The genetic basis of human erythrocyte pyridoxal kinase activity variation

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Thirty years ago we reported that erythrocyte pyridoxal kinase activity of African-Americans was strikingly lower than that of persons with European ancestry in a tissue-specific manner. At the time, it was impossible to elucidate the mechanism by which evolution had selectively lowered the enzyme activity in one cell type but not in others. We have now identified a promoter mutation with potential erythroid-specific properties that could be the basis of a novel mechanism of controlling cell-specific decreased activity of an essential enzyme.

Key words: pyridoxal kinase, erythrocytes, promoter.

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Pyridoxal-5'-phosphate (PLP) is a key co-factor in many enzymatic reactions of intermediary metabolism.[1] The synthesis of PLP requires the conversion of dietary B6 vitamers (pyridoxal, pyridoxine and pyridoxamine) into their active co-enzyme forms. Two enzymes, pyridoxal kinase and pyridoxine oxidase, are involved in this conversion.[2] The pyridoxal kinase enzyme (PdxK; EC 2.7.1.35) phosphorylates all three B6 vitamers into PLP, while pyridoxine oxidase (PNPO; EC 1.4.3.5) allows interconversion of the three 5'-phosphoforms. The main site of PdxK activity is in the liver although it is ubiquitously expressed.[1,3] Thirty years ago we reported that the erythrocyte PdxK activity of African-Americans was strikingly lower than that of persons with European ancestry.[4] Moreover, this racial difference was found to be tissue-specific, with leukocyte and skin fibroblast PdxK enzyme activities being the same in both ethnic groups.[4] Subsequently, these results were confirmed, and it was suggested that the selective pressure of malaria was the cause of the lowered erythrocyte enzyme activities.[5] Diet has been shown to influence PdxK activity, although this alone is not enough to explain the difference seen between African-Americans and Caucasians.[4,6,7] At the time of our original report it was impossible to elucidate the mechanism by which evolution had selectively lowered the enzyme activity in one cell type but not in another. Using more modern methods we are now able to suggest a plausible mechanism by which there is tissue-specific variation in the activity of the human PdxK enzyme.

Design and Methods

Subject recruitment and genomic DNA isolation

Blood was obtained with informed consent from normal hospital employees and from patients attending a Health Appraisal Clinic in a study approved by the Kaiser Permanente and Scripps Research Institute institutional review boards. The ethnic origin of each subject was based on self-identification. DNA was extracted from whole blood using a GENTRA DNA purification kit and used directly for polymerase chain reaction (PCR) amplifications. DNA was obtained for three populations, Caucasian (n=48), African-American (n=167) and Asian (n=81).

PCR amplifications and sequencing

The NCBI sequence AP001752 was used as the reference genomic sequence and NM_003681 for the cDNA sequence for the human *PdxK* gene. The nucleotide immediately 5' to the start ATG codon was designated -1. Oligonucleotide primers were designed for the promoter region and the 11 exons of the *PdxK* gene. The entire PdxK gene was amplified by PCR amplification and purified using spin column purification (Qiagen, CA, USA). Sequencing was then performed by capillary electrophoresis using an ABI PRISM 3100 Genetic Analyzer.

Electrophoretic mobility shift assay (EMSA)

Harvested K562 cells were suspended in cold phosphate-buffered saline and a nuclear extract was obtained using a Qproteome Nuclear protein extraction kit (Qiagen, CA, USA). The protein concentration of the

nuclear extract was determined by the Bradford assay. To perform the EMSA, complementary *PdxK* promoter oligonucleotides of the PdxK insert or *PdxK* wild-type region (5 μM) were annealed, end-labeled with [γ-^{32}P]dATP using polynucleotide kinase (New England Biolabs, MA, USA) and then purified by G50 Sephadex column purification (Pharmacia, NJ, USA). For each probe to be tested, 5 μg of K562 nuclear extract was incubated for 15 mins at room temperature, in a binding buffer containing 10 mM Tris-HCL, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1.0 mM $MgCl_2$, 1.5 μg of poly (dI-dC), and 4.0% glycerol. Then exactly 2×10^3 cpm of a [γ-^{32}P]dATP-labeled *PdxK* promoter probe was added and the mixture was incubated for 1 hr at room temperature. The mixture was subjected to non-denaturing gel electrophoresis at 9 V/cm on a 5% polyacrylamide gel. The gel was vacuum-dried, and radioactivity was detected using a Cyclone PhosphorImager (Packard Instruments, Meridien, CT, USA) equipped with OptiQuant analysis software (Packard Instruments).

Erythrocyte PdxK enzyme assay

Erythrocyte PdxK activity was measured using the method of Chern and Beutler.[8] Briefly, fresh venous blood was collected in acid-citrate dextrose (ACD) vacutainers® (Becton, Dickinson and Co., USA) and stored at 4°C prior to analysis. The blood was filtered through a cellulose column to remove white blood cells and platelets.[9] Hemolysates were prepared by making a 1 to 10 dilution of washed red cells in distilled water. PdxK activity of the hemolysates was determined by measuring the phosphorylation of a [^{3}H]pyridoxine substrate, with unreacted [^{3}H]pyridoxine being absorbed on Dowex-50 ion exchange resin.[8]

Results and Discussion

A number of polymorphisms were discovered in the *PdxK* gene by sequencing (Table 1). Among these we found an insertion event (-306_-305InsGCGCGGCG) in the promoter region. This insert was found to have a significantly lower gene frequency in African-Americans (43.4%) than in either Caucasians (57.3%; χ^2 test, p=0.016) or Asians (74.7%; χ^2 test, p=0.001). Bioinformatic analysis using MatInspector software[10] demonstrated that the insert introduces a putative core promoter binding protein (CPBP) binding site located at -306_-293bp to the *PdxK* start codon. The CPBP transcription factor is known to enhance transcriptional activity by at least four-fold.[11] Interestingly, MatInspector analysis revealed that the putative CPBP site was adjacent to a binding site for an erythrocyte specific transcription factor, erythroid Krüppel like factor (EKLF). This led to the hypothesis that the presence of the insert would lead to enhanced transcription of *PdxK* in erythrocytes only, influencing increased PdxK enzyme activity. To determine whether the *PdxK* insert caused changes in transcription factor binding near the EKLF site, an EMSA was performed. A [γ-^{32}P]dATP labeled *PdxK* promoter probe was made for the *PdxK* wild-type and the *PdxK* insert variants of the *PdxK* promoter. These two probes contained the EKLF binding site with or without the putative CPBP binding site, respectively. Probes were also constructed that contained only the CPBP binding site region. Equal amounts of these probes (2×10^5 cpm) were then incubated with nuclear extracts from the erythroid cell line K562. There was 3.2-fold greater transcription factor binding to the *PdxK* insert probe than to the *PdxK* wild-type probe (Figure 1). Probes specific for the *PdxK* CPBP site showed no transcription factor binding (Figure 1A). These *in vitro* data suggested that the *PdxK* insert causes increased transcription factor binding at the EKLF site and that this increase requires both the CPBP and the EKLF sites. As observations made *in vitro* may not always reflect the entire situation *in vivo*, the *in vivo* effect of the insert was determined by measuring the erythrocyte PdxK enzyme activity of 8 African-American, 16 Caucasian and 5 Asian subjects. In agreement with the original finding, the majority of the African-American samples had a PdxK activity at the lower range of activity documented in either Caucasians or Asians (Figure 2A). The presence of the *PdxK* insert was found to correlate with increased erythrocyte PdxK enzyme activity, regardless of ethnic origin (Figure 2B). Homozygotes for the insert (n=11; 1.05±0.24 mU/gHb) had a significantly higher PdxK activity than had either heterozygotes (n=11; 0.69±0.23 mU/gHb) or homozygote wildtype individuals (n=7; 0.74±0.32 mU/gHb), p=0.0018 and p=0.0319, respectively, by t-test analysis. Overall there was a significant difference between the PdxK activity of individuals homozygous for the insert (n=11; 1.05±0.24 mU/gHb) and that of individuals either homozygous wildtype or heterozygous (n=18; 0.71±0.26 mU/gHb), t-test, p=0.0016. However, variability was observed in the association between genotype and PdxK enzyme activity. It had previously been shown that certain drugs or vitamin B6 supplementation and deficiency can cause changes in erythrocyte PdxK activity.[6,7,12] Retrospective analysis of the assayed individuals revealed that two out of the three individuals who were homozygous wildtype yet had a high PdxK enzyme activity, had been taking a high dose vitamin B6 supplement

Table 1. DNA sequencing screening results of the *PdxK* gene. The number of chromosomes (n) included in the analysis for each population is given in brackets.

PdxK Region	*Polymorphism*	*African-American Allele Frequency (n=334)*	*Caucasian Allele Frequency (n=96)*	*Asian Allele Frequency (n=162)*
Promoter	-306_-305InsGCGCGGCG	43.4%	57.3%*	74.7%°
Exon 9	nt837C→T (S213S)	18%	0%	0%
3'UTR	*13T→C	36%	30%	32%

**χ^2 test, p=0.016 difference compared to African-Americans; °χ^2 test, p=0.001 difference compared to African-Americans.*

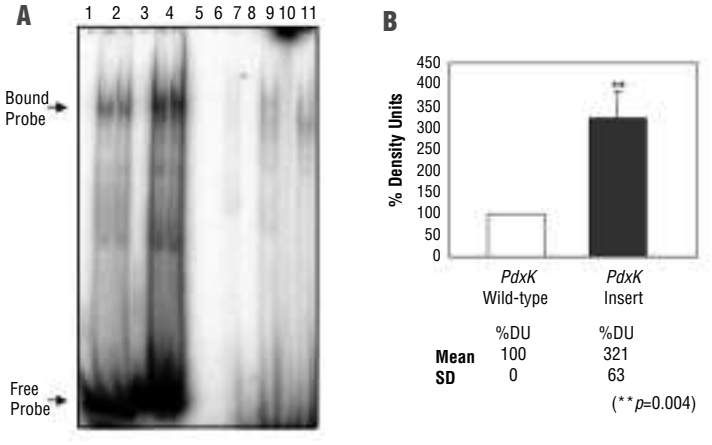

Figure 1. A. DNA-binding activity of the *PdxK* promoter insert. Equal amounts of probe (2×10^3 cpm) were added to 5 μg of K562 nuclear extract. Transcription factor binding was compared between 1, free *Pdxk* wild-type probe; 2, duplicate lanes of *Pdxk* wild-type binding; 3, free *Pdxk* insert probe; 4, duplicate lanes of *Pdxk* insert binding; 5, empty lane; 6, free *Pdxk* wild-type short probe; 7, *Pdxk* wild-type short probe binding; 8, free *Pdxk* insert short probe; 9, *Pdxk* insert short probe binding; 10, free *Pdxk* mutated CPBP insert short probe; 11, *Pdxk* mutated insert probe binding. Duplicate lanes represent independent binding assays performed simultaneously. The short probes have no EKLF site. Binding at the CPBP/EKLF region is indicated by the upper arrow, and free probe is shown by the lower arrow. B. Quantification of relative transcription factor binding. Data are presented as percent density units (%DU) versus *PdxK* wild-type; n=3 experiments in each group (mean ± SD); **$p<0.004$ by *t*-test analysis.

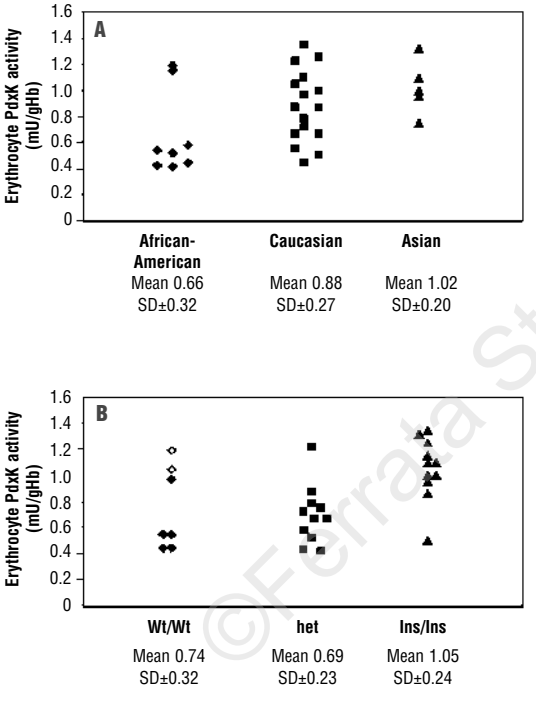

Figure 2. A. Comparison of erythrocyte PdxK enzyme activity between African-Americans, Caucasians and Asians. B. Overall erythrocyte activity of the PdxK enzyme compared to *PdxK* promoter genotype. Open diamonds indicate that the individual was taking >20mg/day pyridoxal supplement. Ins/Ins denotes *PdxK* insertion homozygotes, Het signifies heterozygotes and Wt/Wt indicates *PdxK* wild-type individuals. The mean and standard deviation (SD) of erythrocyte PdxK activity (mU/gHb) of each group is given.

(>20mg/day) for at least 6 months prior to the assay (Figure 2B). All other individuals assayed had been taking a low dose (<5 mg/day) or no vitamin B_6 supplement.

Thus, we have identified an 8bp *PdxK* promoter insertion that is less common in persons of African origin than in those of European or Asian ancestry. Through combined *in silico* and *in vitro* experiments, this insert is predicted to cause increased transcription factor binding of EKLF through the transcriptional enhancer CPBP. The CPBP transcription factor is ubiquitously expressed in humans, while EKLF expression is restricted to fetal liver and bone marrow.[11,13] Therefore, any effect of the insert through CPBP would be tissue-specific for erythrocytes. The insert had a significantly higher gene frequency in Caucasians or Asians than in African-Americans, and this could account for the observed ethnic variation in erythrocyte PdxK activity. We attempted to confirm this hypothesis by assaying the erythrocyte PdxK enzyme activity of individuals genotyped for the *PdxK* insert polymorphism. Individuals homozygous for the insert had significantly higher PdxK activities than had either homozygous wildtype or heterozygous individuals. Some additional variation was observed in the correlation between insert genotype and erythrocyte PdxK activity, which is likely due to dietary or drug influences. These results suggest an explanation for the original difference observed between the erythrocyte Pdxk enzyme activity of African-Americans and Caucasians,[4] with Nature supplying a novel way of selecting for a mechanism that allows cell-specific decreased activity of an essential enzyme.

JF performed the experimental research, intrepreted the data and drafted of the article; EB instigated the initial concept and experimental design, revised the drafted article and gave final approval of the submitted manuscript.

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References

1. McCormick DB. Coenzymes, Biochemistry of. In: Meyers RA, ed. Encyclopedia of Molecular Biology and Molecular Medicine. Germany. 1996: p. 396-406.
2. McCormick DB, Chen H. Update on interconversions of vitamin B-6 with its coenzyme. J Nutr 1999;129:325-7.
3. Ngo EO, LePage GR, Thanassi JW, Meisler N, Nutter LM. Absence of pyridoxine-5'-phosphate oxidase (PNPO) activity in neoplastic cells: isolation, characterization, and expression of PNPO cDNA. Biochemistry 1998;37:7741-8.
4. Chern CJ, Beutler E. Pyridoxal kinase: decreased activity in red blood cells of Afro-Americans. Science 1975; 187: 1084-6.
5. Martin SK, Miller LH, Kark JA, Hicks CU, Okoye VC, Esan GJF. Low erythrocyte pyridoxal-kinase activity in blacks: its possible relation to falciparum malaria. Lancet 1978;1:466-8.
6. Solomon LR, Hillman RS. Vitamin B6 metabolism in human red cells. I. Variations in normal subjects. Enzyme 1978;23:262-73.
7. Coburn SP, Ziegler PJ, Costill DL, Mahuren JD, Fink WJ, Schaltenbrand WE, et al. Response of vitamin B-6 content of muscle to changes in vitamin B-6 intake in men. Am J Clin Nutr 1991; 53:1436-42.
8. Chern CJ, Beutler E. Assay for human erythrocyte pyridoxine kinase. Anal Biochem 1975;67:97-109.
9. Beutler E. Red Cell Metabolism: A Manual of Biochemical Methods. 3rd ed. New York, NY: Grune & Stratton, Inc., 1984.
10. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 1995;23:4878-84.
11. Koritschoner NP, Bocco JL, Panzetta-Dutari GM, Dumur CI, Flury A, Patrito LC. A novel human zinc finger protein that interacts with the core promoter element of a TATA box-less gene. J Biol Chem 1997;272:9573-80.
12. Delport R, Ubbink JB, Vermaak WJH, Becker PJ. Theophylline increases pyridoxal kinase activity independently from vitamin B6 nutritional status. Res Commun Chem Pathol Pharmacol 1993;79:325-33.
13. van Ree JH, Roskrow MA, Becher AM, McNall R, Valentine VA, Jane SM et al. The human erythroid-specific transcription factor EKLF localizes to chromosome 19p13.12-p13.13. Genomics 1997;39:393-5.