Mutations in *PROSC* Disrupt Cellular Pyridoxal Phosphate Homeostasis and Cause Vitamin-B_6-Dependent Epilepsy

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Pyridoxal 5′-phosphate (PLP), the active form of vitamin B_6, functions as a cofactor in humans for more than 140 enzymes, many of which are involved in neurotransmitter synthesis and degradation. A deficiency of PLP can present, therefore, as seizures and other symptoms that are treatable with PLP and/or pyridoxine. Deficiency of PLP in the brain can be caused by inborn errors affecting B_6 vitamer metabolism or by inactivation of PLP, which can occur when compounds accumulate as a result of inborn errors of other pathways or when small molecules are ingested. Whole-exome sequencing of two children from a consanguineous family with pyridoxine-dependent epilepsy revealed a homozygous nonsense mutation in proline synthetase co-transcribed homolog (bacterial), *PROSC*, which encodes a PLP-binding protein of hitherto unknown function. Subsequent sequencing of 29 unrelated indivduals with pyridoxine-responsive epilepsy identified four additional children with biallelic *PROSC* mutations. Pre-treatment cerebrospinal fluid samples showed low PLP concentrations and evidence of reduced activity of PLP-dependent enzymes. However, cultured fibroblasts showed excessive PLP accumulation. An *E.coli* mutant lacking the *PROSC* homolog (Δ*YggS*) is pyridoxine sensitive; complementation with human *PROSC* restored growth whereas *hPROSC* encoding p.Leu175Pro, p.Arg241Gln, and p.Ser78Ter did not. PLP, a highly reactive aldehyde, poses a problem for cells, which is how to supply enough PLP for apoenzymes while maintaining free PLP concentrations low enough to avoid unwanted reactions with other important cellular nucleophiles. Although the mechanism involved is not fully understood, our studies suggest that PROSC is involved in intracellular homeostatic regulation of PLP, supplying this cofactor to apoenzymes while minimizing any toxic side reactions.

Introduction

Pyridoxal 5′-phosphate (PLP) is a highly reactive aldehyde. This allows it to act as the cofactor for over 140 enzyme-catalyzed reactions; however, it also poses a problem for the cell, namely, how to supply enough PLP for all the newly synthesized apo-B_6 enzymes while keeping the cellular level of free PLP so low that it does not react with other nucleophiles (e.g., sulphydryl and amino compounds and proteins that are not B_6 enzymes). Excess intracellular PLP is hydrolysed by phosphatases and, in the liver, pyridoxal is oxidized to pyridoxic acid. However, the mechanisms of regulation of PLP homeostasis within the cell remain an important, and as yet unresolved, issue.[1]

The pathways from dietary B_6 vitamers to brain PLP, the PLP recycling pathways, and the pathway for catabolism of excess PLP are shown in Figure 1. There are several disorders that are known to affect these pathways. In 1995, Waymire et al. showed that mice lacking tissue non-specific alkaline phosphatase (TNSALP) have a reduced concentration of PLP in the brain which disrupts neurotransmitter metabolism and causes seizures.[2] In man, the clinical picture of hypophosphatasia (*ALPL* [MIM: 171760]) caused by a lack of TNSALP is usually dominated by bone disease, but pyridoxine-responsive seizures can occur.[3] In 2004, Gachon et al. showed that, in the mouse, knockout of the three transcription factors that activate pyridoxal kinase leads to low brain levels of PLP, dopamine, and serotonin and to severe epilepsy.[4] In 2005, we described a cohort of infants with neonatal epileptic encephalopathy and changes in cerebrospinal fluid (CSF) concentrations of neurotransmitter amine precursors and

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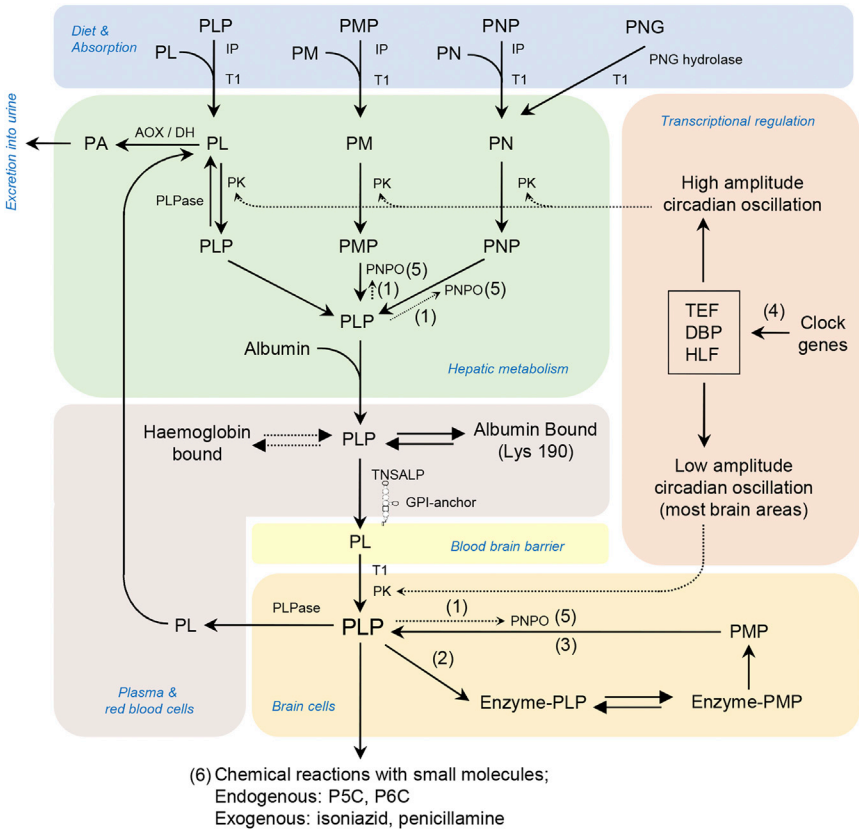

Figure 1. Enzymes and Transporters Involved in Mammalian CNS PLP Synthesis and Homeostasis and Known Human Genetic Vitamin-B_6-Dependent Epilepsies
Pyridoxal 5′-phosphate (PLP); pyridoxamine 5′-phosphate (PMP); pyridoxal (PL); pyridoxine (PN); pyridoxine 5′-phosphate (PNP); pyridoxine-5′-β-D-glucoside (PNG); intestinal phosphatases (IP); transporter (identity unknown; T1); pyridoxal kinase (PK); pyridox(am)ine 5′-phosphate oxidase (PNPO); tissue non-specific alkaline phosphatase (TNSALP); pyridoxal-phosphatase (PLPase); aldehyde oxidase (Mo cofactor)/β-NAD dehydrogenase (AOX/DH). (1) PNPO is controlled by feedback inhibition from PLP. (2) PLP functions as a co-factor, forming Schiff bases with the ε-amino group of lysine residues of proteins. (3) PLP can be formed by recycling the cofactor from degraded enzymes (salvage pathway). (4) PLP levels are maintained, in part, by circadian-clock-controlled transcription factors with PAR bZip transcription factors (DBP, HLF, and TEF) targeting PK. (5) *PNPO* mutations cause a B_6-dependent epilepsy disorder. (6) Disorders resulting in accumulation of L-Δ^1-pyrroline-5-carboxylic acid (P5C) and Δ^1-piperideine-6-carboxylic acid (P6C) (hyperprolinaemia type II and pyridoxine-dependent epilepsy due to mutations in *ALDH4A1* and *ALDH7A1*, respectively) cause decreases in bioavailable PLP as do reactions with exogenous small molecules.

metabolites, indicating deficient activity of aromatic L-amino acid decarboxylase, the PLP-dependent enzyme required for synthesis of dopamine and serotonin.[5] Raised levels of threonine and glycine in the CSF suggested there might be a general defect of B_6-dependent enzymes and, although the infants' seizures did not show much response to treatment with pyridoxine, they responded dramatically to PLP. We were able to show that this cohort of individuals had homozygous mutations in *PNPO* (MIM: 603287), encoding pyridox(am)ine 5′-phosphate oxidase, that substantially reduced the catalytic efficiency of the enzyme. Deficiency of PNPO (MIM: 610090) impairs PLP synthesis and recycling, which is clear from Figure 1. More recently, we have been able to show that individuals with PNPO deficiency can be pyridoxine-dependent rather than only PLP-dependent.[6] In 2006, we showed that pyridoxine-dependent epilepsy (MIM: 266100) is usually caused by accumulation of a metabolite that reacts with PLP, Δ^1-piperideine-6-carboxylate. This metabolite accumulates because of a block in the pipecolic acid pathway of lysine catabolism (ALDH7A1 deficiency);[7] a similar mechanism occurs with the accumulation of Δ^1-pyrroline-5-carboxylate in hyperprolinaemia type II (*HYRPRO2* [MIM: 239510]).[8] Finally, there is a group of disorders in which alkaline phosphatase cannot be anchored because of a defect in the glycosylphosphatidylinositol anchor pathway (GPI-AP deficiencies). Circulating alkaline phosphatase levels are high (hyperphosphatasia), and in some individuals, seizures respond to treatment with pyridoxine.[9]

These days, faced with a child with seizures that show a response to either pyridoxine or to PLP, it is possible to exclude hypophosphatasia and hyperphosphatasia, PNPO deficiency, and ALDH7A1 deficiency by DNA analysis, enzymology, or metabolite analyses (or a combination). When we do this, we are left with a group of children for whom the underlying cause remains obscure. This paper describes the results of investigation of a consanguineous Syrian family in which there have been three children affected by pyridoxine-dependent epilepsy. This led to the implication of a gene of hitherto unknown function, *PROSC* (proline synthetase co-transcribed homolog [bacterial]) (GenBank: NM_007198.3). Subsequent investigation of a cohort of 29 children and young adults with B_6-dependent epilepsy revealed biallelic mutations in *PROSC* in four additional individuals. Analysis of body fluids and fibroblasts of PROSC-deficient individuals suggest that it is an intracellular binding protein that is involved in the homeostatic regulation of free PLP levels.

PROSC is ubiquitously expressed in human tissues and is highly conserved throughout evolution, suggesting an important cellular function.[10] The gene product is a cytoplasmic protein that has a PLP-binding barrel domain similar to the N terminus of bacterial alanine racemase

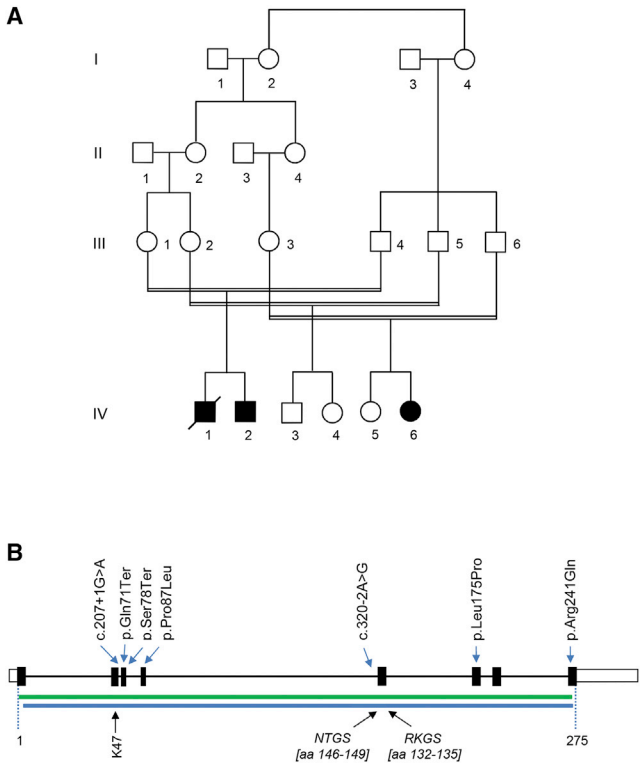

Figure 2. Pedigree of Index Family and Position of *PROSC* Variants

(A) Pedigree of index family (subjects 1–3) and segregation analysis of c.233C>G (p.Ser78Ter) for this family. Affected individuals are homozygous for GG. Analysis of extended family DNA demonstrated that both parents are heterozygous carriers of the identified variant, and all other individuals were found to either be wild-type (CC) or heterozygous (GC) for this variant. Squares represent males, circles represent females, and a double line represents a consanguineous union. Black shapes represent affected individuals, shown subsequently to be homozygous for c.233C>G. The diagonal line through the square indicates that this individual is deceased. Genotypes: II.2, II.4 = GC; III.1, III.2, III.3, III.4, III.5 = GC; III.6 = CC; IV.1, IV.2, IV.6 = GG; IV.3, IV.4 = GC; IV.5 = CC.

(B) Predicted features of *PROSC* and position of mutations. This gene spans 17.17 kb and consists of eight exons. The translated protein is 275 amino acids in length. Blue arrows indicate positions of mutations detected. The green line represents the PLP-binding barrel domain (amino acids 21–250), and the blue line represents the alanine racemase N-terminal domain (amino acids 17–251). K47 is proposed to have an N6-pyridoxal-phosphate modification (by similarity). A cAMP- and cGMP-dependent protein kinase phosphorylation site, RKGS (amino acids 132–135), and a putative N-linked glycosylation site, NTGS (amino acids 146–149), are also predicted. aa, amino acids.

and eukaryotic ornithine decarboxylase[11] to which PLP binds without affecting the quaternary structure,[12] and, although PROSC deficiency in bacteria affects amino acid metabolism, the protein has no definitive enzyme activity.[11] Recently YggS, the *E.coli* homolog, was implicated in PLP homeostasis.[12] In the absence of the PROSC homolog (Δ*YggS* strain), a pyridoxine-containing disc produces a ring of bacterial growth inhibition. We have used this to test the function of human PROSC; we demonstrated that transfection with wild-type *hPROSC* abolished the ring of growth inhibition, whereas *hPROSC*-bearing mutations found in the infants with B_6-responsive epilepsy (p.Leu175Pro [c.524T>C], p.Arg241Gln [c.722G>A], and p.Ser78Ter [c.233C>G]) did not.

Subjects and Methods

Subjects and Samples

All samples from affected individuals and their families were obtained after approval of the study by the ethics committee at the University of Gothenburg and/or the National Research Ethics Service (NRES) Committee (London, Bloomsbury [REC ref. no. 3/LO/0168]). Written informed consent was obtained for all subjects and also for all family members included in the study. Total genomic DNA was extracted from EDTA blood with a Gentra Puregene Blood Kit according to the manufacturer's instructions (QIAGEN) or with an AutoGenFlex STAR automated system according to the manufacturer's protocol. Stored fibroblasts from subjects 2, 4, and 5 were cultured via standard methods. All fibroblasts were tested for mycoplasma contamination prior to any experiments being performed.

Homozygosity Mapping and Whole-Exome Sequencing

Copy-number-neutral loss-of-heterozygosity and whole-exome sequencing analyses were undertaken on the index family. The pedigree of this family is shown in Figure 2A. DNA from two siblings, one affected (IV.6) and one unaffected (IV.5), and one affected cousin (IV.2) were analyzed with a GeneChip Human Mapping 250K Nsp I Array from Affymetrix and analyzed with the software CNAG v.3.0. Several regions of copy-number-neutral loss of heterozygosity were identified, and of these, only one overlapped in both affected individuals but was absent in the healthy sister. This region was subsequently analyzed by whole-exome sequence analysis. DNA was enriched with the Agilent SureSelect Human All Exon and sequenced with the Illumina HiScanSQ. Quality assessment of the next-generation sequencing (NGS) reads was performed by generating quality control statistics with FastQC (see Web Resources). Read alignment to the reference human genome (UCSC Genome Browser hg19, February 2009) was done with the Burrows-Wheeler Aligner[13] with default parameters. Quality score recalibration, indel realignment, and variant calling were performed with the Genome Analysis Toolkit package.[14] Variants were annotated with ANNOVAR.[15] Filtering was done with regard to exonic changes in protein-coding genes located in the loss-of-heterozygosity region on chromosome 8p11.23–p21.2, present in homozygous state in both affected individuals, and finally on predicted function (synonymous changes excluded).

Sanger Sequencing of *PROSC*

Sequence validation of NGS data, subsequent mutation screening of a cohort of 29 children who had vitamin B_6-responsive seizures, and segregation analysis were performed by Sanger sequencing of PCR products amplified from genomic DNA (primers listed in Table S1) for the coding exons and intron/exon boundaries of *PROSC* (ENST00000328195). Amplicons were purified with Shrimp Alkaline phosphatase and Exonuclease I prior to bi-directional sequencing with the Big Dye Terminator Cycle Sequencing System v.1.1 (Applied Biosystems) on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems). Population frequencies of identified sequence changes were obtained from the Ensembl and

ExAC databases (accessed February 8, 2016). Clustal Omega software (see Web Resources) was used to align sequences.

Cloning of Human *PROSC* cDNA from Fibroblasts of Affected Individuals and Sequence Analysis of cDNA

Total RNA was extracted from fibroblasts with an RNeasy Mini Kit (QIAGEN), according to the manufacturer's instructions. cDNA synthesis was carried out with the SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Scientific) and oligo(dT)20 primers. Amplification of *PROSC* cDNA was carried out using the reaction conditions and primers detailed in Table S2. Individual cDNA products were gel purified with the QIAquik Gel Extraction Kit (QIAGEN) and cloned into TOPO 2.1 (Thermo Scientific), and TOP10 competent cells were (Invitrogen) transformed. *PROSC* cDNA inserts were sequenced using the conditions and primers detailed in Table S3.

Quantitative Real-Time PCR

Expression analysis of *PROSC* was performed with fibroblast cDNA generated as described above. Real-time PCR was carried out on a StepOne Real-Time PCR System (Thermo Scientific) with the following TaqMan Gene Expression Assays: PROSC (Hs00200497_m1), β-actin (4333762T), and GAPDH (hs02758991_g1). Relative quantification of gene expression was determined via the $2-\Delta\Delta Ct$ method, with β-actin and GAPDH as reference genes.

Immunoblot Analysis

Proteins in fibroblast lysates were separated electrophoretically on a NuPAGE Novex 4% −12% Bis-Tris Protein Gel (Thermo Fisher) and transferred to a polyvinylidene difluoride (PVDF) membrane using an iBlot Dry Blotting System according to the manufacturer's instructions. Blots were probed with PROSC primary antibody raised in rabbit (1:100 in 5% TBST milk; HPA023646, Sigma) and after incubated with donkey anti-rabbit IgG-HRP (1:5,000; sc-2317, Santa Cruz Biotechnology). The membrane was developed with the Novex ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher) and imaged with a ChemiDoc MP System (Bio-Rad) coupled to ImageLab 4.1 software. Equivalent loading was determined with mouse β-actin primary antibody (1:40,000; A1978, Sigma) and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (1:3,000; P0447, DAKO).

B_6 Vitamers and 4-Pyridoxic Acid Analysis

The method used was essentially as described by Footitt et al.[16] for the measurement of plasma B_6 vitamers with the following minor modifications. Fibroblast pellets were lysed and 10 μL of supernatant added to 110 μL of master mix (containing 40 μL of dH_2O, 60 μL of 0.3N trichloroacetic acid [TCA], and deuterated internal standards: 100 nM each of D2-PLP, D2-PA, and D3-PM, 10 nM D2-PN, and 50 nM D3-PL). In the case of CSF, 50 μL of sample was added to 70 μL of master mix (containing 60 μL of 0.3 N TCA and deuterated internal standards: 100 nM each of D2-PLP, D2-PA, and D3-PM, 10 nM D2-PN, and 50 nM D3-PL). Supernatants were analyzed by liquid chromatography–mass spectrometry (LC-MS/MS) (Waters Acquity Ultra Performance LC system linked to a triple Quadrupole Xevo TQ-S instrument) on an Acquity UPLC HSS T3 column (1.8 μm × 2.1 mm × 50 mm) fitted with a HSS T3 VanGuard guard column (Waters) using a mixture of mobile phase A (3.7% acetic acid with 0.01% HFBA) and B (100% methanol) at a flow rate of 0.4 mL/minute. Details of the mobile phase gradients are shown in Table S4. The quantification of the different analytes was performed using the transitions detailed in Table S5.

Fractionation of Fibroblast Cell Lysates

Fibroblast cell lysate supernatants were fractionated with Amicon Ultra-0.5 mL 3 kDa Centrifugal Filters (Millipore). The B_6 vitamers in the resulting fractions (> 3 kDa, < 3 kDa) and in the unfiltered supernatant were analyzed with the ultra performance (UP)LC-MS/MS method described above and were corrected for the unfiltered protein concentration.

Cloning and Generation of *PROSC* Mutations by Site-Directed Mutagenesis

Wild-type human *PROSC* cDNA in pOTB7 (Thermo Scientific; BC012334.1; clone ID 3546307) was used as a template. Site-directed mutagenesis was carried out with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) in order to generate p.Ser78Ter. The primers used for site-directed mutagenesis are as detailed in Table S5. Wild-type *PROSC* and p.Ser78Ter *PROSC* were subsequently amplified with the following primers: SDHuyggS-fwd (5′-GGAA TTCAGGAGGTATACACCATGTGGAGAGCTGGCAGCAT-3′) and HuyggS-rev (5′-CGCGGATCCGGTACCTTAGTGCTCCTGTGCCA CC-3′) and SDHuyygS-fwd and SHuyggS-rev (5′-ATTATTGGATCC TTATTAGTCGACTTATGCTTTTTCTAGCAGTTCCTGAACGTAG-3′). The resulting PCR products were subcloned into the EcoRV and BamHI sites of the multiple cloning site (MCS) of pBEY279.1[12] to generate pBEY332.1 and pBEY330.4. Wild-type and p.Ser78Ter *PROSC* were subsequently amplified from pBEY332.1 and pBEY330.4 with the following primers: FW_PBAD33_SacI_RBS_ ProsC (5′-TTAGCGAGCTCAGGAGGAATTCACCATGTGGAGAGCT GGCA-3′) and RV_PBAD33_SalI_ProsC (5′-TTATTAGTCGACTT AGTGCTCCTGTGCCACCTCCAGCGGG-3′) and FW_PBAD33_ SacI_RBS_ProsC and RV_PBAD33_salI_PROSC_stop (5′-TTATTAG TCGACTTATGCTTTTTCTAGCAGTTCCTGAACGTAG-3′). This was done to enable sub-cloning of the gene into the SacI and SalI sites of the multiple cloning site of pBAD33 to generate the plasmids pLSP01 and pLSP02. The Q5 Site-Directed Mutagenesis Kit (New England Biolabs) was used essentially as described by the manufacturer to generate the missense variants p.Arg241Gln, p.Leu175Pro, and p.Pro87Leu, with the plasmid pLSP01 as a template to generate the plasmids pLSP07, pLSP03, and pLSP05, respectively. The primers used for site-directed mutagenesis are as detailed in Table S6.

E. coli Complementation Studies

The *E. coli* ΔyggS (VDC6594) was freshly transformed with the empty vector (pBAD33) as a negative control, and pLSP01, pLSP02, pLSP03, pLSP05, and pLSP07 were transformed as described previously.[12] Two sterile discs were set on the top of the agar and 20 μL of PN (0.1 mg/mL or 1 mg/mL) was added to the disc and plates were incubated overnight at 37°C.[12] The sensitivity to pyridoxine is defined as the distance (in cm) from the disc where the ΔyggS strain does not grow.

Results

Homozygosity Mapping and Exome Sequencing of Index Family

In order to identify shared regions of homozygosity and potential candidate genes, DNA of the index family from affected individuals IV.2 and IV.6 and the unaffected sister

Table 1. Genotypes of Subjects with *PROSC* Mutations

Subject	Position	Nucleotide	Predicted Effect	Allele Frequency[a]
1[b]	exon 3	c.233C>G (M) + c.233C>G (P)	p.Ser78Ter	novel
2[b]	exon 3	c.233C>G (M) + c.233C>G (P)	p.Ser78Ter	novel
3[b]	exon 3	c.233C>G (M) + c.233C>G (P)	p.Ser78Ter	novel
4	exon 6	c.524T>C (M) + c.524T>C (P)	p.Leu175Pro	1 in 121,412
5	intron 2	c.207+1G>A (M)	splicing effect	4 in 121,408
	intron 4	c.320-2A>G (P)	splicing effect	novel
6	exon 3	c.211C>T + c.211C>T	p.Gln71Ter	novel
7	exon 4	c.260C>T (M)	p.Pro87Leu	2 in 121,410
	exon 8	c.722G>A (P)	p.Arg241Gln	5 in 121,407

Abbreviations are as follows: M, maternal allele; P, paternal allele.
[a]Data from ExAC; although individuals heterozygous for p.Pro87Leu, p.Leu175Pro, p.Arg241Gln, and c.207+1G>A have been reported on the ExAC database, the frequency of these variants is <0.01% and there are no reports of any homozygous individuals. "Novel," as used here, refers to an absence in the ExAC database.
[b]Related.

of IV.6, i.e., IV.5, were analyzed on a human mapping array. Several overlapping copy-number-neutral loss-of-heterozygosity regions were identified in the affected individuals, however, only one was absent in the healthy sister. However, none of the 77 protein-coding genes in this region (chromosome 8p11.23–p21.2; Table S7) obviously explained the seizures. Characterization of this region via whole-exome sequencing for individuals IV.2 and IV.6 identified 18 exonic single-nucleotide variants (Table S7) present in both affected individuals after filtering. One had a stop-gain predicted function, c.233C>G (p.Ser78Ter) in *PROSC*, a gene of unknown function. This variant had not been reported in the dbSNP, 1000 Genomes, or ExAC databases.

Sanger Sequencing of *PROSC*

Sequencing of *PROSC* in the index family (subjects 1–3; Table 1) showed that this variant segregated within the family (Figure 2A). Genotyping of 237 Swedish and 89 Syrian control individuals (living in Sweden) for c.233C>G identified one heterozygote and no homozygotes. Subsequent sequencing of *PROSC* in a cohort of 29 children with B_6-repsonsive epilepsy (for whom ALDH7A1 and PNPO deficiency had been excluded via genetic and/or biochemical evidence) identified potential pathogenic variants in four additional subjects (Subjects 4–7; Table 1, Figure 2B, Figures S1 and S2), and analysis of parental DNA for these families confirmed that the parents were heterozygous carriers. Whereas the nonsense mutation p.Gln71Ter (c.211C>T) and the splice-site mutation c.320-2A>G have not been reported in the ExAC, dbSNP, or Ensembl databases, the missense mutations p.Pro87Leu (c.260C>T), p.Leu175Pro (c.524T>C), and p.Arg241Gln (c.722G>A) and the splice-site mutation c.207+1G>A have, but only in heterozygous form with a prevalence of <0.01%.

Clinical Histories

The index family is of Syrian descent and includes three affected members with vitamin B_6-dependent seizures in whom PNPO and ALDH7A1 deficiencies were excluded: two siblings (IV.1 and IV.2) and a cousin (IV.6). The pedigree in Figure 2A demonstrates an autosomal-recessive pattern of inheritance. Clinical characteristics of the affected children are summarized in Table 2. Detailed clinical histories of all seven affected individuals are in the Supplemental Note and are tabulated in Table 2.

Comparison of the Clinical Phenotypes

Of the seven individuals with *PROSC* mutations, three had abnormal intrauterine movements (including the one subject whose mother was being treated with pyridoxine throughout pregnancy), four showed signs of fetal distress, and four had a birth head circumference (HC) at or below the 9th percentile. All affected infants presented with seizures on day 1 of life, apart from subject 7, who presented at 1 month of age. The commonest electroencephalogram (EEG) finding was burst suppression (in 5/7 individuals). All responded to pyridoxine treatment with an immediate reduction in seizure frequency and severity; two showed respiratory depression. In the case of subject 1, concerns about respiratory depression led to the pyridoxine being discontinued for several weeks, and this infant died. Some affected infants showed signs of systemic disease in addition to the seizures in the first few days of life: 4/7 infants had metabolic acidosis with raised blood lactate, 2/7 had anemia, and 3/7 had gastrointestinal dysfunction, including abdominal distension, vomiting, necrotizing enterocolitis. Upon follow-up, four affected individuals had their B_6 treatment changed from pyridoxine to PLP and all showed an improvement in seizure control. The majority (5/6) of the surviving subjects require treatment with anticonvulsants (clobazam, carbamazepine, levetiracetam, and/or phenobarbitone) in addition to vitamin B_6, with 3/5 subjects receiving levetiracetam for optimal seizure control. Neonatal MRI scans where available (4/7) and with the exception of the one individual treated in utero showed global

Table 2. Clinical Characteristics of Individuals with Mutations in *PROSC*

	Subject 1	Subject 2[a]	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Gender	M	M	F	F	F	M	M
Current age	died at 4.5 m	9 y	6 y	3 y, 6 m	5 y, 6 m	3 y, 2 m	16 y
Ethnicity (domicile)	Syrian (Sweden)	Syrian (Sweden)	Syrian (Sweden)	Indian (UK)	German (Germany)	Indian (UK)	Italian (Italy)
Consanguinity	+	+	+	+	−	+	−
Birth gestational age (weeks)	32	40	36 and 6 days	36 and 3 days	35 and 1 day	39	39
Abnormal intrauterine movements	−	+	+	+	−	−	−
Fetal distress	+	−	−	+	+	+	−
Apgar scores	5, 10, 10	8, 9, 10	8, 8, 9	7, 8, 8	8, 8, 9	9, 9, 9	9, 9, 10
Birth HC percentile	25%–50%	2%–9%	9%	90%	9%	9%	25%–50%
Anemia at birth	+	−	−	+	−	−	−
Acidosis	+	−	+	+	+	+	−
Raised blood lactate	+	−	−	+	+	+	not known
Respiratory distress	−	−	−	+	+	+	−
Hypertonia (neonatal)	−	+	−	+	−	+	−
Hypotonia (neonatal)	−	−	−	−	+	−	−
Abdominal distension, vomiting, or feed intolerance	+ NEC	−	−	+	+	−	−
Irritability	not known	not known	not known	+	+	+	−
Seizure onset within 24 hr	+	+	+	+	+	+	−[b]
Response to AEDs	−	given with PN; some improvement	given with PLP/PN; some improvement	minimal	partial	given with PLP; better control	partial[c]
EEG: burst suppression	+	−	+	+	+	+	−
EEG: reduced background activity	+	+	+	NR	NR	NR	−
EEG: focal discharge(s)	NR	+	NR	NR	NR	NR	−
EEG: multifocal spikes	NR	NR	NR	NR	+	NR	−
Seizure type:[d] tonic	+	+	NR	NR	+	+	NR
Seizure type:[d] clonic	−	−	−	−	−	+	−
Seizure type:[d] GTC	−	+	+	+	−	+	+
Seizure type:[d] myoclonic	+	+	−	−	+	−	−
Seizure type:[d] lip-smacking and/or grimacing	+	−	−	+	+	NR	NR
Response to pyridoxine	+	+	+	+	+	+	+
Respiratory depression upon treatment with PN in neonatal period	+	−	−	−	+	−	NA
PN withdrawal undertaken (and led to recurrence of seizures)	+ (+)	−	−	−	+ (+)	−	−
Switch from PN to PLP (improved seizure control)	−	−	+ (+)	+ (+)	+ (+)	+ (+)	−
PLP withdrawal (recurrence of seizures)	NA	NA	−	−	+ (+)	+ (+)	NA
Speech delay	NA	+	+	+	+	+	−

(*Continued on next page*)

Table 2. ***Continued***

	Subject 1	Subject 2[a]	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Motor delay	NA	+	+	+	+	–	–
Learning difficulties	NA	+	+	+	+	+	+
Breakthrough seizures with fever	NA	+	+	–	+	+	+
Acquired microcephaly	+	+	+	+	+	+	–
Minor dysmorphic features[e]	+	+	+	not known	+	–	–

Abbreviations are as follows: NA, not applicable; NR, not reported; m, months; y, years; AEDs, antiepileptic drugs; GTC, generalized tonic-clonic; NEC, necrotising enterocolitis; HC, head circumference; EEG, electroencephalogram; PN, pyridoxine; PLP, pyridoxal 5′-phosphate.
[a]Subject was treated prophylactically; the mother was given 100 mg pyridoxine daily through pregnancy.
[b]Subject presented at 1 month of age.
[c]Anticonvulsant treatment stopped at the age of 8 years.
[d]Refers to seizure type at time of presentation.
[e]2/4 subjects had a thin upper lip, 1/4 had a long philtrum, 1/4 had upslanting palpebral fissures, 1/4 had a convergent squint, and 1/4 had periorbital fullness.

underdevelopment of the brain and periventricular germinolytic cysts (Figure 3, Table 3). Follow-up scans showed persistent white matter abnormalities, and HC measurements revealed acquired microcephaly. Although all subjects showed some degree of developmental delay (Table 2), one attended normal school and leads a normal life. Minor dysmorphic features were evident for four of the affected individuals.

Analysis of Impact of *PROSC* Sequence Variants found in Subjects with B_6-Dependent Epilepsy

Sequence alignment (Figure S2) of PROSC and COG0325 family members to which PROSC belongs shows that two of the missense changes identified, p.Leu175Pro and p.Arg241Gln, affect residues conserved across mammalian species and in bacteria and yeast. Pro87, however, is only conserved across higher organisms. Analysis of mRNA in fibroblasts indicated that p.Leu175Pro (c.524T>C), in subject 4, decreases *PROSC* expression ($p < 0.0001$) (Figure 4A, Figure S3), resulting in no detectable PROSC in fibroblasts (Figure 4B). PROSC is also not detectable in fibroblasts of subject 2 (Figure 4B), with c.233C>G (p.Ser78Ter) presumably resulting in nonsense-mediated mRNA decay (Figure 4A). Fibroblasts were not available to allow us to study p.Gln71Ter or the effects of a combination of p.Pro87Leu with p.Arg241Gln further.

Analysis of cDNA from subject 5 revealed that c.207+1G>A and c.320-2A>G affect DNA splicing (Figure 4C) and result in decreased mRNA expression (Figure 4A). The largest cDNA product originates from c.207+1G>A and is due to inclusion of intron 2 (Figure S4) and results in nonsense-mediated decay due to the introduction of a premature stop codon (p.Val70Ilefs*6; Figure S4), while the smallest cDNA product arises due to skipping of exon 2 and an in-frame deletion of 36 amino acids (p.Asp34_Tyr69del) and corresponds to a protein of ≈26 kDa (Figure 4B). Sequencing of the intermediate product revealed that c.320-2A>G does not cause skipping of exon 5, but results in deletion of the first ten amino acids of this exon and use of a cryptic splice site (p.Ala107_Thr116del).

Biochemical Profiles of Individuals with *PROSC* Mutations

The response of individuals with *PROSC* mutations to B_6 treatment indicated CNS PLP deficiency, and biochemical analyses (where available) supported this. Subjects 4 and 5 had low CSF PLP concentrations prior to or just after starting B_6 supplementation (Table 4), and subjects 2, 4, and 5 displayed features of deficiency of aromatic L-amino acid decarboxylase (AADC),[19] a B_6-dependent enzyme, including elevated CSF concentrations of 3-ortho-methyldopa, L-dopa, and/or 5-hydroxytryptophan (Table 5), and urinary organic acid analysis showed raised vanillactate. Glycine, the substrate for the B_6-dependent glycine cleavage enzyme, was on occasions slightly elevated when subjects 4 and 5 were off B_6 supplementation (Table S8), as was alanine, a substrate of B_6-dependent alanine transaminase (Table S8). When subjects were on B_6 supplementation, CSF amino acid levels normalized with the exception of tyrosine (substrate of the PLP-dependent enzyme tyrosine aminotransferase), which was slightly raised on several occasions in subject 2 (Table S9).

Pre-treatment CSF profiling of subject 5 revealed that homocarnosine, a histidine dipeptide carbonyl scavenger,[20] was undetectable (Table S10).

Vitamin B_6 Profiles of Plasma and Fibroblast Samples from PROSC-Deficient Individuals

Analysis of B_6 vitamer profiles of plasma samples from subjects on B_6-supplementation revealed that PROSC-deficient individuals had high plasma PLP levels but, unlike individuals with PNPO deficiency,[16] do not accumulate pyridoxine, pyridoxamine, pyridoxine phosphate, and pyridoxamine phosphate (Table S11), suggesting PNPO activity is not impaired. Plasma PLP levels, although not as high as those seen in hypophosphatasia caused by mutations in *ALPL*,[21] were 4–7 times higher than those

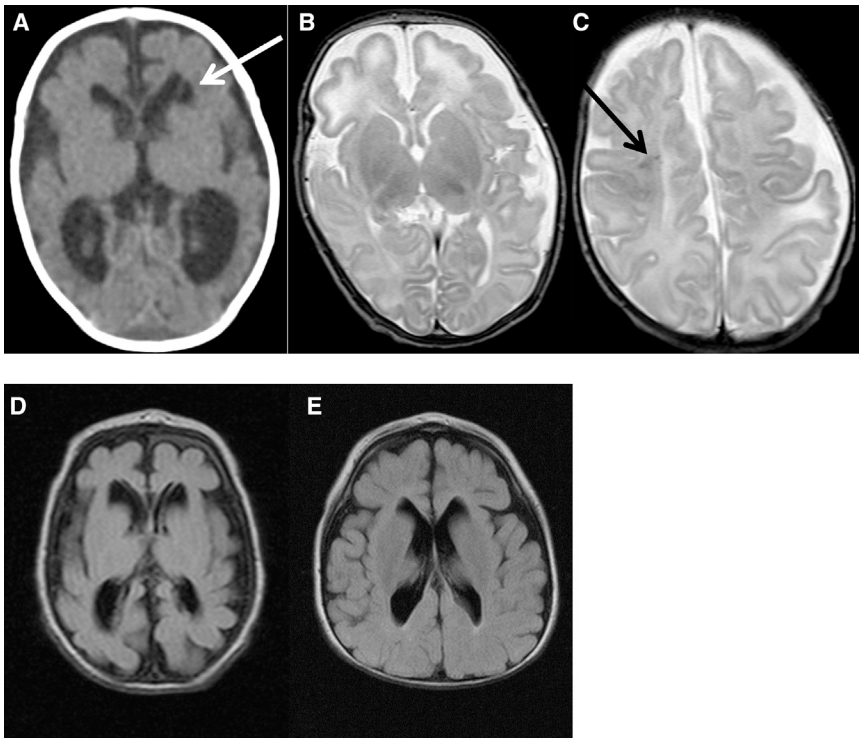

Figure 3. CT and MRI Brain Features of Individuals with Mutations in *PROSC*

(A) Computed tomography (CT) head scan (axial) of subject 1 at 2 months of age showing underdevelopment of brain with broad gyri and shallow sulci, as well as a cyst adjacent to the left frontal horn (white arrow).

(B and C) MRI scan (axial T2 weighted) of subject 4 at 2 months of age showing underdevelopment of brain with broad gyri and shallow sulci, as well as subcortical and deep white matter edema and white matter petechial hemorrhages (black arrow).

(D) MRI scan (axial, T2 flair) from subject 5 at 16 days of age showing global underdevelopment of brain with coarse gyral pattern and bilateral large cysts adjacent to the frontal horns.

(E) MRI scan (axial, T2 flair) from subject 5 at 1 year and 6 months of age showing global underdevelopment of the brain with a coarse gyral pattern, more severe at the frontal poles, and underdevelopment of white matter.

reported for individuals with PNPO and PDE deficiencies and receiving comparable supraphysiological doses of B_6 (Table S11; unpublished clinical diagnostic data, Neurometabolic Unit, National Hospital for Neurology and Neurosurgery, Queen Square). TNSALP activity was normal (Table S12) in all PROSC-deficient individuals for whom it was measured, and no pathogenic sequence variants in *ALPL* were detected in whole-exome sequencing data available for subjects 1 and 2.

Similarly to plasma, PLP/protein ratios were also found to be elevated in fibroblasts from PROSC-deficient individuals (when grown in standard media [containing pyridoxine]) and were 2–3 times greater than those of controls (Figure 4D). The concentration of pyridoxal was significantly elevated in cells from subject 2 (Figure S5), and this might have arisen from phosphatase activity on elevated PLP concentrations. Comparison of other B_6 vitamers in affected and control fibroblasts was unremarkable.

PLP is a highly reactive aldehyde[22] and can bind not only via Schiff base linkages to the epsilon amino groups on lysine residues at the active site of B_6-dependent enzymes, but can also react with other residues of proteins[23], with metabolites such as Δ^1-piperideine 6-carboxylate,[7,8] with many amino acids, particularly cysteine (forming thiazolidine adducts),[24,25] and with histidine dipeptides.[20] Therefore, the PLP distribution between low (<3 kDa) and high (>3 kDa) molecular weight fractions from control and PROSC-deficient fibroblasts was investigated. This was similar between fractions (Figure S6), suggesting that supra-physiological cellular concentrations of PLP react non-specifically with both proteins and small molecules.

Complementation of the Pyridoxine-Sensitive Phenotype of ΔyggS *E. coli*

To further explore the role of PROSC in PLP homeostasis, we looked for complementation of the pyridoxine-sensitive phenotype of the Δ*yggS E.coli*[12] by wild-type and mutant human *PROSC* (encoding p.Ser78Ter, p.Pro87Leu, p.Leu175Pro, and p.Arg241Gln) (Figure 5, Table S13). Wild-type human PROSC and p.Pro87Leu, which is not conserved between human PROSC and *E.coli* YggS, restored growth. The missense variants p.Leu175Pro and p.Arg241Gln, however, which affect highly conserved amino acids across the YggS/PROSC/YBL036C family (COG0325), did not complement the Δ*yggS* pyridoxine toxicity phenotype, nor did the nonsense variant p.Ser78Ter. Interestingly p.Arg241Gln appeared to exacerbate the toxicity effect of pyridoxine on the ΔyggS *E. coli* mutant.

Discussion

Homozygosity mapping and exome sequencing of a consanguineous family led to the discovery of a homozygous nonsense variant, p.Ser78Ter, in *PROSC*, a gene of hitherto unknown function; this variant segregates with a phenotype of prenatal- and/or neonatal-onset seizures that respond to treatment with pyridoxine and PLP. The phenotype is expressed in all three affected children but not in nine heterozygotes and two homozygous wild-type individuals across three generations. In a cohort of individuals with B_6-responsive epilepsy, in which known causes of B_6-dependent epilepsy had been excluded,

Table 3. MRI Brain Features of Individuals with Mutations in *PROSC*

Subject	Age	MRI Feature				
		Global Underdevelopment of Brain with Broad Gyri and Shallow Sulci	Microcephaly with Global Underdevelopment of White Matter	Cyst(s)	White Matter Edema Deep White Matter Petechial Hemorrhages[a]	Normal
1	2 m	+	+[b]	one (close to L frontal horn)	–	–
2	3.5 m	–	–	–	–	+[c]
	23 m	–	–	–	–	+[c]
3	10 d	+	+[b]	one (close to L frontal horn)[d]	–	–
	2 y, 2 m	+	+	one (close to L frontal horn)[d]	–	–
4	10 d	+	+[b]	–	+	–
	2 m	+	+[b]	–	–	–
	2 y	+	+	–	–	–
5	16 d	+[e]	+[b]	two (bilateral, adjacent to frontal horns)[d]	–	–
	7 m	+[f]	+[b]	have coalesced with ventricular system	–	–
	1 y, 6 m and 3 y, 11 m	+[g]	+	coalesced	–	–
6	2 m	–	–	–	–	+[h]
7	10 y	–	–	–	–	+[h]

Abbreviations are as follows: m, months; d, days; y, years; L, left.
[a]Asymmetrical.
[b]Too young to determine white matter development.
[c]Treated in utero and from birth.
[d]With associated hemorrhage.
[e]With failure of operculisation.
[f]Magnetic resonance spectroscopy showed a low N-acetyl asparate (NAA)/creatine ratio, a high choline/creatinine ratio, and high lactate peaks in parietal white matter.
[g]More severe in frontal regions.
[h]Not reviewed in London.

homozygous or compound heterozygous mutations were found in *PROSC* in four of the 29 children and young adults (14%). Little is known about the COG0325 family of proteins to which PROSC belongs. Although structural studies of the yeast and *E. coli* family proteins[26] have shown that these proteins are similar to the N terminal of alanine racemase and ornithine decarboxylase and bind PLP in a similar manner, they are monomeric and have no racemase activity toward any of the 20 protein amino acids or their D-enantiomers.[11,12] Indeed, PLP binding does not affect the quaternary structure of the protein.[12]

As with pyridoxine-dependent epilepsy due to ALDH7A1 (antiquitin) deficiency[27,28] and pyridoxine/PLP-dependent epilepsy due to PNPO deficiency,[6] the clinical phenotype is dominated by the B_6-dependent seizure disorder, although systemic signs such as anemia, enterocolitis, electrolyte abnormalities, and lactic acidosis might be present in the neonate. The birth HC was at or below the 9th percentile for four out of the seven subjects with PROSC deficiency; birth HC is usually normal in individuals with PNPO deficiency and ALDH7A1 deficiency. Also, in PROSC deficiency, we noted MRI features showing global underdevelopment of the brain (broad gyri and narrow sulci) and later underdevelopment of white matter. Periventricular cysts could be present in the neonatal scans. In the child treated during pregnancy, these changes were not seen but acquired microcephaly was evident on follow-up for him and all but one of the other PROSC-deficient individuals. All affected children had some degree of developmental delay, whereas some individuals with PNPO deficiency and 25% of individuals with ALDH7A1 deficiency have a normal neurodevelopmental outcome.[28,29]

Analysis of CSF concentrations of neurotransmitter amine precursors and metabolites on PROSC-deficient subjects off treatment showed elevation of 3-O-methyldopa (2/3 subjects) and 5-hydroxytryptophan (2/2 subjects)—and also consistent abnormalities in PNPO deficiency—but minimal or no reduction in homovanillic acid (HVA) or 5-hydroxyindoleacetic acid (5HIAA). HVA and 5HIAA were low in the first-described individuals with PNPO deficiency[5] but not consistently in subsequently described

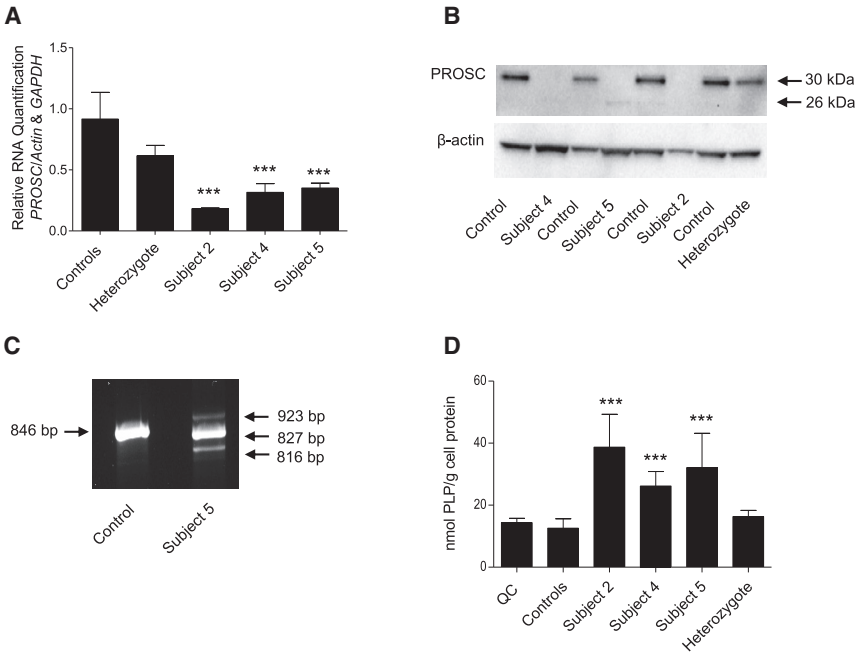

Figure 4. Loss of *PROSC* Expression Affects Intracellular PLP Homeostasis

(A) qRT-PCR showing *PROSC* expression in affected individuals and wild-type. Reference genes: *β-Actin* and *GAPDH*. Data are presented as means ± SD. Statistical analysis was performed with Student's two tailed t test; ***$p < 0.001$. Controls, $n = 8$. Samples from affected individuals and heterozygotes, $n = 3$.

(B) Western blot analysis of control fibroblasts, fibroblasts from PROSC-deficient individuals and a Ser78Ter PROSC heterozygote. β-Actin was used as a control. Full-length wild-type PROSC is 30 kDa, faint band (subject 5) is 26 kDa.

(C) *PROSC* cDNA products from wild-type and subject 5 fibroblasts.

(D) Total fibroblast PLP of controls, PROSC-deficient individuals, and Ser78Ter heterozygote. PLP was quantified relative to D2-PLP internal standard and normalized (mg cell protein). Data presented as means ± SD. Statistical analysis performed with Student's two tailed t test; comparison of controls with PROSC-deficient samples; ***$p < 0.0001$. QC, quality control sample. QC, $n = 5$. Controls, $n = 18$. PROSC-deficient subjects and heterozygote, $n > 3$.

individuals with PNPO deficiency.[30] There might be a route of production of dopamine, and hence its metabolite, HVA, that does not require AADC,[31] and this pathway might be active to a variable degree in individuals with PROSC deficiency and PNPO deficiency. In the one PROSC-deficient subject in whom CSF homocarnosine was measured, it was undetectable. This might indicate that this carbonyl scavenger is reacting with free PLP; whether this will prove to be a specific marker for PROSC-deficiency remains to be seen.

The plasma PLP concentration in PROSC-deficient individuals receiving pharmacological doses of pyridoxine/PLP was higher than we see in ALDH7A1 deficiency or PNPO deficiency but not as high as seen in hypophosphatasia.[21] Unlike PNPO deficiency, there was no accumulation of pyridoxine, pyridoxamine, pyridoxine phosphate, or pyridoxamine phosphate in the plasma of the PROSC-deficient individuals. So, the plasma B_6 vitamer profile on treatment could be a pointer to PROSC deficiency.

The mutations in *PROSC* in the children with B_6-dependent epilepsy include nonsense, missense, and splice-site mutations. The missense varaints p.Leu175Pro and p.Arg241Gln affect residues that are highly conserved from bacteria through yeast to man. However, subject 7, who had a milder disease with onset of seizures at 1 month rather than on day 1, had a mutation on one allele that affected a residue (Pro87) that is only conserved across higher species.

We used fibroblasts to study the effects of the *PROSC* mutations found in the affected individuals on *PROSC* RNA and protein and B_6 vitamer levels. The nonsense mutation, c.233C>G (p.Ser78Ter), led to a reduction in *PROSC* mRNA expression, as would be expected from nonsense-mediated decay, but reduced *PROSC* mRNA and PROSC protein were also seen in the fibroblasts of the child homozygous for p.Leu175Pro. The splice-site mutations were confirmed to give rise to aberrant splicing. In fibroblasts cultured in normal medium, the amount of PLP present in the cells of the PROSC-deficient individuals was 2–3 times that present in controls; an excess was present in both high molecular weight and low molecular weight cell lysate fractions, suggesting that the excess PLP present was binding to proteins and small molecules indiscriminately.

An *E. coli* strain lacking the PROSC homolog (Δ*YggS*) shows growth inhibition when exposed to a high concentration of pyridoxine, and this phenotype was rescued by transfection with wild-type human *PROSC* but not by *hPROSC* encoding the p.Leu175Pro, p.Arg241Gln, and p.Ser78Ter variants found in affected individuals. One mutation, however, did allow hPROSC to rescue the pyridoxine sensitivity of Δ*YggS,* namely p.Pro87Leu. This is unsurprising as Pro87 is not conserved between human PROSC and *E. coli* YggS. Previous studies on *E.coli* Δ*YggS* showed an abnormality of the metabolism of amino acids and 2-oxoacids that were interpreted as indicating deficiency of coenzyme A (CoASH) and were reversed by supplementation with pantothenate.[11] It is possible that in the absence of PROSC, free PLP rises to a concentration at which it reacts with and inactivates CoASH.

Several mechanisms have been proposed to explain how the cell ensures that PLP is guided to apo-B_6 enzymes and does not react inappropriately with small molecules and proteins.[32–37] It has been suggested that PNPO and

Table 4. CSF B_6 Vitamer Profiles of PROSC-Deficient Individuals

	Age	B_6 (Dose)	PLP	PL	PA	PN	PNP	PMP	PM
Ref. range	1 y–18 y[a]	none	11–34	16–56	0.09–3	<0.03[b]	NM	<5.4[b]	0.3–0.9
Subject 4	6 d[c]	PN (100 mg/day)	<4*[d]	NM	NM	NM	NM	NM	NM
Subject 5	17 m	none[e]	6,* 5*	15,* 15*	ND	0.05, 0.08	0.9, 0.8	ND	0.1, 0.2
Subject 6	20 m	PN (40 mg/kg/day)	43	NM	NM	NM	NM	NM	NM

Data were not available for subjects 1–3 and 7. All units nmol/L, except for PNP, which is stated in concentration units. Values outside the reference range marked by an asterisk. Abbreviations are as follows: ND, not detected; NM, not measured; y, years; d, days; m, months; Ref., reference; PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine; PLP, pyridoxal 5′-phosphate; PNP, pyridoxine 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PA, 4-pyridoxic acid.
[a]Albersen et al., 2015.[17]
[b]Limit of quantification for PN/PMP (Albersen et al., 2012).[18]
[c]Measurement was taken 2 days after starting pyridoxine.
[d]Reference range for local lab (26–69).
[e]Subject received tapered PLP withdrawal trial. CSF was collected 48 hr after last dose. Duplicate measurements of same sample are given for subject 5.

pyridoxal kinase, which are regulated by PLP feedback inhibition,[32] channel PLP to certain apo-B_6-enzymes.[36,37] Our data suggest PROSC is involved in cellular PLP homeostasis in man. Although exact mechanisms remain uncertain, we postulate that PROSC is a PLP-carrier that prevents PLP from reacting with other reactive molecules, supplies it to apo-enzymes, and protects it from intracellular phosphatases (Figure S7). In human PROSC deficiency, there is clear evidence of low CSF PLP concentrations, which are accompanied by deficient activity of PLP-holoenzymes (e.g., AADC) and by B_6-dependent epilepsy. There is also evidence of carbonyl scavenger depletion in vivo and evidence, in cultured cells, of uncontrolled build-up of PLP with excessive binding to proteins and small molecules.

Treatment of PROSC-deficient individuals with PLP produced better seizure control than treatment with pyridoxine. The plasma B_6 vitamer profile of a subject on pyridoxine showed very high concentrations of pyridoxal and pyridoxic acid (Table S11). This suggests that the PLP produced in the liver from the pyridoxine supplement by the sequential actions of pyridoxal kinase and PNPO is, in the absence of PROSC, vulnerable to degradation by intracellular phosphatases and aldehyde oxidase. All but one of the individuals with PROSC deficiency required treatment with antiepileptic drug(s) as well as B_6. This suggests that the pathophysiology of the seizure disorder involves more than CNS PLP deficiency; it is possible that the inappropriate reaction of PLP with proteins and small molecules contributes to the pathogenesis of the seizures. It is also possible that variants in other genes will be found to affect the phenotype of PROSC deficiency.

Table 5. CSF Monoamine Metabolite, Pterin, and Folate Results of PROSC-Deficient Subjects

	Subject 2	Subject 4	Subject 5[a]	Subject 5[a]	Subject 6
Age	19 m	6 d	21 d	17 m	20 m
B_6 supplementation	yes[b]	no	no	no	yes
Homovanillic acid	755 (295–932)	310* (324– 1,098)	886 (484–1446)	770 (364–870)	556 (154–867)
5-Hydroxyindoleacetic acid	223 (114–336)	376 (199–608)	538 (302–952)	387* (155– 359)	158 (89–367)
3-Ortho-Methyldopa	46 (<300)	586* (<300)	508* (0–310)	84* (0–50)	NM
L-Dopa	22 (<25)	NM	200* (0–15)	<2 (0–15)	NM
5-Hydroxytryptophan	105* (<10)	NM	32* (0–20)	28* (0–15)	NM
5-MTHF	89 (64–182)	120 (72–305)	97 (62–287)	113 (37–214)	NM
Tetrahydrobiopterin	10* (15–40)	71 (27–105)	12* (25–121)	35 (20–61)	NM
7,8-Dihydrobiopterin	12 (12–30)	17* (0.4–13.9)	8 (0–18)	4 (0–18)	NM
Total neopterin	NM	124* (7–65)	17 (6–59)	6 (5–53)	NM

Subjects 2, 4, and 5 display some biochemical features of aromatic L-amino acid decarboxylase (AADC) deficiency: elevated concentrations of 3-ortho-methydopa, L-dopa, and 5-hydroxytryptophan and low homovanillic acid. Subject 5 also had raised urinary vanillactic acid on several occasions, although this normalized (additional details are in the case histories in the Supplemental Note). Results are expressed as nmol/L. Values marked by an asterisk are outside of the reference range. Age related reference ranges are given in parentheses. Abbreviations are as follows: d, day; m, month; 5-MTHF, 5-methyl-tetrahydrofolate; NM, not measured.
[a]Plasma L-aromatic amino acid decarboxylase (AADC) activity markedly decreased at 1 m (12 pmol/mL/min; reference range, 47–119), marginally decreased at 7 m (44 pmol/mL/min; reference range, 47–119) and normal at 17 m (50 pmol/mL/min; reference range, 33–79); no mutations were found in *AADC*.
[b]Treated prophylactically.

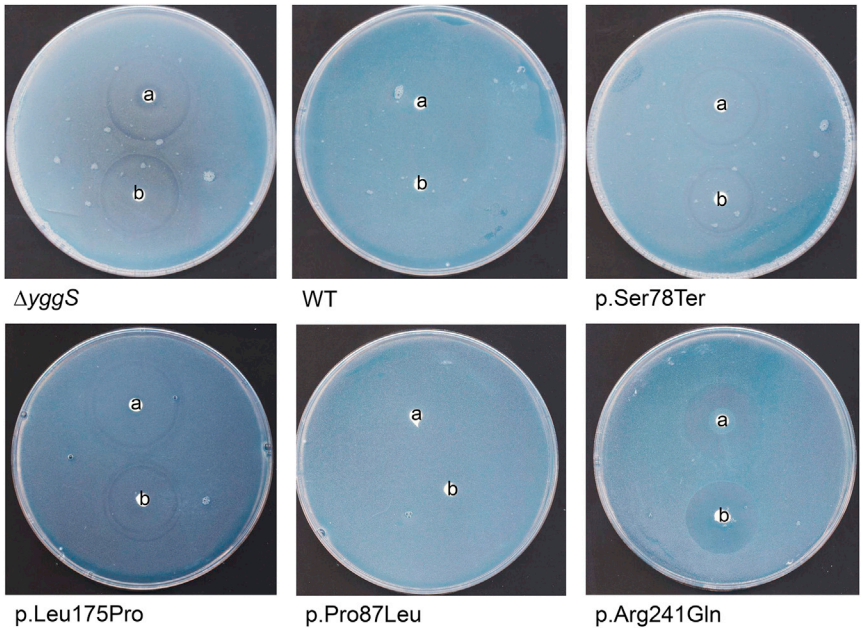

Figure 5. Complementation of *E. coli* Δ*yggS* with Human Wild-Type and Mutant *PROSC*

E. coli Δ*yggS* were transformed with an empty vector (pBAD33) as a negative control, human wild-type *PROSC*, and the mutant versions encoding p.Ser78Ter, p.Leu175Pro, p.Pro87Leu, or p.Arg241Gln and grown in the presence of discs impregnated with (1) 20 μL 0.1 mg/mL pyridoxine or (2) 20 μL 1 mg/mL pyridoxine. Pyridoxine produces a ring or rings of growth inhibition around the disc. This is prevented by transfection with wild-type *PROSC* or *PROSC* encoding the p.Pro87Leu variant. It is not prevented by transfection with *PROSC* encoding p.Ser78Ter, p.Leu175Pro, or p.Arg241Gln. The latter exacerbates growth inhibition, producing a filled circle rather than a ring.

Supplemental Data

Supplemental Data include a Supplemental Note, seven figures, and thirteen tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.10.011.

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Web Resources

ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalo/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
Ensembl Genome Browser, http://www.ensembl.org/index.html
ExAC Browser, http://exac.broadinstitute.org/
FastQC, http://www.bioinformatics.babraham.ac.uk/projects/fastqc
OMIM, http://www.omim.org/
UCSC Genome Browser, http://genome.ucsc.edu

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