowska I, Plochocka D, et al. Several mutations including two novel mutations of the glucose-6-phosphate dehydrogenase gene in Polish G6PD deficient subjects with chronic nonspherocytic hemolytic anemia, acute hemolytic anemia, and favism. Hum Mutat. 1999;14:477-484.
32. Iwai K, Hirono A, Matsuoka H, et al. Distribution of glucose-6-phosphate dehydrogenase mutations in Southeast Asia. Hum Genet. 2001;108:445-449.
33. Beutler E, Kuhl W, Saenz GF, Rodriguez W. Mutation analysis of glucose-6-phosphate dehydrogenase (G6PD) variants in Costa Rica. Hum Genet. 1991;87:462-464.
34. Beutler E. Discrepancies between genotype and phenotype in hematology: an important frontier. Blood. 2001;98:2597-2602.
35. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Cryst. 1991;24:946-950.
36. Merritt EA, Bacon DJ. Raster3D: photorealistic molecular graphics. Methods Enzymol. 1997;277:505-524.

CHAPTER 7

HK Utrecht: missense mutation in the active site of human hexokinase associated with hexokinase deficiency and severe nonspherocytic hemolytic anemia

Richard van Wijk[1], Gert Rijksen[2], Eric G. Huizinga[3], H. Karel Nieuwenhuis[2], and Wouter W. van Solinge[1]

[1]Department of Clinical Chemistry, University Medical Center Utrecht, Utrecht, The Netherlands, [2]Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands, [3]Department of Crystal & Structural Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands.

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Abstract

Hexokinase deficiency is a rare autosomal recessive disease with a clinical phenotype of severe hemolysis. We report a novel homozygous missense mutation in exon 15 (c.2039C>G, HK [hexokinase] Utrecht) of *HK1*, the gene that encodes red blood cell-specific hexokinase-R, in a patient previously diagnosed with hexokinase deficiency. The Thr680Ser substitution predicted by this mutation affects a highly conserved residue in the enzyme's active site that interacts with phosphate moieties of adenosine disphosphate, adenosine triphosphate (ATP), and inhibitor glucose-6-phosphate. We correlated the molecular data to the severe clinical phenotype of the patient by means of altered enzymatic properties of partially purified hexokinase from the patient, notably with respect to Mg^{2+}-ATP binding. These kinetic properties contradict those obtained from a recombinant mutant brain hexokinase-I with the same Thr680Ser substitution. This contradiction thereby stresses the valuable contribution of studying patients with hexokinase deficiency to achieve a better understanding of hexokinase's key role in glycolysis.

Introduction

Hexokinase (HK) catalyses the phosphorylation of glucose to glucose-6-phosphate (G6P) using adenosine triphosphate (ATP) as a phosphoryl donor. HK-I is the predominant HK isozyme in tissues that depend strongly on glucose use for their physiological functioning, such as brain, muscle and erythrocytes. HK-I displays unique regulatory properties in its sensitivity to inhibition by physiological levels of the product G6P and, moreover, relief of this inhibition by inorganic phosphate (P_i).[1] The recent determination of structures for human[2] and rat[3] HK-I has provided substantial insight into ligand binding sites and subsequent modes of interaction.[2-7]

Erythrocytes contain a specific subtype of HK-I (HKr)[8] that is encoded by the *HK-I* gene.[9] Erythroid-specific transcriptional control results in a unique red blood cell-specific cDNA that differs from HK-I cDNA at the 5'-end.[10-12] Hexokinase deficiency is a rare disease with nonspherocytic hemolytic anemia as the predominant clinical feature. Seventeen families with hexokinase deficiency have been described to date[13] and only one patient has been characterized at the cDNA level.[14] We now report on the molecular defect underlying hexokinase deficiency in a previously reported Dutch family.[15]

Study Design

Patient Z62 was born from a consanguineous marriage, that is, from first cousins, and was originally reported in 1983.[15] She presented with neonatal jaundice and transfusion-dependent hemolysis. Residual HK activity in the patient's erythrocytes, platelets, and lymphocytes was about 25% of normal,[15] in agreement with a generalized HK deficiency caused by a mutation in *HK1*. DNA was isolated from peripheral white blood cells according to standardized procedures. The erythroid-specific promoter, red blood-cell specific exon 1, and exons 2 to 18 of *HK1* of the patient and a healthy control subject were amplified by polymerase chain reaction (PCR) (primer sequences available on request). cDNA nucleotide and amino acid numbering starts at the HK-I start codon, and sequence variations are described according to the mutation nomenclature system.[16] DNA sequence analysis was performed with the appropriate primers as described.[17]

Results and discussion

DNA sequence analysis of *HK1* of the patient and a control subject revealed several base changes compared with the reference sequences (GenBank AF016349-016365) (Table 1).

Table 1. DNA sequence variations in *HK1*.

	Control subject	patient Z62
–426_–424insG[a]	$(G)_4$	$(G)_4$
IVS1+66C>G[b]	G/G	G/G
IVS1+85C>G	G/G	G/G
Exon 2, c.78G>C (Leu25)	G/C	C/C
IVS12+31G>A	A/A	A/A
Exon 15, c.2039C>G (Thr680Ser)	C/C	G/G
IVS15+27C>T	C/T	C/C
IVS17+41T>C	C/C	C/C

[a] Relative to the HKr initiator methionine (GenBank AF029306); [b] relative to HKr exon 1 (GenBank AF016349).

Base variations for which both the patient and control subject were homozygous could represent either sequence discrepancies or polymorphisms. The c.78G>C and IVS15+27C>T are postulated dimorphic changes for Leu25 and an intronic polymorphism, respectively. The c.2039C>G missense mutation in exon 15, however, was a likely candidate

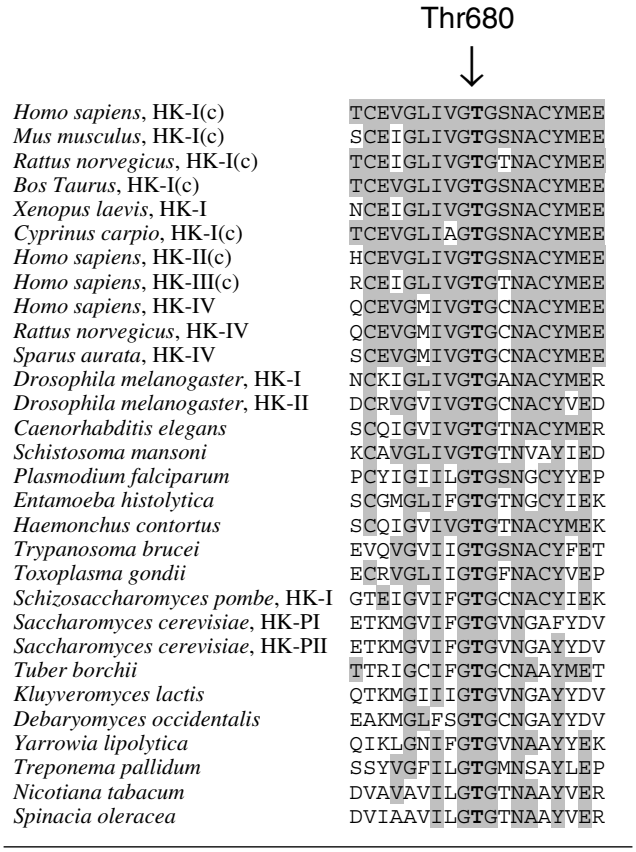


Figure 1. Conservation of Thr680. Amino acid alignment of the region surrounding human HK-I residue Thr680 among 30 hexokinases. Where applicable, specific isozymes are denoted. Conserved residues compared with human HK-I are in shaded gray. The "c" in parentheses refers to the C-terminal domain of HK.

to cause hexokinase deficiency because the consequent Thr680Ser substitution it encodes affects a critical residue in the active site[2,3,7,18] that is, moreover, evolutionary conserved (Figure 1). Subsequent screening of the patient's family for c.2039C>G affirmed cosegregation of this mutation with lowered HK activity (Figure 2) whereas in a healthy control population (n = 50), this mutation was not encountered (not shown). We designated the variant HK found in this family HK Utrecht.

Structural changes due to a conservative threonine-to-serine substitution are likely to be limited since serine can form the same hydrogen bonds as threonine. However, the consequent removal of the methyl group could affect hydrogen bond formation by increasing

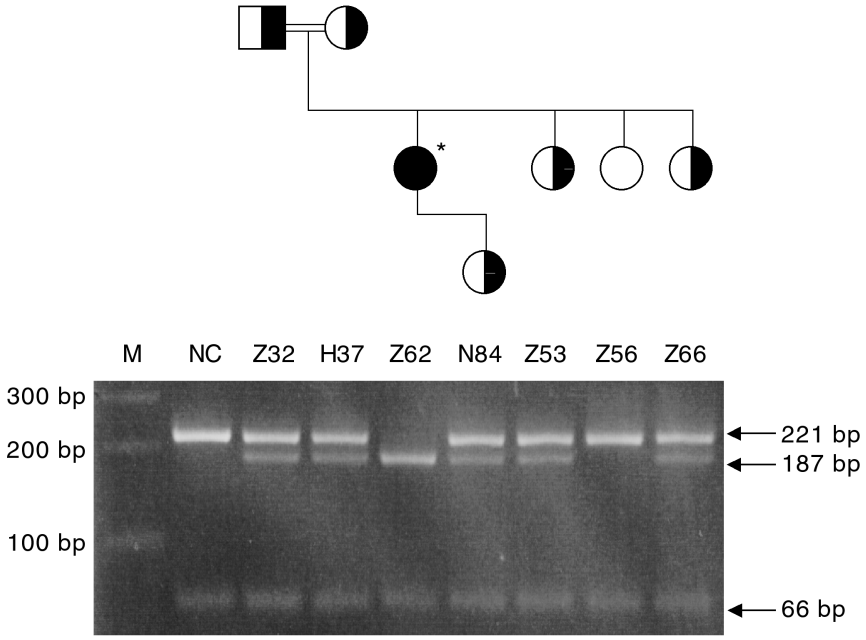


Figure 2. Cosegregation of the novel c.2039C>G (Thr680Ser) missense mutation with lowered HK activity. In the family pedigree chart, individuals heterozygous or homozygous for the c.2039C>G mutation are indicated by half-filled and filled symbols, respectively. Symbols represent the corresponding family members in the bottom part of the figure. Lanes in the agarose gel for each family member are directly below the symbol for that individual. The agarose gel below shows a 301-bp PCR product encompassing exon 15, which was amplified from genomic DNA and subjected to *Aci*I digestion. c.2039C>G creates an additional restriction site upon the two normally present in this fragment. Thus, digestion of the wild-type allele results in fragments of 14, 221, and 66 bp, whereas the extra *Aci*I recognition sequence yields additional fragments of 34 and 187 bp. Digestion fragments are indicated by arrows on the right (14 and 34 bp fragments are not shown). Homozygosity for c.2039C>G is confirmed in patient Z62. The patient's father (Z32), mother (H37), sisters (Z53 and Z66),[15] and daughter N84 were all heterozygous, whereas sister Z56 didn't carry the mutation. All family members heterozygous for the Thr680Ser substitution displayed reduced HK activity in their red blood cells, ranging from 0.64–0.89 U/gHb (reference value: 1.34 ± 0.42 U/gHb).[15] HK activity for sister Z56 was 1.22 U/gHb. *indicates Z62; M, marker; NC, healthy control.

the rotational freedom of the side chain or, alternatively, by introducing a water molecule at the site previously occupied by the methyl group. Small conformational changes could thus have a significant effect on catalysis, because enzymes typically require a delicate balance of interactions for optimal activity.

In view of its central position in the active site (Figure 3), Thr680 has been proposed to hydrogen bond to the β- and γ-phosphoryl group of ATP during catalysis.[7] Thus, altered binding of phosphate-containing ligands is likely an important factor in deficient enzyme function. Indeed, enzymatic properties of partially purified mutant red cell HK from the

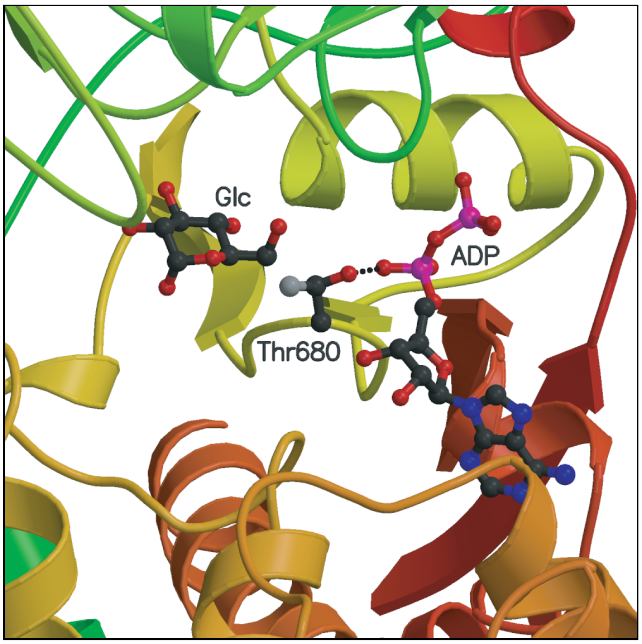


Figure 3. Thr680 is a critical residue in the active site of HK (color version, addendum page 169). Schematic drawing of the active site of HK-I shows Thr680, bound glucose, and adenosine diphosphate (ADP) in ball-and-stick representation (atom coloring: carbon black; oxygen, red; nitrogen, blue; and phosphor, magenta). The carbon atom that is absent in the Thr680Ser mutant is colored gray. Illustrated is the central position occupied by Thr680 in the active site where it is located at the tip of a loop and makes a hydrogen bond with the α-phosphoryl group of ADP. This figure was generated from the atomic coordinates of the HK-I ADP/glucose complex[7] (protein data bank entry 1DGK) using computer programs Molscript[22] and Raster3D.[23]

patient showed near normal affinity for glucose[15] but a 2-fold decrease in affinity for Mg^{2+}-ATP and a markedly (3- to 9-fold) decreased affinity for inhibitor glucose-1,6,-diphosphate.[15] Surprisingly, these results contradict with kinetic data obtained from a recombinant human brain HK-I Thr680Ser mutant as expressed in *Escherichia coli*.[18] The k_{cat} value of this mutant decreased only 2.5-fold and was, in fact, characterized solely by a slightly higher affinity for glucose, whereas the K_m(ATP), K_i(ATP), and K_i for the G6P analog 1-5-anhydroglucitol-6-phosphate were similar when compared to wild-type HK-I.[18] These unexpected discrepancies must be attributed either to the different sources of the enzyme, that is, *E. coli* versus human red blood cells, or to the different N-terminal ends of HKr and HK-I. It highlights the differences in fate and function of mutant enzyme in vivo as underscored by a recent case of glucose-6-phosphate dehydrogenase deficiency.[19]

The 2-fold higher K_m for Mg^{2+}-ATP[15] reflects a relatively modest effect of the Thr680Ser

substitution on ATP binding to the active site. This change in K_m may not affect *in vitro* HK activity, which is measured at excess Mg^{2+}-ATP (5 mM),[20] but may have a significant effect *in vivo* because intracellular ATP concentrations are much lower (0.6 mM)[21] and similar to the K_m. In the study conducted by Ouwerkerk et al[21], blood from patient Z62 was used to measure intracellular Mg^{2+}-ATP concentrations in oxygenated and deoxygenated red blood cells. In oxygenated red blood cells, Mg^{2+}-ATP concentrations were slightly higher in the patient (1.04 mM) when compared with the healthy control (0.61 mM) and equal to the reticulocyte control (1.11 mM).[21] Likewise, in deoxygenated red blood cells comparable Mg^{2+}-ATP concentrations were measured in the patient (0.57 mM) and healthy control (0.62 mM),[21] which, in fact, denotes a strongly decreased Mg^{2+}-ATP concentration, when compared to a reticulocyte control (1.79 mM)[21] and considering the high degree of reticulocytosis (40–50%) in the patient. Therefore, we postulate that at physiological concentations of Mg^{2+}-ATP, the 2-fold increase in K_m for Mg^{2+}-ATP results in a significantly decreased HK activity in the patient's red blood cells, which is reinforced by deoxygenation. These considerations are in agreement with the severe clinical phenotype as observed in patient Z62 and even true if HK is expressed and maintained at a normal level. The residual (*in vitro*) activity of 25%, however, suggests an additional effect on either protein expression or enzyme stability.

The identification of a homozygous c.2039C>G (Thr680Ser) missense mutation in patient Z62 places previously determined enzymatic properties of the mutant enzyme into perspective and stresses the valuable contribution of studying patients with HK deficiency toward a better understanding of the key role played by HK in the glycolytic pathway and, in particular, in red cell metabolism.

References

1. Wilson JE. Hexokinases. Rev Physiol Biochem Pharmacol. 1995;126:65-198.
2. Aleshin AE, Zeng C, Bourenkov GP, et al. The mechanism of regulation of hexokinase: new insights from the crystal structure of recombinant human brain hexokinase complexed with glucose and glucose-6-phosphate. Structure. 1998;6:39-50.
3. Mulichak AM, Wilson JE, Padmanabhan K, Garavito RM. The structure of mammalian hexokinase-1. Nat Struct Biol. 1998;5:555-560.
4. Aleshin AE, Zeng C, Bartunik HD, Fromm HJ, Honzatko RB. Regulation of hexokinase I: crystal structure of recombinant human brain hexokinase complexed

with glucose and phosphate. J Mol Biol. 1998;282:345-357.

5. Fang T-Y, Alechina O, Aleshin AE, Fromm HJ, Honzatko RB. Identification of a phosphate regulatory site and a low affinity binding site for glucose 6-phosphate in the N-terminal half of human brain hexokinase. J Biol Chem. 1998;273:19548-19553.
6. Sebastian S, Wilson JE, Mulichak A, Garavito RM. Allosteric regulation of type I hexokinase: A site-directed mutational study indicating location of the functional glucose 6-phosphate binding site in the N-terminal half of the enzyme. Arch Biochem Biophys. 1999;362:203-210.
7. Aleshin AE, Kirby C, Liu X, et al. Crystal structures of mutant monomeric hexokinase I reveal multiple ADP binding sites and conformational changes relevant to allosteric regulation. J Mol Biol. 2000;296:1001-1015.
8. Murakami K, Blei F, Tilton W, Seaman C, Piomelli S. An isozyme of hexokinase specific for the human red blood cell (HK_R). Blood. 1990;75:770-775.
9. Andreoni F, Ruzzo A, Magnani M. Structure of the 5' region of the human hexokinase type I (HKI) gene and identification of an additional testis-specific HKI mRNA. Biochim Biophys Acta. 2000;1493:19-26.
10. Murakami K, Piomelli S. Identification of the cDNA for human red blood cell-specific hexokinase isozyme. Blood. 1997;89:762-766.
11. Ruzzo A, Andreoni F, Magnani M. Structure of the human hexokinase type I gene and nucleotide sequence of the 5' flanking region. Biochem J. 1998;331:607-613.
12. Murakami K, Kanno H, Miwa S, Piomelli S. Human HK_R isozyme: organization of the hexokinase I gene, the erythroid-specific promoter, and transcription initiation site. Mol Genet Metab. 1999;67:118-130.
13. Kanno H. Hexokinase: gene structure and mutations. Baillieres Best Pract Res Clin Haematol. 2000;13:83-88.
14. Bianchi M, Magnani M. Hexokinase mutations that produce nonspherocytic hemolytic anemia. Blood Cells Mol Dis. 1995;21:2-8.
15. Rijksen G, Akkerman JW, van den Wall Bake AW, Hofstede DP, Staal GE. Generalized hexokinase deficiency in the blood cells of a patient with nonspherocytic hemolytic anemia. Blood. 1983;61:12-18.
16. Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat. 2000;15:7-12.
17. van Wijk R, Nieuwenhuis K, van den Berg M, et al. Five novel mutations in the gene for human blood coagulation factor V associated with type I factor V deficiency. Blood. 2001;98:358-367.

18. Zeng C, Aleshin AE, Hardie JB, Harrison RW, Fromm HJ. ATP-binding site of human brain hexokinase as studied by molecular modeling and site-directed mutagenesis. Biochemistry. 1996;35:13157-13164.
19. Hirono A, Kawate K, Honda A, Fujii H, Miwa S. A single mutation 202G>A in the human glucose-6-phosphate dehydrogenase gene (*G6PD*) can cause acute hemolysis by itself. Blood. 2002;99:1498.
20. Rijksen G, Staal GE. Purification and some properties of human erythrocyte hexokinase. Biochim Biophys Acta. 1976;445:330-341.
21. Ouwerkerk R, van Echteld CJ, Staal GE, Rijksen G. Intracellular free magnesium and phosphorylated metabolites in hexokinase- and pyruvate kinase-deficient red cells measured using ^{31}P-NMR spectroscopy. Biochim Biophys Acta. 1989;1010:294-303.
22. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Cryst. 1991;24:946-950.
23. Merritt EA, Bacon DJ. Raster3D: photorealistic molecular graphics. Methods Enzymol. 1997;277:505-524.

CHAPTER 8 Summary, general discussion, and future prospects

The clinical hallmark of the patients described in this thesis is chronic hemolysis. Regardless of the kind of underlying inherited enzymopathy, the here described deficiencies of hexokinase (HK), pyruvate kinase (PK), and glucose-6-phosphate dehydrogenase (G6PD) ultimately resulted, albeit to a various extent, in shortened red blood cell survival because "metabolically defective red blood cells are old before their time, and suffer from metabolic progeria" (Valentine W.N. and Paglia D.E. Blood Cells. 1980; 6: 819-829). All three mentioned enzymes are key enzymes of the Embden-Meyerhof Pathway (HK and PK) and the Hexose Monophosphate Shunt (G6PD). The underlying mechanisms by which these deficiencies cause hemolysis, however, are distinct. The main cause of hemolysis in either HK or PK deficieny is a depletion of the cellular ATP content whereas the G6PD-deficient erythrocyte is more susceptible to oxidative damage. To induce a clinically significant hemolysis, red blood cell enzyme function must be significantly impaired under physiological conditions. Thus, in compound heterozygous patients overt hemolysis assures that, at least, one gene product is severely defective. The focus of this thesis was to identify the molecular mechanisms by which inherited enzymopathies lead to impaired enzyme function. This constitutes the primary basis of the associated hemolytic disease. The molecular defects underlying the above-mentioned enzyme deficiencies were found to affect correct human gene expression **(chapter 1)** either at the level of transcriptional regulation **(chapter 2)**, pre-mRNA processing **(chapter 3)**, or protein structure **(chapters 4 and 5)** and function **(chapters 6 and 7)**.

Enzymopathy due to defects at the level of transcriptional regulation

In **chapter 2** we described a severely PK-deficient patient. In reticulocyte RNA from this patient we detected a monoallelic pattern of gene expression. This led to the identification of a novel transcriptional regulatory element in the erythroid-specific promoter of *PKLR*. RNA from the patient demonstrated sole expression of the paternal c.1529G>A missense *PKLR* allele. On the DNA level, however, the boy was heterozygous for this missense mutation. Moreover, 3 novel *in cis* DNA sequence variations were identified on the maternal allele in the erythroid-specific promoter of *PKLR*: –324T>A, –248delT, and –83G>C. We established the functional consequences of each individual mutation using *in vitro* transfection assays in K562 cell line. Hence, we demonstrated that –324T>A was a non-functional mutation whereas –248delT represented a non-functional polymorphism. This polymorphism had previously been associated with PK deficiency by others because it disrupts a binding site for the erythroid-specific transcription factor GATA-1. We clearly demonstrated, however, that the concerning GATA-1 binding site was non-functional. The –83G>C mutation caused effective downregulation of the *PKLR* promoter, thereby explaining the pattern of

monoallelic gene expression of the *in trans* allele. Systematic mutagenesis of the region surrounding nt –83 disclosed the presence of a novel *cis*-regulatory element, which we designated PKR-RE1. To confirm that this novel element was actively involved in DNA-protein interaction we performed electrophoretic mobility shift assay using K562 nuclear extract. These experiments demonstrated *in vitro* DNA-protein interaction at the core of PKR-RE1, involving an as-yet-unidentified *trans*-acting factor. Because no known protein is known to recognize the PKR-RE1 motif, we concluded that PKR-RE1 may be involved in a novel mechanism of erythroid-specific *trans* activation, mediating the effects of factors necessary for regulation of PK gene expression during red cell differentation and maturation. The monoallelic expression of the 1529A allele rendered this particular patient 'pseudo-homozygous' for c.1529G>A, a missense mutation that predicts the substitution of arginine by glutamine at residue 510. It was, therefore, reasonable to assume that the PK-deficient phenotype, *i.e.* severe, transfusion-dependent hemolytic anemia, of the patient described in **chapter 2** could be attributed to the sole synthesis of the Arg510Gln PK-R variant. As demonstrated in **chapter 5**, the introduction of a glutamine residue at position 510 in the A/C domain interface causes reduced conformational freedom between these two domains. This may explain the very low activity and striking instability that is usually associated with this common PK-deficient variant. It is of interest that the severe clinical picture of the 'pseudo-homozygous' patient in **chapter 2** was remarkably different from that of patient MB86, described in **chapter 4**. The latter, mildly affected patient was homozygous for the c.1529G>A mutation and had never been transfusion-dependent. This may indicate that in spite of the envoked structural perturbation, some residual enzymatic function is still maintained in the Arg510Gln PK-R variant. Thus, the amount of residual enzymatic activity would be higher in the homozygous patient because about twice the amount of aberrant protein is synthesized from the two defective alleles whereas in the 'pseudo-homozygous' patient only one allele is actively involved in protein synthesis.

Chapter 2 emphasizes the need for an as-complete-as-possible molecular characterization in general. Molecular diagnostic approaches that are confined to the protein coding region of the gene or use only cDNA transcribed from a patient's RNA, in this case would either have missed the transcriptional mutant or misdiagnosed the patient as homozygous for the c.1529G>A missense mutation.

Enzymopathy due to defects at the level of pre-mRNA processing

The synthesis of only one species of mutant PK, in spite of a compound heterozygous genotype, was also demonstrated in another patient with severe PK deficiency. In this case the production of mutant Arg479His PK-R was due to aberrant processing of *PKLR* pre-

mRNA. This patient, described in **chapter 3**, was compound heterozygous for two mutations which were both likely to affect correct splicing. One novel mutation abolished the invariant dinucleotide of intron 5 (IVS5+1G>A) whereas the other mutation (c.1436G>A) altered the exonic consensus sequence of IVS10 and, in addition, encoded an arginine to histidine substitution at residue 479. A novel approach was employed to study the effects of both mutations on pre-mRNA processing. The main feature of this approach constituted the use of *ex vivo* produced nucleated erythroid cells from the patient as a source of RNA. This approach allowed for a more profound analysis of the effects on pre-mRNA processing, as demonstrated by the detection of multiple aberrant transcripts, associated with both mutations but also, for the first time, in wild-type *PKLR* pre-mRNA. As anticipated, one of the predominant transcripts associated with the IVS5+1G>A mutation lacked the upstream exon 5. Unexpected, however, was the identification of the second major transcript associated with this mutation. This nonsense transcript lacked both exons 5 and 6 (see below). No functional protein was expected nor detected (by Western blotting) from either transcript, which rendered the IVS5+1A allele *de facto* a null allele. The c.1436G>A mutation in this patient was mainly associated with a strong reduction in transcript levels although the majority of these transcripts were normally spliced. Such a strong reduction of transcripts may be the reflection of increased instability of the major part of mutant transcripts derived from the 1436A allele. However, because the processes of transcriptional regulation and pre-mRNA processing are intimately related, an additional effect of the c.1436G>A mutation on transcription cannot be excluded. Altogether, the PK-deficient phenotype in this patient could mainly be attributed to the presence of strongly reduced amounts of Arg479His PK-R. We confirmed this by Western blot. The sole presence of Arg479His PK-R in this patient enabled a direct comparison with the crystal structure and enzymatic properties of the same recombinant PK variant as expressed in *Escherichia coli*. The structural data were in agreement with our own observations but a striking difference was noted with regard to the heat stability of both mutant PK variants. We had demonstrated that the patient's PK showed reduced thermal stability *in vitro*. This was in agreement with a remarkable sensitivity to heat displayed by the patient during febrile episodes or even prolonged exposure to sunlight. These two observations suggested a decreased stability of the Arg479His PK tetramer. The recombinant Arg479His PK variant, however, showed nearly unaffected thermal stability. This discrepany resembles a similar contradiction between enzymatic properties of mutant human enzyme and its recombinant analog observed in a case of hexokinase deficiency **(chapter 7)** and will be further discussed below (***Enzymopathy due to defects at the protein level – protein function***). It emphasized, however, that knowledge obtained from recombinant (mutant) enzymes should be interpreted

with care when applied to their human analogs.
As mentioned above, the composition of one transcript identified in the patient described in **chapter 3** was quite unusual. During pre-mRNA processing, two consecutive exons (exons 5 and 6) had been simultaneously skipped from the pre-mRNA transcribed from the allele with the IVS5+1G>A mutation. These two events were likely related and led us to propose a mechanism in which correct processing of exon 6 in wild-type *PKLR* pre-mRNA depends on the presence of exon 5 in the primary transcript. In other words, in wild type *PKLR* pre-mRNA, exon 5 contains regulatory splicing elements necessary for proper processing of exon 6. It is obvious that due to a mutation at the intron 5 donor splice site and the subsequent removal of the entire exon 5, the putative regulatory elements were automatically skipped from the pre-mRNA, leading to inefficient inclusion of the sixth exon. The presence of a splice enhancer element in *PKLR* exon 5 automatically implies that other base changes in this exon, for example the missense mutations as described in **chapter 4**, may be capable of disturbing correct pre-mRNA processing. For further discussion, see ***Future prospects***.

Enzymopathy due to defects at the protein level – protein structure
The major molecular defect underlying the hereditary enzymopathies are single-base changes that predict the substitution of a structurally and functionally important amino acid. This is especially true in case of PK and G6PD deficiency **(chapter 1)** and our results obtained from a large group of patients with PK deficiency, described in **chapter 4**, are in agreement with this observation of missense mutations being predominant. The 28 patients studied and reported in **chapter 4** originated from 26 different families. We identified 24 different alleles associated with PK deficiency and 14 of these mutations were novel. Of the 24 mutant allels, 20 carried a missense mutation (83%), including 12 novel ones. This is slightly higher than the global distribution of missense mutations which is approximately 70% **(chapter 1)**. The four remaining mutations were nonsense mutations (two mutations, including one novel) or predicted to affect pre-mRNA processing (two mutations, including one novel). The frequently detected c.331G>A mutation was of particular interest. This mutation predicts the unfavorable subsitution of glycine by arginine at residue 111 (PK Utrecht) and was identified in five unrelated Dutch families. This meant that the novel c.331G>A mutation constituted a major cause of PK deficiency in The Netherlands. More specific, together with the common mutations c.1456C>T and c.1529G>A, c.331G>A accounted for nearly 50% of mutant *PKLR* alleles in The Netherlands. When applied to diagnostic procedures, we recommend that an efficient initial screening for *PKLR* mutations in Dutch PK-deficient patients should include these 3 mutations.
Ideally, the effects of missense mutations on protein structure and function should be

examined using purified mutant PK from the patient. However, apart from the practical limitations, a mutant enzyme purified from red blood cells of the patient may be unstable or lack enzymatic activity, thereby hampering further biochemical characterization. The variant G6PD Utrecht described in **chapter 6** is an illustration of the latter. Furthermore, in case of PK deficiency most patients are compound heterozygous. Hence, when both genes are actively producing a mutant monomer, five different tetrameric forms can be assembled. This limits the value of biochemical characterization considerably. An alternative approach comprises the expression of the recombinant mutant enzyme. In that case, especially with regard to the evaluation of enzymatic properties, **chapters 3 and 7** have demonstrated that significant differences in enzymatic behaviour may be observed when comparing recombinant human enzymes with enzymes obtained from the patient. An initial approach to elucidate the mechanism by which amino acid substitutions may lead to impaired enzymatic function involves the evaluation of the concerning residue and the interactions in which it is involved in the three-dimensional model of the crystal structure. The tetrameric crystal structure of human erythrocyte R-state PK has recently been elucidated and we used this model in **chapter 4** to study the consequences of the novel amino acid changes on structure and function of PK. By doing so we obtained additional structural support to affirm the association of the respective mutations with PK deficiency. The effects of some mutations were quite clear because they concerned the substitution of amino acids buried in the hydrophobic core of the protein. Others altered the conformation of the B domain near the active site or directly affected the ATP binding site. Provided that stable protein is synthesized, these mutations may significantly alter PK enzymatic activity. Because the allosteric regulation of PK is considered to be regulated by interdomain and intersubunit interactions **(chapter 1)**, a group of mutations that affected those regions were of particular interest. One of these amino acid changes altered the C/C' subunit interface, Arg569Gln, whereas three mutations predicted a disruption of the A/A' subunit interface: Arg337Trp, Gly358Glu, and Arg385Lys. More specific, the mutations regarding residues Arg337 and Arg385 may disrupt the formation of salt bridges, crucial for the T- to R-state allosteric transition of the tetrameric enzym. Again, provided that stable PK monomers are synthesized. The Gly358Glu substitution was the only mutation that could be directly correlated to the patient's phenotype because of homozygosity of this patient for the causative c.1073G>A mutation. The patient was severely affected and transfusion-dependent, thereby confirming the detrimental effects of this mutation at the A/A' subunit interface. Unfortunately, further biochemical characterization of enzymatic properties of this mutant PK was not possible because the, Syrian, patient was not available for further study. Our investigations reported in **chapter 4** confirmed the association of the common

c.1456C>T mutation with, in general, mild clinical expression of PK deficiency. This mutation encodes a PK-R variant characterized by an arginine to tryptophan substitution at residue 486 in the A/C subdomain interface. In this critical region of PK, replacing Arg486 with tryptophan is an amino acid change with potentially dramatic consequences. Interestingly, in the three-dimensional model, a neighbouring residue of Arg486 is Gly364. While the drastic Arg486Trp change causes little structural perturbation and, consequently, leaves the enzymatic properties nearly unaffected, a similar drastic amino acid substitution at residue 364 (Gly364Asp) caused severe PK deficiency and ultimately led to death in a homozygous patient **(chapter 5)**. This illustrates that the impairment of enzymatic function is strongly dependent not only on the location of the replaced amino acid but also on the amino acid with which it is replaced. A similar example is described in **chapter 6** and concerns two different G6PD variants that are both characterized by a perturbation of the same critical region of the dimeric enzyme (see below). In **chapter 5**, the above-mentioned mutation that predicted the Gly364Asp substitution concerned a novel c.1091G>A mutation, identified in the homozygous state in the deceased patient. Because, at that time, the human crystal structure had not yet been elucidated, the predicted structure of human R-state PK-R was developed based on the homologous crystal structures of R-like muscle PK from cat and rabbit. A detailed structural analysis was made of the effects of this mutation by molecular modeling of aspartate at residue 364 of the PK monomer. We were able to demonstrate that, located at the A/C domain interface, aspartate at residue 364 in the A domain interacts with the backbone carbonyl of Gln482 of the C domain and introduces an alternative inter-domain salt bridge with Arg488. As a result, the critical conformation of the A/C interface is disrupted. Our results regarding the inter-domain interactions at the A/C interface being critical for stability of the protein as well as the deleterious effect of the Gly364Asp substitution were recently confirmed by the elucidation of the crystal structure of human Gly364Asp PK-R (Valentini G. *et al*. Journal of Biological Chemistry. 2002; 17: 23807-23814). The homozygous patient described above was the first child from the mother's second marriage. The mother had the misfortune to re-marry a second husband who was, again, heterozygous for PK deficiency. Her first husband was also heterozygous and the child born from this first marriage was compound heterozygous for the c.1091G>A and the c.1529G>A mutations. This child was severely affected and required a blood transfusion every three months at the time this study was conducted. He showed clinical symptoms that were comparable with the clinical symptoms of two other patients from a second family who were also compound heterozygous for the same combination of mutations in *PKLR* **(chapter 5)**. A comparison of these PK deficiency-associated phenotypes may lead to the conclusion that the co-inheritance of the 1529A allele confers some residual enzyme stability or

function that is lost upon inheritance of two 1091A alleles. This is in agreement with the beneficial 'additive effect' of the Arg510Gln PK-R variant as proposed above.

Enzymopathy due to defects at the protein level – protein function

Dramatic changes in the enzyme's structure brought about by significant structural perturbation are obviously also likely to result in altered enzymatic activity, most frequently in a negative manner. The location and interactions of the affected amino acid, as well as the nature of the introduced substitution are important determinants for the resulting molecular perturbation and its impact on protein function. This has been mentioned above with regard to two drastic substitutions in PK (Gly364Asp and Arg486Trp) that led to differently impaired enzyme function, and is further illustrated by the study described in **chapter 6**. In this study, two patients with severe G6PD deficiency were investigated at the molecular level. One patient suffered from severe chronic hemolytic anemia whereas in the other patient only low level, subclinical, chronic hemolysis could be detected. Molecular analysis revealed that both patients carried a variant G6PD gene on their X-chromosomes. The respective mutations had arisen *de novo*, either in early embryonic development or in the maternal germline. The amino acid substitutions Pro409Ser (G6PD Utrecht) and Val431Gly, predicted by the mutations, both affected the G6PD dimer interface. Formation of the G6PD dimer is crucial for G6PD enzymatic activity and nearly all mutations that affect the dimer interface cause the most severe form of G6PD deficiency, the one that is associated with chronic nonspherocytic hemolytic anemia (class I G6PD deficiency) (**chapter 1**). We studied the two mutations in the three-dimensional model of human G6PD. This revealed that Pro409 and Val431 were also located in the dimer interface and moreover, being located on different subunits, interacted directly across the subunit interface. It was shown that Pro409 makes inter-subunit contacts with Tyr424, Tyr428, and Val431, and that mutation to serine would disrupt some of these contacts. Likewise, inter-subunit contacts of Val431 with Pro409 were likely to be disrupted upon mutation of Val431 to glycine. Moreover, the Pro409Ser and Val431Gly mutations both introduced a residue that allows for greater main chain flexibility, which could destabilize subunit interactions. We concluded that both the Pro409Ser and Val431Gly substitutions could weaken dimer interactions, thereby compromising the stability and enzymatic activity of G6PD. On one hand, these observations, when combined with the different phenotypes of both patients, pointed out the importance of both the position as well as the nature of amino acid replacement with regard to the extent of chronic hemolysis. On the other hand, however, they also clearly demonstrated that the ultimate outcome of identical amino acid substitutions with regard to the clinical phenotype is not solely dependent on the amino acid substitution itself because the phenotype of the patient

with the Val431Gly substitution described in **chapter 6**, differed markedly from the only other patient described to date that carried this G6PD variant. This other patient displayed persistent chronic jaundice since birth, splenomegaly, mild chronic anemia, and reticulocytosis. In contrast, the patient we described showed no neonatal jaundice and had been clinically normal until he presented with favism at age 29 months. Therefore, like the observed differences in expression of the Arg510Gln PK-R variant **(chapters 2 and 4)**, we concluded that the clinical picture also depended on other, genetic, factors.

Chapter 6 demonstrates that differences in chronic hemolysis exist among patients with different class I G6PD variants and even among patients with the same G6PD variant. This underscores the need for a re-evaluation of the G6PD deficiency classification system **(chapter 1)** which is based on residual G6PD enzymatic activity and clinical features.

The homozygous PK-deficient patient in **chapter 5** died of severely impaired enyzme function caused by a dramatic perturbation of a critical inter-domain interface. The G6PD-deficient patients described in **chapter 6** displayed different phenotypes upon a drastic molecular perturbation of the G6PD subunit interface. In **chapter 7** we showed that also subtle alterations in crucial areas of the enzyme can cause a severe impairment of enzyme function *in vivo*, leading to chronic hemolysis. The patient described in **chapter 7** had been diagnosed with severe HK deficiency 20 years ago and we recently elucidated the molecular basis underlying this rare case of HK deficiency. Previously, the enzymatic properties of purified mutant HK from the patient had been biochemically characterized and had revealed a two-fold decrease in affinity for Mg^{2+}-ATP and a markedly (3- to 9-fold) decreased affinity for inhibitor glucose-1,6,-diphosphate. We could easily correlate these features to the now established molecular defect in the homozygous patient. We detected a novel c.2039C>G missense mutation which predicted the substitution of active site residue Thr680 by serine (HK Utrecht). Although structural changes due to this conservative substitution were likely to be limited, structural analysis using the three-dimensional model of human HK1 displayed a central position of the absolutely conserved Thr680 in the active site. In the active site, Thr680 interacts with phosphate moieties of ADP/ATP and glucose-6-phosphate. Hence, the removal of a methyl group upon mutation from threonine to serine could cause small conformational changes, resulting in altered binding of phosphate-containing ligands and illustrating the delicate balance of interactions that enzymes typically require for optimal activity. The only 2-fold higher K_m for Mg^{2+}-ATP reflected the relatively modest effect of the Thr680Ser substitution on ATP binding to the active site. We anticipated a more significant effect *in vivo*, however, because of the severe clinical picture of the patient. A plausible explanation was provided by intracellular Mg^{2+}-ATP concentrations of the patient, as measured in previous studies. These concentrations were much lower *in vivo*, and close to

the K_m, than the excess Mg^{2+}-ATP concentrations used for HK activity measurements *in vitro*. We postulated that at physiological concentrations of Mg^{2+}-ATP, the two-fold increase in K_m for Mg^{2+}-ATP results in significantly decreased HK activity in the patient's red blood cells.

Strikingly, again the enzymatic properties of the human red blood cell HK contradicted with published kinetic data obtained from a recombinant human brain HK (HK-I) that harboured the same mutation. The recombinant mutant HK was characterized solely by a slightly higher affinity for glucose, whereas the K_m(ATP), and K_i(G6P) were unaffected. These unexpected discrepancies in kinetic behaviour between human mutant red blood cell HK and recombinant mutant HK-I may be attributed to the different sources of the respective enzyme, *i.e. Escherichia coli versus* human red blood cells. An alternative explanation, however, may be provided by the differences in N-terminal ends of red blood cell HK and HK-I. Human red blood cell HK and HK-I are transcribed from the same gene and both proteins are identical except for their N-terminal ends. Similarly, human PK-R and PK-L are transcribed from the same gene and both proteins are identical except for a unique peptide that is only present at the N-terminal end of PK-R. The differences in enzymatic properties between the two HK mutants is reminiscent of similar discrepancies between two Arg479His PK-R mutants as observed in **chapter 3**. In this case, to avoid partial proteolysis of the recombinant enzyme, a truncated mutant PK-R was produced. This protein lacked 49 amino acids from its N-terminal end, including the erythroid-specific peptide. Although *in vitro* enzymatic activity was essentially unaffected in case of the truncated PK-R (*de facto* PK-L), the observed discrepancies between two otherwise identical proteins might point at a functional role for the erythroid-specific N-terminal peptides of HK and PK *in vivo*.

It has been suggested that in PK, structure and function are so finely tuned that even moderate molecular alterations may significantly perturb cell metabolism. Among the single amino acid changes described in **chapter 4**, one was postulated to cause little, if any, structural perturbation. This concerned a conservative leucine to valine substitution at the surface of the protein. The underlying mutation, c.814C>G (Leu272Val), was detected by chance in a asymptomatic individual who, moreover, displayed normal PK enzymatic activity. Enzymatic activities as determined under artificial conditions in the laboratory, may not always truely reflect the behaviour of aberrant enzymes *in vivo*, and the Leu272Val PK-R variant may cause a very mild form of PK deficiency. The individual carrying this variant originated from an African country, Ghana, and in this area of the world, (severe) PK deficiency is very rare. Interestingly, very recently it was reported that PK deficieny in mice protects against malaria (Min-Oo, G. *et al*. Nature Genetics. 2003; 35: 357-362). The defense mechanism itself remains unclear but the PK-deficient mice displayed splenomegaly,

constitutive reticulocytosis, and were resistant against *Plasmodium chabaudi* infection. Thus, PK deficiency contributes to the complex genetic control mechanism in humans that controls the susceptibility of infection with *Plasmodium* species as well as the ultimate outcome of the disease. It is therefore tempting to speculate that the Leu272Val substitution, as identified in the Ghanese individual, represents a PK-R variant which is characterized by only slightly modified enzymatic properties, such that cellular survival is ensured but infection with *Plasmodium* species is withstood. Perhaps this is achieved in conjunction with other alleles that alter red blood cell metabolism and that have been retained by positive selection in areas of the world where malaria is an endemic disease. These other alleles include sickle-cell anemia, β-thalassemia, and G6PD deficiency.
It is worth noting that the PK deficiency itself in the malaria-resistant mice could be attributed to a single Ile90Asn amino acid substitution, which was encoded by the c.269T>A missense mutation. Coincidentally, we had identified the same missense mutation in two, related, compound heterozygous patients with severe PK deficiency, described in **chapter 4**.

Future prospects
Several aspects of the studies conducted in this thesis may be the subject of future research. Of considerable interest is the identification of PKR-RE1, a novel transcriptional regulatory element in the erythroid-specific promoter of *PKLR*, described in **chapter 2**. Electrophoretic mobility shift assay has demonstrated the binding of a *trans*-acting factor to this element and transfection experiments have demonstrated its functional importance. Obviously, a major aim for further study will be the identification of the putative transcription factor. Thus far, the core binding motif resembles no known transcription factor binding elements. This could imply that a novel *trans*-acting factor is involved or, alternatively, a known factor with yet unrecognized binding properties. The putative transcription factor has been demonstrated to play a crucial role in *PKLR* erythroid-specific transcription but may do so also in other, erythroid, genes. Future research should, therefore, consider a broad perspective and include other, erythroid-specific, promoters.
Discussing the putative aberrant effects of enzyme function is based upon the assumption that the respective protein is actually synthesized and stable enough *in vivo* to allow dimer (G6PD) or tetramer (PK) assembly. However, numerous examples are available in the literature that show that mutants proteins can be unstable. For a comprehensive understanding of the effect of a mutation, the quantitative determination of (mutant) protein would be of surplus value. Another important factor to consider in this respect is the, additional, effect that mutations may have on pre-mRNA processing. Such putative effects on RNA splicing have always been a major focus in our research and, if possible, the

appropriate experiments have always been conducted to exclude any such additional effect **(chapters 5 and 7)**. Regarding the results of the RNA experiments conducted in **chapter 3**, we hypothesized the presence of an exonic splice enhancer element (ESE) in *PKLR* exon 5, required for the efficient inclusion of exon 6 in wild-type *PKLR* pre-mRNA. Future experiments using recombinant constructs and *in vitro* splicing assays may aim to confirm and identify the putative ESE(s) in *PKLR* exon 5, and the exact sequence motif(s) it comprises. The importance of considering additional effects on pre-mRNA processing is underscored by the recent general appreciation of the role of ESEs in constitutive splicing. Because ESEs constitute degenerate sequence motifs that may be spread throughout the exon, their important functional role implies that every other mutation that does not directly disrupt any splice site consensus sequence may still be a candidate mutation to affect correct pre-mRNA processing. In fact, the number of missense and synonomous mutations that does so is increasing. Because RNA was unavailable from most patients described in **chapter 7**, additional effects on pre-mRNA processing have not been taken into account. Especially with regard to amino acid substitutions Val134Asp, Ala154Thr, and Gly165Val which are predicted by missense mutations in *PKLR* exon 5, it should be excluded that the identified mutations do not cause additional effects on pre-mRNA processing. Considering the fact that ESEs may be present in many, if not all, human exons, a comprehensive approach to investigate the mechanism of disease should include RNA analysis.

The sophisticated allosteric mode of regulation of PK complicates the evaluation of the effect of molecular pertubation on the enzyme's structure and function. A limitation in this respect is the often-mentioned fact that no T-state and R-state crystal structures are available from the same species. The latter would enable a more profound analysis of the effects of the appropriate mutations in both the T- and R-state enzymes, like for instance the amino acid substitutions Arg337Trp and Arg385Lys described in **chapter 4**. The recent elucidation of the crystal structure of R-state human erythrocyte PK has significantly contributed to a better understanding of the structure and function of PK. It would be of even greater benefit to the scientific community if both human crystal structures would become available.

In Africa, (severe) PK deficiency is very rare but malaria is an endemic disease. The recent report that PK deficiency in mice protects against malaria combined with the identification of a novel, putatively mild, Leu272Val PK-R variant in an individual from Ghana **(chapter 4)** may indicate that the two are connected. The c.814C>G mutation (Leu272Val) was not detected in a Caucasian normal control population and it will be of interest to screen a representative African control population. This may resolve the question whether the African *PKLR* 814G allele is either very rare, or a polymorphic variant that has remained undetected because of only subtle effects on enzymatic activity.

Twice, discrepancies were observed between the enzymatic properties of mutant enzymes (PK and HK) obtained either from the patient's enzyme or from a recombinant enzyme **(chapters 3 and 7)**. These differences may be attributed to the different sources of the enzyme but it is intriguing that both recombinant enzymes lacked the erythroid-specific N-terminal peptide. This may indicate that the N-terminal peptide has a functional role *in vivo* and future studies may be directed towards the elucidation of this putative role.

The only cure available to patients with an inherited enzymopathy is bone marrow transplantation. This is only rarely performed because of its negative side effects. Instead, blood transfusion is the therapy of choice to alleviate the anemia and to protect patients from fatal hemolysis. With regard to PK, the PK-M2 isozyme is gradually replaced by the red blood cell-specific PK-R isozyme during erythroid differentiation and maturation **(chapter 1)**. Persistent PK-M2 expression throughout erythroid maturation might be able to compensate, in part, for a defective PK-R isozyme and thereby ameliorate the hemolysis. Patients with sickle-cell anemia display a similar amelioration of clinical symptoms due to persistent expression of fetal hemoglobin (HbF). In certain PK-deficient patients, a persistent expression of PK-M2 has been detected but the beneficial effect is unclear. Also, the reason why this 'compensation' occurs in some patients but not in others is unknown. From a therapeutical point of view it might be worth to first resolve these questions, followed, if appropriate, by a detailed analysis of the transcriptional control mechanisms that drives PK-M2 expression throughout erythroid differentiation and maturation. Eventually, this may lead to clues regarding the means to upregulate PK-M2 expression in patients with severe PK deficiency.

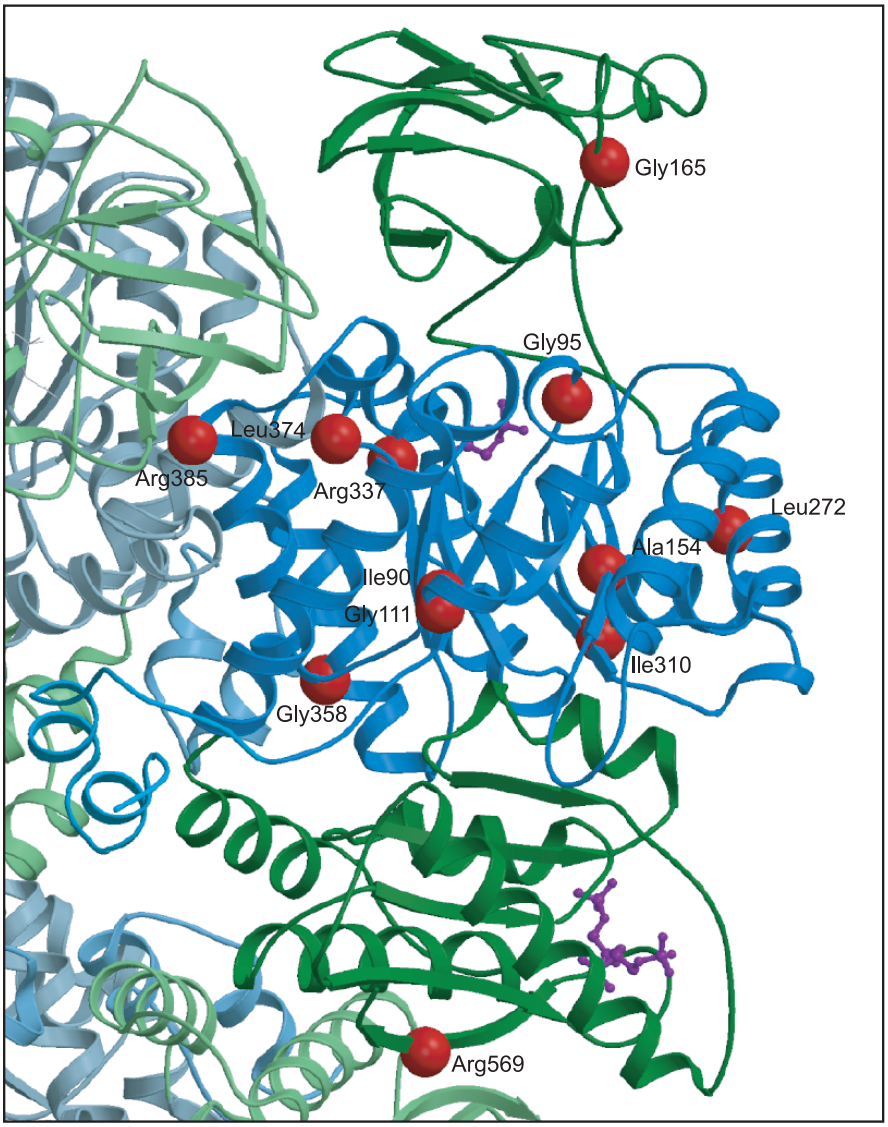
Concluding remarks

The variety of clinical features associated with the various enzymopathies, regardless of the underlying molecular mechanism, have unequivocally made clear that the phenotype of hereditary red blood cell enzymopathies, and perhaps genetic disease in general, is not solely dependent on the molecular properties of mutant proteins but rather reflects a complex interplay between physiological, environmental and other (genetic) factors. Putative phenotypic modifiers include differences in genetic background, concomitant functional polymorphisms of other glycolytic enzymes (many enzymes are regulated by their product or other metabolites), post-translational modification, ineffective erythropoiesis, and different splenic function.

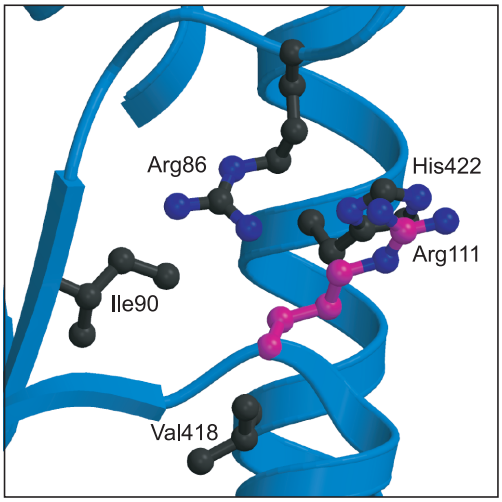
It was the focus of this thesis, as a first step towards a better understanding of the correlation between genotype and phenotype, to identify the molecular basis of disease in hereditary human red blood cell enzymopathies. In summary, the investigated mutations cause disease

by means of a variety of mechanisms and they affect several aspects of gene expression. They may either abolish gene expression completely **(chapter 2)**, or lead to aberrant RNA splicing events **(chapter 3)**. Once a mutant enzyme is synthesized it may be unstable because of significant structural perturbation **(chapters 4 and 5)**, or unable to assemble its proper active conformation **(chapter 6)**. Even subtle changes may still significantly impair an enzyme's function, especially at physiological conditions **(chapter 7)**. It is not even excluded and far from unlikely that some mutations exert their effect *via* multiple of these molecular mechanisms. With regard to the patients described in this thesis we are far from understanding the factors that contribute to their complex phenotypes but, hopefully, we have shed that much light on the underlying molecular mechanisms of disease so that we can state: "That is why the patient is ill".

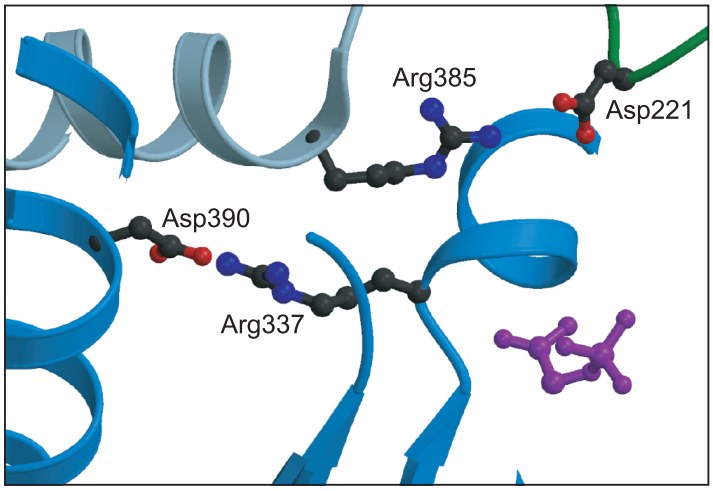
Addendum ‘Color Figures’



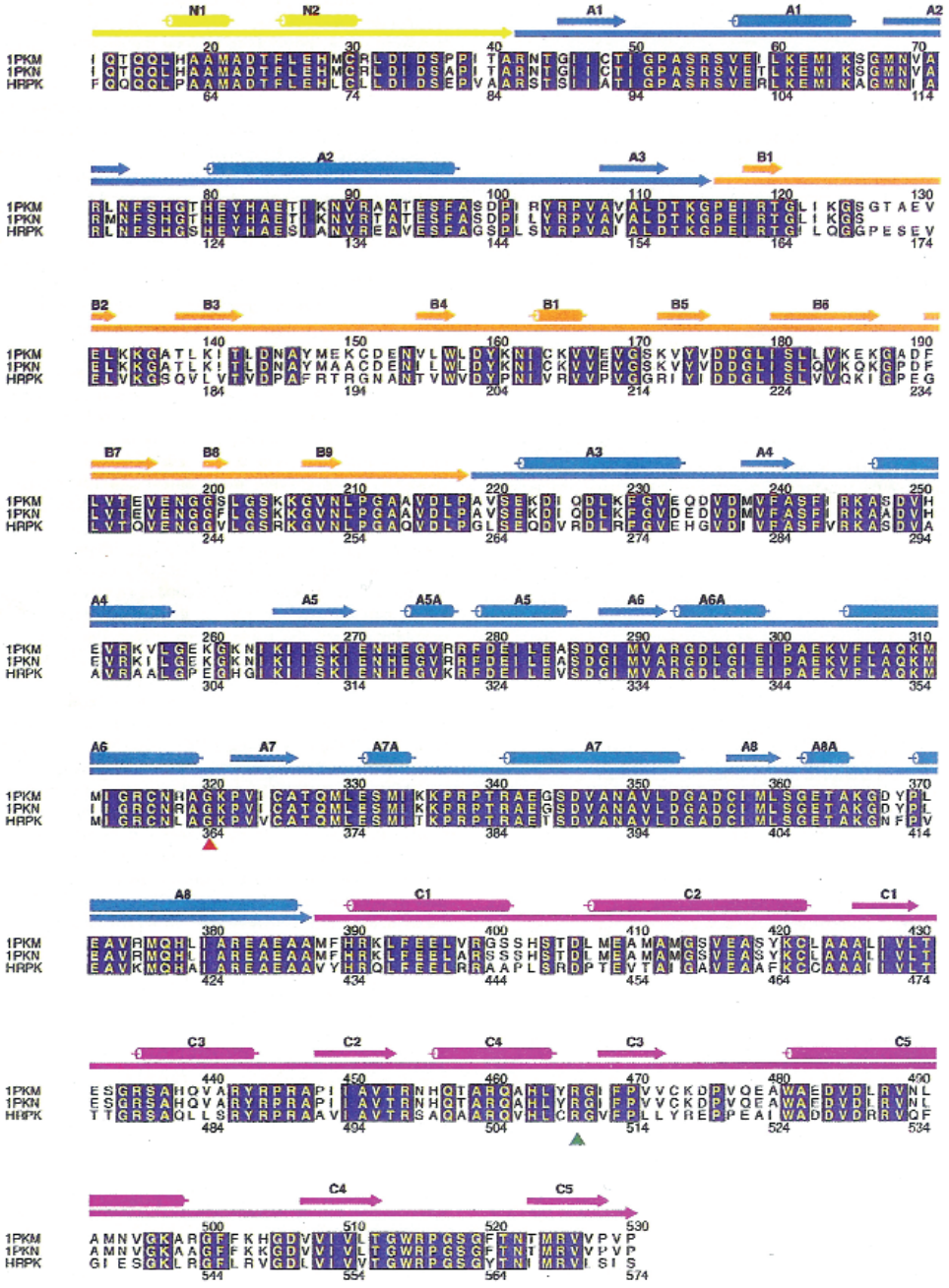
Chapter 4, Figure 1. PK monomer with depicted positions of the substituted amino acids described in this study. The figure highlights one PK subunit in the tetrameric structure. The residues affected by the novel missense mutations are shown as red spheres. The A domain is colored blue. The B (top) and C domains (bottom) are colored green. The N-terminal domain is colored light-blue. The substrate analog phosphoglycolate and the allosteric effector fructose-1,6-diphosphate are colored purple and drawn in ball-and-stick mode in their binding pockets in the A and C domains, respectively.



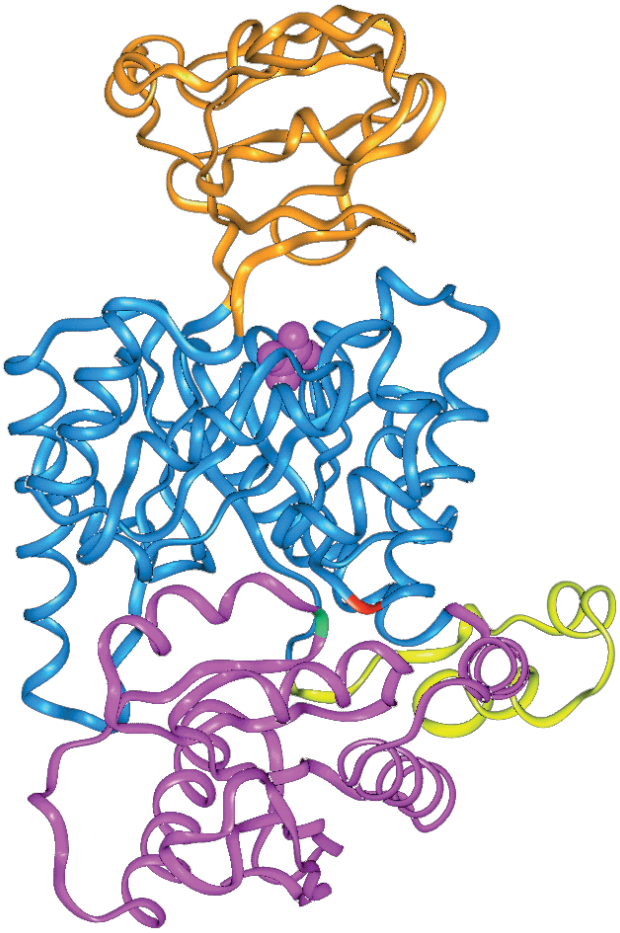
Chapter 4, Figure 2. Modeling of the Gly111Arg substitution. Ribbon drawing of the neighborhood of the Gly111Arg substitution with selected residues shown in ball-and-stick. The modeled conformation of Arg111 is depicted with magenta carbon atoms. Arginine side chains can adopt several orientations, but only one is shown. In all possible conformations of Arg111, sterical clashes or unfavorable charge interactions would occur between its side chain and one or more of the neighboring residues depicted.



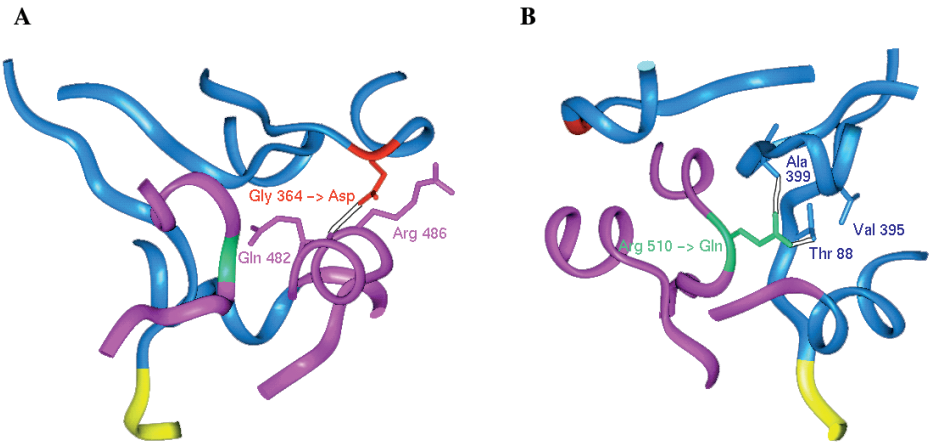
Chapter 4, Figure 3. Structural role of Arg337 and Arg385. Arg337 is located close to the substrate-binding site in the A domain (blue) and forms a salt bridge with Asp390 of the same subunit. Arg385, located in the A domain of an opposing subunit (gray), forms a salt bridge with Asp221 of the B domain (green). In the crystal structure, the substrate binding site is occupied by the substrate analog phosphoglycolate drawn in purple ball-and-stick representation. Mutation Arg337Trp would cause severe sterical hindrance and abolish charge interactions. The more conservative mutation of Arg385Lys would not cause sterical hindrance and would retain in part the potential for hydrogen bonding and charge interactions. Due to their position close in domain and subunit interfaces close to the active site these mutations will likely affect catalysis and allosteric regulation.



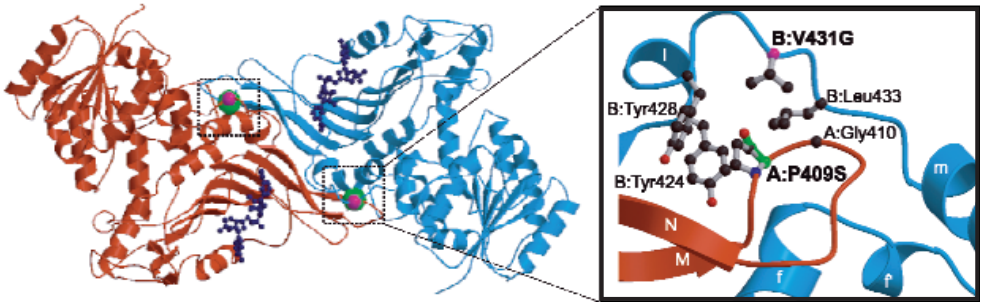
Chapter 5, Figure 1. Alignment of muscle-type pyruvate kinase from *Felis domesticus* (1PKM) and *Oryctolagus cuniculus* (1PKN) with R-type PK from human (HRPK). The alignment was generated with AMPS, as described in the Materials and Methods and drawn with the program ALSCRIPT.[35] Amino acids that are completely conserved among the three PKs are shown with yellow letters in boxes shaded with magenta. Only residues of human R-type PK (56-574) corresponding to 1PKM (12-530) are shown. The secondary structure elements, α-helixes and β-strands, assigned to 1PKM are shown above the sequences as cylinders and filled arrows, respectively. The domain organization of PK is represented by filled arrows right under the secondary structure elements. Color code: the N-terminal domain N is yellow; the catalytic domain A is blue, domain B is orange, and the C-terminal twisted αβ-type domain C is pink. The numbers above the sequences are in accordance with the numbering in 1PKM, whereas the numbers beneath the sequences refer to the codon numbering of HRPK. The red-filled triangle indicates the position of residue 364 (glycine to aspartic acid mutation), and the green filled triangle indicates the position of residue 510 (arginine to glutamine mutation).



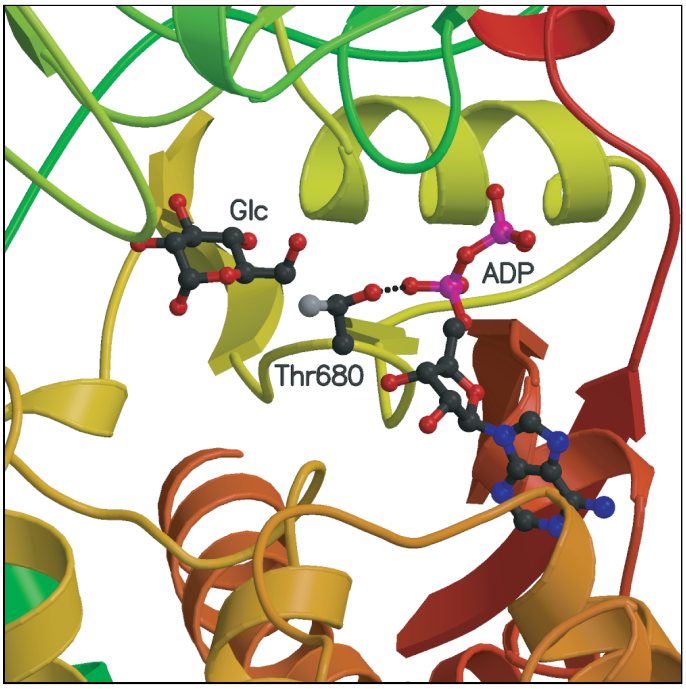
Chapter 5, Figure 4.
Cα-ribbon trace of the model of human R-type PK monomer. The color code is as for Figure 1: the N-terminal domain N is yellow; catalytic domain A is blue, domain B is orange, and the C-terminal twisted αβ-type domain C is pink. The positions of the amino acids 364 (G_{1091} to A mutation) and 510 (G_{1529} to A mutation) are indicated by red and green color, respectively. The active site bound pyruvate molecule taken from 1PKN[9] is represented by pink space filling.



Chapter 5, Figure 5.
Close-up view of areas around the amino acids 364 (A) and 510 (B). Residues in close contact with the mutated residues, translated from codons 364 and 510, are shown with side chains, whereas the residues further apart are shown in Cα-ribbon trace representation as in Figure 4. The color code is as for Figure 1. (A) and (B) are shown with the mutated aspartic acid and glutamine replacing the wild-type glycine and arginine, respectively. Hydrogen bonds less than 3.0 Å are shown as white lines.



Chapter 6, Figure 3. Ribbon representation of a G6PD dimer with green spheres indicating the position of Pro409 and magenta spheres indicating the position of Val431. Subunits A and B are colored red and blue, respectively. The structural NADP molecules are shown in dark blue ball-and-stick representation. Dashed rectangles depict the two regions in the G6PD dimer interface disrupted by the Pro409Ser and Val431Gly substitutions. Note that although residues Pro409 and Val431 are located close together, they in fact reside on different subunits. (Inset) Close-up view of the vicinity of Pro409 (here shown on subunit A) and Val431 (subunit B) with selected residues shown in ball-and-stick (oxygen, red; nitrogen, blue; carbon, black). The carbon atoms of the mutated residues are shown in green (Ser409) and magenta (Gly431). The mutations were modeled using the program O.[14] The figure was generated from the atomic coordinates of protein data bank entry 1QKI[8] using computer programs Molscript[35] and Raster3D.[36]



Chapter 7, Figure 3. Thr680 is a critical residue in the active site of HK. Schematic drawing of the active site of HK-I shows Thr680, bound glucose, and adenosine diphosphate (ADP) in ball-and-stick representation (atom coloring: carbon black; oxygen, red; nitrogen, blue; and phosphor, magenta). The carbon atom that is absent in the Thr680Ser mutant is colored gray. Illustrated is the central position occupied by Thr680 in the active site where it is located at the tip of a loop and makes a hydrogen bond with the α-phosphoryl group of ADP. This figure was generated from the atomic coordinates of the HK-I ADP/glucose complex[7] (protein data bank entry 1DGK) using computer programs Molscript[22] and Raster3D.[23]

Samenvatting in het Nederlands

Inleiding

Vanaf het moment dat de volwassen rode bloedcel het beenmerg verlaat is hij optimaal in staat om zuurstof te binden, te transporteren, en af te geven aan de weefsels. Dit is de belangrijkste taak van de erythrocyt gedurende zijn ongeveer 120-dagen durende verblijf in de bloedbaan. De biconcave vorm van de rode bloedcel is zeer geschikt voor het uitwisselen van zuurstof en kooldioxide. Aangezien dit laatste tot in de kleinste microcapilairen plaatsvindt, moet de erythrocyt in staat zijn om sterk van vorm te veranderen. Dit is mogelijk door het ontbreken van een kern en cytoplasmatische organellen, en dus de mogelijkheid tot eiwitsynthese.
De rode bloedcel gebruikt energie voor een aantal cellulaire processen die van vitaal belang zijn. Dit zijn onder meer (1) het in stand houden van de glycolyse; (2) het in stand houden van de electrolytische gradiënt tussen plasma en het cytoplasma van de rode bloedcel; (3) de synthese van glutathion; (4) purine en pyrimidine metabolisme; (5) het in een gereduceerde, functionele, staat houden van hemoglobine; (6) het beschermen van metabole enzymen, hemoglobine, en membraan eiwitten tegen oxidatieve schade; en (7) het in stand houden van de fosfolipiden asymetrie van de erythrocytaire celmembraan. Omdat kern en mitochondrieën ontbreken, is de volwassen rode bloedcel niet in staat om energie te genereren via de oxidatieve Krebs Cyclus. Daarom is hij voor het genereren van energie in de vorm van adenosine trifosfaat (ATP), en reducerend vermogen in de vorm van purine nucleotiden: gereduceerd nicotinamide adenine dinucleotide (NADH) en nicotinamide adenine dinucleotide fosfaat (NADPH), volledig afhankelijk van de anaerobe omzetting van glucose via de Embden-Meyerhof route (glycolyse) en de oxidatieve Hexose Monofosfaat Shunt.
Bij de hierboven beschreven metabole routes en processen zijn vele enzymen betrokken. Enzymopathieën van de rode bloedcel tasten uiteindelijk de cellulaire integriteit van de rode bloedcel aan en verkorten diens levensduur. Aldus leiden zij tot een hemolytische anemie. Het overgrote deel van deze enzymopathieën is erfelijk. Om een klinisch relevante hemolyse te veroorzaken moet de functie van de erythrocyt onder fysiologische omstandigheden aanzienlijk zijn verminderd. Klinisch gezien kunnen de erythrocytaire enzymopathieën in twee groepen worden ingedeeld. De eerste groep betreft deficiënties van enzymen van de glycolyse, en enzymen betrokken bij het purine en pyrimidine metabolisme. Deze stoornissen resulteren uiteindelijk in een tekort aan ATP, hetgeen aanleiding geeft tot een chronisch hemolytische anemie. De meest voorkomende oorzaak van een dergelijke anemie is een deficiëntie van pyruvaat kinase (PK). De tweede groep heeft betrekking op stoornissen in de Hexose Monofosfaat Shunt, welke de hoeveelheid

gereduceerd glutathion constant houdt. De enzymopathieën van deze groep zijn geassocieerd met een periodieke hemolyse, geïnduceerd door oxidatieve stress, medicijngebruik, of infecties. De meest voorkomende oorzaak in deze groep zijn deficiënties van glucose-6-fosfaat dehydrogenase (G6PD).
In het algemeen geldt dat de mate van hemolyse wordt bepaald door het relatieve belang van het aangedane enzym, maar ook door de veranderde eigenschappen van een mutant enzym met betrekking tot functie of stabiliteit (of beide). Daarnaast speelt de mogelijkheid tot compensatie van een enzymdeficiëntie door overexpressie van een isoenzym, of het gebruik van alternatieve metabole routes eveneens een rol in het uiteindelijke fenotype van patiënten met een erfelijke hemolytische anemie.

Doel van het onderzoek

Het fenotype van patiënten met erfelijke enzymopathieën van de rode bloedcel is zeer variabel. Een van de doelstellingen van de laboratoriumgeneeskunde is om het verband vast te stellen tussen het genotype van een patiënt en diens fenotype. Daartoe is het kennen van de moleculaire pathogenese van essentieel belang. Het is immers de basis van de ermee samenhangende hemolytische anemie. Dit betekent dat naast het identificeren van de oorzakelijke mutatie op DNA-niveau, de betrokken moleculaire mechanismen via welke deze aanleiding geeft tot een verminderde enzymfunctie in kaart moeten worden gebracht. Deze kunnen zich op verschillende niveau's in genexpressie afspelen; transcriptie, vorming van een correct mRNA, translatie, en eiwit. Het combineren van de genetische informatie met klinische, biochemische, en structurele data resulteert in een beter begrip van de moleculaire pathogenese en draagt zo bij aan het antwoord op de vraag: "Waarom is de patiënt ziek?".

Samenvatting en conclusies

De in dit onderzoek beschreven enzymopathieën van de rode bloedcel betreffen deficiënties van HK, PK, en G6PD. Deze drie enzymen zijn allen belangrijke regulator enzymen van de glycolyse (HK en PK) en de Hexose Monofosfaat Shunt (G6PD). De onderzochte mutaties in het DNA beïnvloedden de expressie van het betreffende gen op het niveau van de regulatie van transcriptie **(hoofdstuk 2)**, het vormen van een correct mRNA **(hoofdstuk 3)**, of de structuur **(hoofdstukken 4 en 5)** en functie **(hoofdstukken 6 en 7)** van het eiwit.

Erythrocytaire enzymopathie ten gevolge van verstoorde regulatie van transcriptie

In **hoofdstuk 2** wordt een ernstig PK-deficiënte patiënt beschreven. Uit RNA-analyse bleek dat bij deze patiënt slechts het paternale allel tot expressie kwam waarop een puntmutatie (c.1529G>A) aanwezig was. Op DNA-niveau was deze patiënt echter heterozygoot voor deze mutatie. Daarnaast bleek hij eveneens heterozygoot voor drie mutaties *in cis* op het maternale allel; –324T>A, –248delT, en –83G>C. Deze laatste drie mutaties waren niet eerder beschreven.

Met behulp van *in vitro* transfectie studies in K562 cellen werd het effect van elke mutatie, afzonderlijk en gezamenlijk, onderzocht. –324T>A bleek een niet-functionele mutatie en –248delT een niet-functioneel polymorfisme. De –83G>C mutatie daarentegen was in staat de promoter activiteit drastisch te reduceren. Dit verklaarde de expressie van enkel het paternale, *in trans,* allel. Mutagenese van de nucleotiden in de directe nabijheid van nucleotide –83 toonde vervolgens de aanwezigheid aan van een onbekend, functioneel transcriptie regulator element in de erythroid-specifieke promoter van het *PKLR* gen: PKR-RE1. Met behulp van Electrophoretic Mobility Shift Assay met K562 nucleair extract werd bevestigd dat er ook DNA–eiwit interactie plaatsheeft op dit element, waarbij een onbekend eiwit blijkt te binden aan PKR-RE1. Van geen van de bekende eiwitten is bekend dat zij binden aan het bindingsmotief van PKR-RE1. Daarom concluderen wij dat dit regulator element mogelijk betrokken is bij een nieuw mechanisme van erythroïd-specifieke *trans* activatie.

Feitelijk was bovenstaande patiënt ‘pseudo-homozygoot’ voor de c.1529G>A puntmutatie, welke codeert voor een arginine naar glutamine aminozuur substitutie op positie 510 (Arg510Gln). Dus bepaald dit mutante PK voor een belangrijk deel het PK-deficiënte fenotype. Zoals beschreven in **hoofdstuk 5**, veroorzaakt de Arg510Gln verandering een drastische afname van de vrijheid in conformatie tussen het A- en het C-domein. Dit verklaart de zeer lage enzymatische activiteit en de instabiliteit welke deze PK variant vaak kenmerkt. Het is opvallend dat het ernstige klinische beeld van de patiënt zoals beschreven in **hoofdstuk 2** aanmerkelijk verschilde van dat van patiënt MB86, beschreven in **hoofdstuk 4**. Deze patiënt was daadwerkelijk homozygoot voor c.1529G>A, vertoonde een zeer mild klinisch beeld en was niet transfusie-afhankelijk. Wellicht duidt dit verschil erop dat er ondanks de drastische structurele veranderingen nog steeds een bepaalde mate van enzymatische activiteit bestaat in de Arg510Gln PK variant. Deze restactiviteit zou dan uiteraard, ongeveer tweemaal, hoger uitkomen in de homozygote patiënt dan in de ‘pseudo homozygote’ patiënt.

Erythrocytaire enzymopathie ten gevolge van defect mRNA

De aanwezigheid van slechts één mutant PK, ondanks heterozygotie voor de onderliggende mutatie op DNA-niveau, bleek ook bij een andere patiënt met een ernstige PK-deficiëntie. In dit geval bleek het enig aanwezige PK een variant te zijn met een Arg479His substitutie. Deze patiënt, beschreven in **hoofdstuk 3**, was dubbel heterozygoot voor twee puntmutaties die beiden zeer waarschijnlijk een negatief effect zouden hebben op de vorming van een correct mRNA. De eerste, niet eerder beschreven, mutatie veranderde de invariante dinucleotide aan het 5'-einde van intron 5 (IVS5+1G>A). De andere mutatie bevond zich in exon 10 (c.1436G>A) en veranderde de 'splice site' consensus sequentie aan het 5'-einde van intron 10. Deze laatste mutatie codeerde daarnaast eveneens voor de Arg479His substitutie. Om het effect van beide mutaties op pre-mRNA niveau te bestuderen werd gebruik gemaakt van een *ex vivo* benadering. Hierbij werden uit perifeer bloed van de patiënt kernhoudende erythroïde cellen gekweekt waaruit vervolgens RNA werd geïsoleerd. Dat deze benadering een toegevoegde waarde had bleek uit het feit dat er meerdere afwijkende transcripten konden worden aangetoond die elk afzonderlijk het gevolg waren van een van beide mutaties. Bij een aanzienlijk deel van de transcripten was, ten gevolge van de IVS5+1G>A mutatie, exon 5 niet geïncludeerd. Daarnaast bleek dat naast exon 5, ook exon 6 in een groot deel van de transcripten ontbrak (zie onder). Omdat van deze beide transcripten geen eiwit werd verwacht noch aangetoond met behulp van Western Blot, was het IVS5+1A allel in feite een nul-allel.

De c.1436G>A mutatie bij deze patiënt bleek voornamelijk geassocieerd met een sterke afname van het aantal transcripten. Van het merendeel hiervan waren de introns echter op een correcte wijze verwijderd. Deze mRNA's codeerden dus voor een kleine hoeveelheid Arg479His PK, hetgeen het fenotype van deze patiënt bepaalde. De recent gepubliceerde kristal-structuur van recombinant humaan Arg479His PK bevestigde onze bevindingen voor wat betreft het geringe effect van de mutatie op de stabiliteit van de PK monomeer. Opvallend was echter dat recombinant Arg479His PK niet temperatuur-gevoelig bleek terwijl het PK van de patiënt dat wel was; zowel *in vitro* als *in vivo* bleek het PK van de patiënt zeer gevoelig voor een verhoging van de temperatuur. Dit laatste uitte zich door een toename van de hemolyse tijdens perioden met koorts maar ook, bijvoorbeeld, na een lang verblijf in de (warmte van) de zon. Een dergelijke tegenstrijdigheid in enzymatische eigenschappen tussen mutante enzymen van, respectievelijk, recombinante en menselijke herkomst werd eveneens geconstateerd in **hoofdstuk 7** en zal later verder worden besproken (zie ***Erythrocytaire enzymopathie ten gevolge van verstoorde eiwitfunctie***). Het benadrukt dat enzymatische eigenschappen van recombinante (mutante) enzymen met de nodige voorzichtigheid dienen te worden geïnterpreteerd.

Zoals hierboven vermeld was de samenstelling van één transcript vrij opmerkelijk. In dit transcript ontbrak als gevolg van de IVS5+1G>A mutatie niet alleen exon 5 maar bovendien bleek ook het naastgelegen exon 6 uit het pre-mRNA te zijn verwijderd. Het was aannemelijk dat het ontbreken van beide exons verband hield met elkaar. Wij postuleren een mechanisme waarbij het efficiënt includeren van exon 6 in wild-type *PKLR* mRNA afhankelijk is van de aanwezigheid van exon 5 in het primaire transcript. Met andere woorden, in exon 5 bevinden zich splice-regulator elementen die noodzakelijk zijn voor een efficiënte inclusie van exon 6 in *PKLR* mRNA. Omdat de IVS5+1G>A mutatie tot exclusie van exon 5 leidt, worden deze splice-regulator elementen daarmee automatisch eveneens verwijderd uit dit pre-mRNA. Dit geeft vervolgens weer aanleiding tot een inefficiënte inclusie van exon 6. De mogelijke aanwezigheid van dergelijke splice-regulator elementen betekent dat andere (punt)mutaties in exon 5 die coderen voor een aminozuur substitutie (**hoofdstuk 4**) mogelijk eveneens tot een defect mRNA leiden.

Erythrocytaire enzymopathie ten gevolge van verstoorde eiwitstructuur

De belangrijkste genetische oorzaak van erfelijke enzymopathieën van de rode bloedcel vormen puntmutaties die coderen voor een substitutie van structureel en functioneel belangrijke aminozuren. Onze resultaten aangaande een grote group patiënten met PK deficiëntie is daarmee in overeenstemming. In **hoofdstuk 4** beschrijven we een genetisch onderzoek onder 28 patiënten die afkomstig waren uit 26 niet-gerelateerde families. We identificeerden 24 verschillende mutaties die geassocieerd waren met PK deficiëntie. Hiervan waren er 14 niet eerder beschreven. Twintig van de 24 verschillende mutaties (83%) waren puntmutaties die coderen voor een aminozuur substitutie, waaronder een niet eerder beschreven c.331G>A mutatie. Deze puntmutatie codeert voor een arginine in plaats van glycine op positie 111 (PK Utrecht) en in vijf niet-gerelateerde Nederlandse families bleek deze mutatie voor te komen. Samen met de eveneens frequent voorkomende c.1456C>T en c.1529G>A mutaties was de c.331G>A mutatie daarom een van de belangrijkste oorzaken van PK deficiëntie in Nederland.

Om meer inzicht te krijgen in het effect van bepaalde aminozuur substituties op de structuur en functie van PK hebben wij in **hoofdstuk 4** de locatie en interacties van het desbetreffende aminozuur in de drie-dimensionale structuur van humaan wild-type rode bloedcel-PK bestudeerd. In een aantal gevallen bleek de substitutie het hydrofobe binnenste van het eiwit ernstig te verstoren. Andere aminozuur substituties hadden betrekking op het B-domein en de ATP-bindingsplaats. Aangezien beiden deel uitmaken van het actieve centrum van PK zullen mutaties in dit gebied tot een verlies aan enzymatische activiteit leiden. Een bepaalde groep puntmutaties in het bijzonder betrof substituties van aminozuren die zich bevinden op

het scheidingsvlak tussen twee subunits in de PK tetrameer. Samen met de inter-domein interacties zijn inter-subunit interacties verantwoordelijk voor de allostere regulatie van PK. Een van deze aminozuur substituties betrof een verstoring van C/C' subunit interacties (Arg569Gln) terwijl drie andere mutaties een verandering van A/A' subunit interacties tot gevolg hadden (Arg337Trp, Gly358Glu, en Arg385Lys). Met name Arg337Trp en Arg385Lys verhinderen zeer waarschijnlijk de vorming van een zoutbrug die van cruciaal belang is voor de allostere transitie van PK.

Onze resultaten uit **hoofdstuk 4** bevestigden eveneens het mild klinische beeld zoals dat over het algemeen wordt waargenomen bij patiënten met de veel voorkomende c.1456C>T (Arg486Trp) mutatie. Arg486 bevindt zich op het scheidingsvlak tussen het A- en C-domein binnen de PK monomeer. Op ditzelfde scheidingsvlak bevindt zich Gly364. Het is opvallend dat de Arg486Trp substitutie nauwelijks effect heeft op de enzymatische eigenschappen van PK, terwijl eenzelfde drastische substitutie van een nabijgelegen aminozuur (Gly364Asp) wel een ernstige PK deficiëntie tot gevolg heeft en in een homozygote patient zelfs tot de dood heeft geleid. De Gly364Asp substitutie werd gecodeerd door een niet eerder beschreven c.1091G>A puntmutatie. Omdat op het moment van dit deel van het onderzoek de kristalstruktuur van humaan rode bloedcel-PK nog niet bekend was ontwikkelden wij een theoretisch drie-dimensionaal model dat was gebaseerd op de kristalstructuur van spier-PK. Aan de hand van dit model bleek dat een aspartaat op positie 364 de interactie tussen het A- en C-domein ernstig verstoorde door het creëren van een extra zoutbrug. Zeer recent werden deze bevindingen bevestigd middels de kristalstructuur van humaan Gly364Asp PK. Eerder genoemde homozygote patiënt was het eerste kind uit het tweede huwelijk van haar moeder. Deze had uit haar eerste huwelijk een kind dat dubbel heterozygoot was voor twee *PKLR* mutaties, c.1091G>A en c.1529G>A. Dit kind vertoonde een ernstig fenotype en was transfusie-afhankelijk. Wederom wekte het verschil in fenotype de suggestie dat de overerving van het c.1529G>A allel wellicht nog enige enzymatische activiteit met zich meebracht daar waar homozygotie voor het c.1091G>A allel lethaal was (zie ***Erythrocytaire enzymopathie ten gevolge van verstoorde regulatie van transcriptie***).

Erythrocytaire enzymopathie ten gevolge van verstoorde eiwitfunctie

Uit het bovenstaande mag worden geconcludeerd dat naast de positie en de interacties van een aminozuur ook de aard van het aminozuur waarmee het wordt vervangen een belangrijke rol speelt in het uiteindelijke effect van dergelijke puntmutaties. Dit bleek ook het geval bij twee patiënten met G6PD deficiëntie, beschreven in **hoofdstuk 6**. Terwijl de ene patiënt zich kenmerkte zich door een ernstige chronische hemolytische anemie, vertoonde de andere slechts een milde, subklinische, chronische hemolyse. Beide patiënten waren hemizygoot

voor een *de novo* mutatie in *G6PD*. De mutaties codeerden voor een niet eerder beschreven Pro409Ser (G6PD Utrecht) en Val431Gly substitutie in G6PD. G6PD is enzymatisch actief in dimere vorm en zowel Pro409 als Val431 bevinden zich op het scheidingsvlak tussen twee G6PD monomeren. Vrijwel alle mutaties die het proces van dimerisatie verstoren leiden tot de klinisch meest ernstige vorm van G6PD deficiëntie. Deze klasse I G6PD deficiënties worden gekenmerkt door een chronische hemolyse. Beide aminozuur substituties werden bestudeerd aan de hand van het drie-dimensionale model van de kristalstruktuur van humaan G6PD. Dit liet zien dat aminozuren Pro409 en Val431 zich niet alleen bevinden op voornoemd scheidingsvlak maar dat ze daarnaast ook interactie met elkaar aangaan in wild-type G6PD. Zowel Pro409Ser als Val431Gly verstoren de interacties tussen twee G6PD monomeren waardoor de stabiliteit en enzymatische activiteit van G6PD ernstig wordt verminderd. Gezien de fenotypische verschillen toont dit, aan de ene kant, wederom het belang aan van de locatie en aard van aminozuur substitutie. Aan de andere kant laten deze resultaten ook zien dat het fenotype niet alleen afhankelijk is van de aminozuur substitutie omdat het (milde) fenotype van de patiënt met de Val431Gly G6PD variant sterk verschilde van dat van de enig andere patiënt die tot nu toe is beschreven met deze G6PD variant. Deze patiënt vertoonde vanaf geboorte een chronische icterus, milde chronische anemie, reticulocytose, en splenomegalie. De door ons beschreven patiënt vertoonde geen neonatale icterus en had geen klinische verschijnselen tot hij 29 maanden oud was en zich toen presenteerde met favisme. Het fenotype wordt dus door meerdere, al dan niet genetische factoren, (mede) bepaald.

Zowel in **hoofdstuk 5** als in **hoofdstuk 6** bleek het veelal ernstige fenotype het gevolg van een drastische aminozuur substitutie die een cruciaal deel van het eiwit betrof. Dat ook een subtiele verandering tot een ernstig verlies aan *in vivo* activiteit kan leiden, hetgeen uiteindelijk resulteerd in een ernstige chronische hemolyse, wordt beschreven in **hoofdstuk 7**. Bij een patiënt waarbij meer dan 20 jaar geleden de, zeldzame, diagnose HK deficiëntie werd gesteld, hebben wij de oorzakelijke mutatie in het DNA geïdentificeerd. Het betrof een niet eerder beschreven puntmutatie (c.2039C>G) waardoor threonine op positie 680 wordt vervangen door serine (HK Utrecht). Dit is een zeer conservatieve verandering van een echter evolutionair zeer geconserveerd aminozuur. Aan de hand van het drie-dimensionale model van de kristalstructuur van humaan HK blijkt dat Thr680 zich centraal in het actieve centrum van HK bevindt en daar interacties aangaat met fosfaatgroepen van ADP/ATP en glucose-6-fosfaat. Deze resultaten correleerden goed met eerder bepaalde enzymatische eigenschappen van partieel gezuiverd mutant HK van de patiënt. Het mutante HK van de patiënt kenmerkte zich namelijk door een tweevoudig verlaagde affiniteit voor Mg^{2+}-ATP, en een aanzienlijk verlaagde affiniteit voor de allostere remmer glucose-1,6-difosfaat. Waarschijnlijk betekent

het verlies van een methyl-groep een kleine verandering van de conformatie hetgeen resulteert in een veranderde binding van fosfaat-groep dragende liganden.
De tweevoudig verlaagde affiniteit voor Mg^{2+}-ATP impliceerde een relatief gering effect op ATP binding. Dit was in tegenspraak met het ernstige klinisch beeld waardoor de suggestie werd gewekt dat het *in vivo* effect van de mutatie ernstiger is. Daarom vergeleken we eerder bij deze patiënt bepaalde intracellulaire Mg^{2+}-ATP concentraties. Deze concentraties waren vele malen lager *in vivo*, en vrijwel gelijk aan de K_m, dan de Mg^{2+}-ATP concentraties zoals die worden gebruikt bij *in vitro* activiteits bepalingen. Het is daarom zeer waarschijnlijk dat een tweevoudig verlaagde affiniteit voor Mg^{2+}-ATP bij fysiologische concentraties een veel ernstiger effect zal hebben op de enzymatische activiteit van HK.
De resultaten uit **hoofdstuk 7** duidden, net als in **hoofdstuk 3**, op een verschil in enzymatische eigenschappen tussen, in dit geval, partieel gezuiverd HK van de patiënt en recombinant geproduceerd HK-I met dezelfde mutatie. Het recombinante Thr680Ser HK-I kenmerkte zich door een normale K_m(ATP) en K_i(glucose-6-fosfaat). Mogelijk worden deze opvallende verschillen veroorzaakt door de verschillende bronnen waaruit het enzym werd geïsoleerd (humane rode bloedcellen *versus Escherichia coli*). Het is echter ook mogelijk dat het verschil in N-terminus tussen rode bloedcel-HK en HK-I hierbij een rol speelt. Zowel rode bloedcel-HK als HK-I worden door hetzelfde gen gecodeerd maar alleen rode bloedcel-HK bezit een uniek peptide aan het N-terminale uiteinde. Dit vertoont opvallende overeenkomsten met de weefsel-specifieke expressie van rode bloedcel-PK en lever-PK door *PKLR*. Ook rode bloedcel-PK bezit daardoor een uniek peptide aan de N-terminus. Het verschil in enzymatische eigenschappen tussen beide mutante HK's doet denken aan eenzelfde verschil in eigenschappen tussen de twee mutante PK's met een Arg479His substitutie **(hoofdstuk 3)**. Hierbij speelt het rode bloedcel-specifieke peptide mogelijk een rol omdat ook in het geval van PK dit peptide bij het recombinant mutante enzym ontbrak. Ondanks een normale *in vitro* enzymatische activiteit van beide recombinant geproduceerde 'rode bloedcel-enzymen' suggereren de waargenomen verschillen bij beide enzymen een mogelijk functionele rol voor het rode bloedcel-specifieke peptide *in vivo*.

De erfelijke enzymopathieën van de rode bloedcel leiden tot een grote diversiteit in fenotypen. De onderliggende moleculaire pathogenese blijkt slechts voor een deel verantwoordelijk voor het fenotype en naast de afwijkende eigenschappen van een mutant enzym spelen overige fysiologische-, genetische-, en omgevingsfactoren een rol. In dit onderzoek is de moleculaire pathogenese van een aantal erfelijke enzymopathieën van de rode bloedcel in kaart gebracht om zodoende een beter inzicht te krijgen in de genotype–fenotype relatie. Samenvattend blijkt dat de onderzochte mutaties op verschillende

manieren bepaalde aspecten van gen-expressie verstoren. Gen expressie kan volledig worden geblokkeerd (**hoofdstuk 2**) of er wordt enkel afwijkend mRNA gesynthetiseerd (**hoofdstuk 3**). Wanneer een mutant eiwit toch tot expressie komt kan het instabiel zijn door ernstige verstoringen van de structuur (**hoofdstukken 4 en 5**) of niet in staat zijn om zijn juiste conformatie aan te nemen (**hoofdstuk 6**). Ook subtiele veranderingen blijken echter een sterke verlaging van de enzymactiviteit te kunnen veroorzaken, met name onder fysiologische omstandigheden (**hoofdstuk 7**). Het is niet uitgesloten en zelfs waarschijnlijk dat sommige mutaties via meerdere moleculaire mechanismen hun uiteindelijke effect sorteren. Met betrekking tot het complexe fenotype van de patiënten zoals beschreven in dit proefschrift is er dan ook nog veel onderzoek nodig om tot een beter begrip van de genotype–fenotype relatie te komen. Hopelijk heeft het onderzoek zoals beschreven in dit proefschrift echter wel een dusdanig licht geworpen op de onderliggende moleculaire pathogenese van de onderzochte enzymopathieën van de rode bloedcel dat we kunnen zeggen: “daarom is de patiënt ziek”.

Epiloog

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List of publications

W. W. van Solinge, B. Lind, R. van Wijk, H. C. Hart, and R. J. Kraaijenhagen. Clinical expression of a rare β-globin gene mutation co-inherited with haemoglobin E-disease. *European Journal of Clinical Chemistry and Clinical Biochemistry (1996); 34: 949-954*

W. W. van Solinge, R. J. Kraaijenhagen, G. Rijksen, R. van Wijk, B. B. Stoffer, M. Gajhede, and F. C. Nielsen. Molecular modelling of human red blood cell pyruvate kinase: structural implications of a novel G_{1091} to A mutation causing severe nonspherocytic hemolytic anemia. *Blood (1997); 90: 4987-4995*

M. C. Montefusco, R. Asselta, R. van Wijk, S. Duga, and M. L. Tenchini. Concerns about the mutations identified in a case of familial coagulation factor V deficiency: factor V Stanford. *Thrombosis and Haemostasis (2000); 84: 1131-1132*

R. van Wijk, H. K. Nieuwenhuis, H. M. van den Berg, E. G. Huizinga, B. B. van der Meijden, R. J. Kraaijenhagen, and W. W. van Solinge. Five novel mutations in the gene for human blood coagulation factor V associated with type I factor V deficiency. *Blood (2001); 98: 358-367*

A. Buijs, P. Poddighe, R. van Wijk, W. van Solinge, E. Borst, L. Verdonck, A. Hagenbeek, P. Pearson, and H. Lokhorst. A novel *CBFA2* single-nucleotide mutation in familial platelet disorder with propensity to develop myeloid malignancies. *Blood (2001); 98: 2856-2858*

R. van Wijk, M. C. Montefusco, S. Duga, R. Asselta, E. Santagostino, W. van Solinge, M. Malcovati, M. L. Tenchini, and P. M. Mannucci. Coexistence of a novel homozygous nonsense mutation in exon 13 of the factor V gene with the homozygous Leiden mutation in two unrelated patients with severe factor V deficiency. *British Journal of Haematology (2001); 114: 871-874*

J. A. Remijn, R. van Wijk, P. G. de Groot, and W. W. van Solinge. Nature of the fibrinogen Aα gene *Taq*I polymorphism. *Thrombosis and Haemostasis (2001); 86: 935-936*

J. J. Vlietman, J. Verhage, H. L. Vos, R. van Wijk, J. A. Remijn, W. W. van Solinge, and F. Brus. Congenital afibrinogenaemia in a newborn infant due to a novel mutation in the fibrinogen Aα gene. *British Journal of Haematology (2002); 119: 282-283*

K. M. K. de Vooght, R. van Wijk, H. K. Nieuwenhuis, J. K. Ploos van Amstel, G. Rijksen, and W. W. van Solinge. [From gene to disease; hereditary non-spherocytic haemolytic anaemia caused by pyruvate kinase deficiency]. *Nederlands Tijdschrift voor Geneeskunde (2002); 146: 1828-1831*

R. van Wijk, G. Rijksen, E. G. Huizinga, H. K. Nieuwenhuis, and W. W. van Solinge. HK Utrecht: missense mutation in the active site of human hexokinase associated with hexokinase deficiency and severe nonspherocytic hemolytic anemia. *Blood (2003); 101: 345-347*

R. van Wijk, W. W. van Solinge, C. Nerlov, E. Beutler, T. Gelbart, G. Rijksen, and F. C. Nielsen. Disruption of a novel regulatory element in the erythroid-specific promoter of the human *PKLR* gene causes severe pyruvate kinase deficiency. *Blood (2003); 101: 1596-1602*

J. A. Remijn, R. van Wijk, H. K. Nieuwenhuis, P. G. de Groot, and W. W. van Solinge. Molecular basis of congenital afibrinogenaemia in a Dutch family. *Blood Coagulation and Fibrinolysis (2003); 14: 299-302*

R. van Wijk, E. G. Huizinga, I. Prins, A. Kors, G. Rijksen, M. Bierings, and W. W. van Solinge. Distinct phenotypic expression of two de novo missense mutations affecting the dimer interface of glucose-6-phosphate dehydrogenase. *Blood Cells Molecules and Diseases (2004); 32: 112-117*

R. van Wijk, A. C. W. van Wesel, A. A. M. Thomas, G. Rijksen, and W. W. van Solinge. *Ex vivo* analysis of aberrant splicing induced by two donor site mutations in *PKLR* of a patient with severe pyruvate kinase deficiency. *British Journal of Haematology, accepted for publication*

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 1 juni 1966 te Nijkerk. Na het behalen van het VWO diploma aan de Rooms Katholieke Scholengemeenschap “Eemland College Noord” in 1984, werd in datzelfde jaar begonnen met de studie Sociale Geografie aan de Universiteit Utrecht. In 1985 werd deze studie verruild voor de Medische Laboratoriumopleiding aan de Hogeschool Utrecht. Na het behalen van het diploma werd in 1989 een aanstelling verkregen bij Algemeen Christelijk Ziekenhuis Eemland te Amersfoort. Van 1993 tot 1999 was de auteur daar werkzaam als specieel analist op het gebied van de bijzondere hematologie en moleculaire diagnostiek. Als assistent in opleiding in het Universitair Medisch Centrum Utrecht werd onder leiding van Prof. Dr. W. W. van Solinge en Prof. Dr. J. J. M. Marx in de periode januari 2000 tot december 2003 het onderzoek verricht dat in dit proefschrift is beschreven. Momenteel is de auteur werkzaam als onderzoeker bij de Rode Bloedcel Pathologie Onderzoeksgroep binnen het Centraal Diagnostisch Laboratorium van het Universitair Medisch Centrum Utrecht.