Human Molecular Genetics, 2000, Vol. 9, No. 19 2853–2858

Hyperammonemia with reduced ornithine, citrulline, arginine and proline: a new inborn error caused by a mutation in the gene encoding Δ^1-pyrroline-5-carboxylate synthase

Matthias R. Baumgartner[1], Chien-an A. Hu[1], Shlomo Almashanu[2], Gary Steel[2], Cassandra Obie[2], Bernard Aral[3], Daniel Rabier[3], Pierre Kamoun[3], Jean-Marie Saudubray[3] and David Valle[1,2,+]

[1]Department of Pediatrics, McKusick-Nathans Institute of Genetic Medicine and [2]Howard Hughes Medical Institute, Johns Hopkins University, Baltimore, MD 21205, USA, [3]Laboratoire de Biochimie Médicale B and [4]Service des Maladies Métaboliques, Department of Pediatrics, Hôpital Necker-Enfants Malades, 75743 Paris, France

Received 18 July 2000; Revised and Accepted 15 September 2000

Δ^1-pyrroline-5-carboxylate synthase (P5CS), a bifunctional ATP- and NADPH-dependent mitochondrial enzyme, catalyzes the reduction of glutamate to Δ^1-pyrroline-5-carboxylate, a critical step in the biosynthesis of proline, ornithine and arginine. Recently, we reported the cloning and expression of human and murine *P5CS* cDNAs. Previously, we showed that mammalian *P5CS* undergoes alternative splicing to generate two isoforms differing only by a 2 amino acid insert at the N-terminus of the γ-glutamyl kinase active site. The short isoform has high activity in the gut, where it participates in arginine biosynthesis and is inhibited by ornithine. The long isoform, expressed in multiple tissues, is necessary for the synthesis of proline from glutamate and is insensitive to ornithine. Here, we describe a newly recognized inborn error due to the deficiency of P5CS in two siblings with progressive neurodegeneration, joint laxity, skin hyperelasticity and bilateral subcapsular cataracts. Their metabolic phenotype includes hyperammonemia, hypoornithinemia, hypocitrullinemia, hypoargininemia and hypoprolinemia. Both are homozygous for the missense mutation, R84Q, which alters a conserved residue in the P5CS γ-glutamyl kinase domain. R84Q is not present in 194 control chromosomes and dramatically reduces the activity of both P5CS isoforms when expressed in mammalian cells. Additionally, R84Q appears to destabilize the long isoform. This is the first documented report of an inborn error of P5CS and suggests that this disorder should be considered in the differential diagnosis in patients with neurodegeneration and/or cataracts and connective tissue disease.

INTRODUCTION

Δ^1-pyrroline-5-carboxylate synthase (P5CS), a bifunctional ATP- and NADPH-dependent mitochondrial enzyme, catalyzes the reduction of glutamate to Δ^1-pyrroline-5-carboxylate (P5C), a critical step in the biosynthesis of proline, ornithine and arginine (Fig. 1) (1,2). P5C is the immediate precursor for both proline and ornithine, in reactions catalyzed by P5C reductase and ornithine δ-aminotransferase (OAT), respectively (1,2). In *de novo* arginine biosynthesis ornithine is further metabolized to citrulline and arginine by the enzymes of the urea cycle (2). This pathway is active in the epithelium of small intestine, particularly in early postnatal life (3,4). The physiological significance of arginine biosynthesis is highlighted by the observations that mice with targeted disruption of OAT die as a result of arginine deficiency in the neonatal period and that human infants with inherited deficiency of OAT develop hypoargininemia and hyperammonemia (5,6). Recently, we reported the cloning and expression of human and murine *P5CS* cDNAs (7). We showed that these mammalian *P5CS* genes undergo alternative splicing to generate two isoforms differing only by a 2 amino acid insert at the N-terminus of the γ-glutamyl kinase (γ-GK) active site. The short P5CS isoform (P5CS.short) has high activity in gut, where it catalyzes an essential step in the arginine biosynthetic pathway. P5CS.short is inhibited by ornithine, providing a mechanism for regulation of arginine synthesis. The long isoform of P5CS (P5CS.long) is expressed in multiple tissues and is necessary for the synthesis of proline from glutamate. This isoform is insensitive to ornithine inhibition and, thus, allows proline synthesis to proceed normally, even when ornithine levels are pathologically elevated (2).

Here, we describe a newly recognized inborn error due to deficiency of P5CS in two siblings with progressive neurodegeneration, joint laxity, skin hyperelasticity, bilateral subcapsular cataracts and a pattern of metabolic abnormalities consistent with impaired proline and ornithine synthesis. A

[+]To whom correspondence should be addressed. Tel: +1 410 955 4260; Fax: +1 410 955 7397; Email: dvalle@jhmi.edu

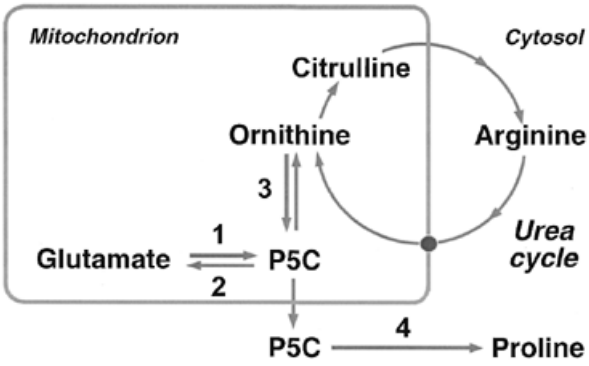

Figure 1. The metabolic pathways involved in the synthesis of ornithine, arginine and proline from glutamate. The enzymes indicated by the numbers are: 1, P5CS; 2, P5C dehydrogenase; 3, OAT; and 4, P5C reductase.

preliminary report of these patients by some of the authors has also been published (8).

RESULTS

The pattern of metabolic abnormality in the patients suggested a defect at the level of P5CS. Unfortunately, the activity of this enzyme is undetectable in control fibroblasts (9), although *P5CS* mRNA is present (data not shown). Therefore, to investigate the possibility of a P5CS defect in these patients, we performed RT–PCR amplification of *P5CS* mRNA from the cultured skin fibroblasts of these patients. We found that both are homozygous for a G→A transition at position 251 (where +1 is the A of the initiation methionine) resulting in the substitution of glutamine for arginine at position 84 (R84Q) and creating a new restriction site for the endonuclease *Sty*I (CCGAGG→CCAAGG). We found no other sequence abnormalities over the entire length of the cDNA. To verify the 251G→A mutation, we amplified a 91 bp fragment from genomic DNA of the patients and their parents and confirmed R84Q by direct sequencing (Fig. 2a) and by digestion with *Sty*I (Fig. 2b). Both parents are heterozygous for R84Q.

To determine whether R84Q is a common variant, we used the *Sty*I digest assay to screen 100 North American and 94 North African (Algerian, Moroccan and Tunisian) chromosomes. We did not find a single carrier among all screened (data not shown). Consistent with this observation, R84, which is in the N-terminal third of the γ-GK domain of P5CS, is conserved in the orthologous enzymes of mouse, *Drosophila*, *Caenorhabditis elegans* and *Vigna aconitifolia.*

To test directly the functional significance of R84Q we expressed it in Chinese hamster ovary (CHO-K1) cells. CHO-K1 cells are proline auxotrophs lacking endogenous P5CS and OAT activities (10–12). We transfected these cells with constructs expressing human wild-type or R84Q-P5CS.short or long cDNAs and selected stable transformants.

First, as an *in vivo* test of the functional consequences of R84Q we compared the growth of stable transformants in medium with or without proline. In Pro^+ medium, the growth of all clones was essentially the same (Fig. 3). In Pro^- medium, the growth of clones expressing the wild-type short or long isoforms was the same as that in Pro^+ medium. In contrast, the

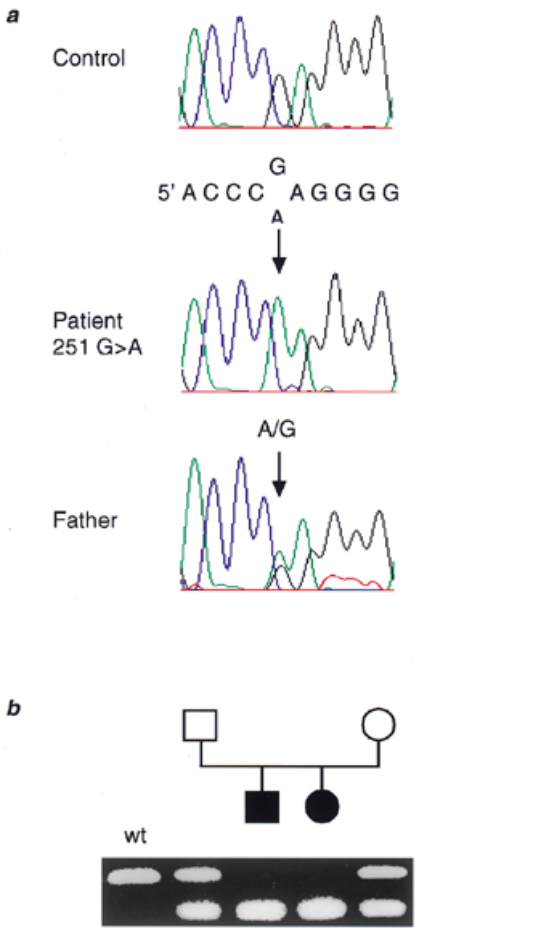

Figure 2. P5CS mutation analysis: G251→A (R84Q). (**a**) Direct sequences of amplified genomic DNA in this region. The normal sequence is shown in the top panel, the homozygous patient in the middle and the heterozygous father in the lower panel. (**b**) Restriction digest of a 91 bp fragment amplified from genomic DNA. In the wild-type, *Sty*I does not cut, leaving the 91 bp fragment. In the homozygous patients, *Sty*I cuts and yields two overlapping fragments of 43 and 48 bp. The parents are both heterozygous.

growth of clones expressing either the short or long P5CS-R84Q isoform was severely reduced in Pro^- medium (Fig. 3).

To confirm these intact cell results, we used the same stable transformants to examine *P5CS* mRNA, protein and enzymatic activity. As expected for stable transformants, expression levels varied from clone to clone. In the clones expressing wild-type P5CS the amount of *P5CS* mRNA expression correlated roughly to the amount of protein and enzyme activity detected (Fig. 4a and b). Expression of *P5CS* mRNA and protein in R84Q.short transformants was comparable to that of wild-type. In contrast, enzyme activity in R84Q.short transformants was reduced, particularly for a given expression of *P5CS* mRNA or protein (Fig. 4a). We obtained similar results with transformants expressing the long *P5CS* cDNAs (Fig. 4b). The clones expressing R84Q.short had some residual activity (mean of three clones 8.9% of wild-type), whereas in clones expressing R84Q.long activity was undetectable. Although the R84Q.long transformants expressed mRNA, no P5CS protein

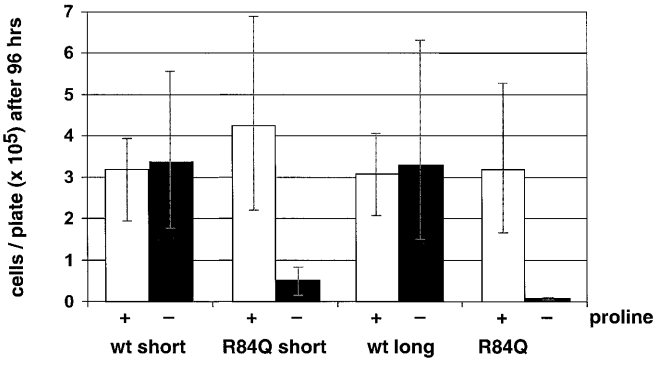

Figure 3. Growth of transformed CHO-K1 clones in Pro^+ and Pro^- media. The columns indicate the mean and range of number of cells/plate after 4 days for three individual clones, all of which express the indicated *P5CS* allele at the RNA level.

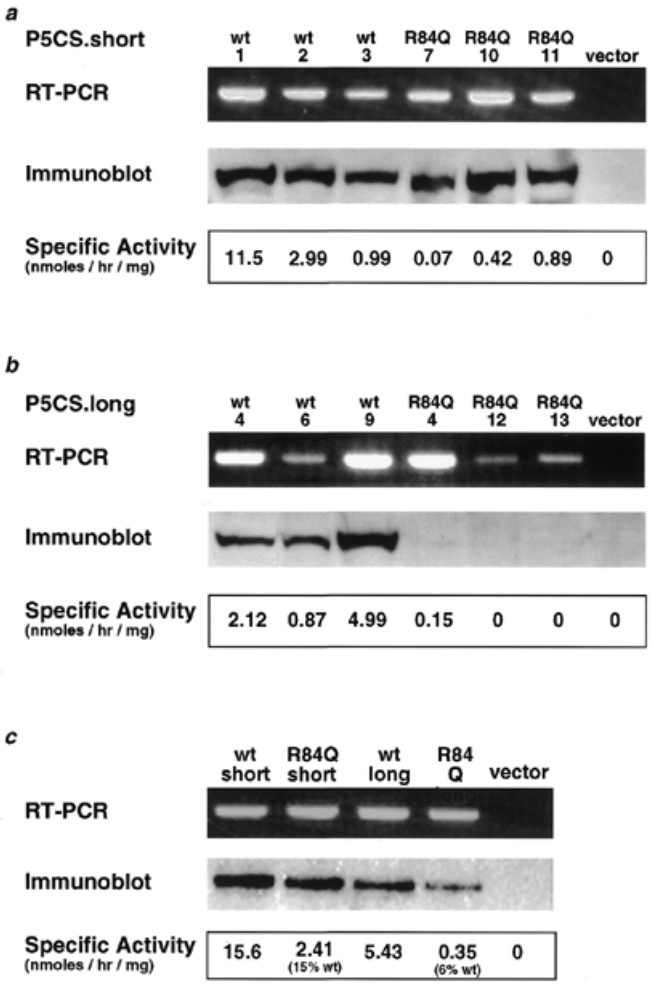

Figure 4. RNA, protein and activity expression in stable transformants (same clones as used for the growth study) and in transient transfections. *P5CS* RNA was measured by semi-quantitative RT–PCR; P5CS protein by immunoblot analysis with an anti-P5CS antiserum and P5CS enzymatic activity using a radioisotopic assay (9). (**a**) RNA, protein and enzyme activity in three stable P5CS.short and three P5CS-R84Q.short transformants. (**b**) RNA, protein and enzyme activity in three stable P5CS.long and three P5CS-R84Q.long transformants. (**c**) RNA, protein and enzymatic activity in transient transfections with the indicated *P5CS* alleles.

was detectable by immunoblot analysis (Fig. 4b). This differs from R84Q.short and suggests that the missense substitution destabilizes the long but not the short P5CS isoform.

Because of the wide variation in expression in the stable clones, we repeated the experiment with transient transfections and obtained similar results (Fig. 4c). In this assay, R84Q.long had some residual activity, and a reduced, but not absent amount of protein.

DISCUSSION

Our results show that these two affected sibs are homozygous for a missense mutation (R84Q) that impairs the function of both the long and short isoforms of P5CS. A preliminary report of molecular studies on these patients identifying a different P5CS missense mutation, L396S, appeared elsewhere (8). We did not find this mutation in RT–PCR amplified fibroblast RNA from these patients nor in two independently obtained samples of genomic DNA from the patients and their parents.

P5CS catalyzes an essential step in the pathways by which proline, ornithine and arginine are synthesized from glutamate (Fig. 1) (1,2). Impaired synthesis of these amino acids is consistent with many of the clinical and metabolic abnormalities in our patients. In tissues with a high proline requirement for protein synthesis and with limited access to amino acids in the extracellular fluid, endogenous synthesis of proline and, hence, P5CS activity may be especially critical (13). For example, chondrocytes and fibroblasts synthesize large amounts of collagen and, together, proline and hydroxyproline derived from proline, comprise >20% of the amino acid residues in fibrillar collagens (14). Thus, an intracellular deficiency of proline in these cells may result in impaired collagen synthesis that, in turn, could account for the connective tissue symptoms (lax joints, hyperelastic skin) in these patients. Similar phenotypic features are present in patients with Ehlers Danlos syndrome, a heterogeneous group of disorders caused by mutations in collagen genes (15).

At least two pathophysiologic explanations can be considered for the development of cataracts in patients with P5CS deficiency. First, deficient proline synthesis may impair protein synthesis in the lens epithelium and/or fibrocytes. The lens is an avascular tissue bathed in aqueous humor with a proline concentration that is ~15% that of plasma (16,17). Lens epithelial cells and fibrocytes synthesize large amounts of lens crystallins and a variety of cytoskeletal proteins (18). These proteins are essential for lens transparency, and inherited defects in crystallins (19,20) or lens structural proteins (21) cause cataracts. Alternatively, cataract formation in P5CS deficiency may result from disruption of a metabolic function of P5C other than as a proline precursor. The lens is sensitive to oxidative stress and utilizes a glutathione redox cycle to maintain a reduced state (18). Phang (22) has shown that P5C/proline interconversions can serve as an NADPH/$NADP^+$-linked redox couple. Furthermore, P5C is present in the aqueous humor of mammals, including primates, at concentrations roughly equal to or greater than plasma, and P5C-metabolizing enzymes (reductase, dehydrogenase) are present in lens epithe-

lium (23). Thus, it is possible that P5C metabolism contributes to the antioxidant defense of lens and that a defect in this system results in cataract.

The pathophysiology of the neurodegeneration in our patients could be explained by a block in proline synthesis in the central nervous system (CNS). P5CS activity is present in the brain (9) and proline is thought to act as an inhibitory neurotransmitter in the CNS (24). The extracellular fluid concentration of proline in the CNS is virtually unmeasurable as judged by cerebral spinal fluid proline concentrations (1). These observations suggest that certain cells in the CNS depend on endogenous synthesis of proline and may be particularly sensitive to P5CS deficiency.

Lack of P5CS also is consistent with the disturbances in ammonium and arginine metabolism in our patients. P5CS catalyzes the first committed step in *de novo* arginine biosynthesis (Fig. 1). P5C is subsequently converted to ornithine by OAT and then to citrulline and arginine by the enzymes of the urea cycle (2). The arginine biosynthetic pathway is active in the epithelium of small intestine, particularly in early postnatal life (3,4). The physiologic significance of this pathway is highlighted by the observations that mice with targeted disruption of OAT die as a result of arginine deficiency in the neonatal period and that human infants with inherited deficiency of OAT develop hypoargininemia and hyperammonemia (5,6). The paradoxical fasting hyperammonemia in our patients is consistent with a relative deficiency of arginine and its urea cycle precursors. In the fasting state, urea cycle intermediates (ornithine, citrulline and arginine) are low and limit ureagenesis and ammonium detoxification. Following a meal, arginine, derived from dietary protein, temporarily corrects this deficit and enhances urea cycle function with the result that plasma ammonium decreases despite the nitrogen load in the meal.

We conclude from these considerations that deficiency of P5CS likely accounts for the entire clinical and metabolic phenotype observed in our patients. We recognize, however, that our patients are the product of a consanguineous union and both could have an additional genetic disorder that was not detected in either, despite comprehensive evaluation. Identification of additional patients will be necessary to confirm our hypothesis. The metabolic phenotype of P5CS deficiency is easily missed. The combination of low levels of ornithine, citrulline, arginine and proline plus paradoxical hyperammonemia should suggest this disorder. Early recognition would allow a trial of treatment with citrulline and proline.

MATERIALS AND METHODS

Patient report

The affected siblings were born to asymptomatic first cousin Algerian parents after an uneventful pregnancy (8). The oldest, a boy, is 21 and his affected sister is 12 years old. They have two healthy brothers, a third died in the neonatal period from unknown causes. Both siblings presented in early infancy with failure to thrive, vomiting and progressive neurological dysfunction. By the age of 2 years, they were noted to have bilateral subcapsular cataracts (8). Currently, both are hypotonic with lax joints and hyperelastic skin. They lost the ability to walk at the age of 12 years due to their motor handicap. Both are moderately mentally retarded (IQ ~50).

Metabolic studies repeatedly displayed hyperammonemia [60–120 μM; normal level (n.l.) <50 μM], hypoornithinemia (33 ± 15 μM; n.l. 72 ± 25 μM), hypocitrullinemia (6 ± 5 μM; n.l. 26 ± μM), hypoargininemia (35 ± 12 μM; n.l. 77 ± 20 μM) and hypoprolinemia (91 ± 18 μM; n.l. 180 ± 47 μM). Paradoxically, the plasma ammonium in these patients fell by 15% after meals (8). In normal individuals, postprandial ammonium levels increase modestly (8).

RT–PCR, genomic PCR and *Sty*I digest assay

We extracted RNA and genomic DNA from cultured skin fibroblasts and/or blood using the Puregene RNA and DNA isolation kits (Gentra Systems, Minneapolis, MN). We performed RT–PCR using 5–10 μg fibroblast RNA and a cDNA cycle kit (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. To search for mutations, we used overlapping primer pairs to amplify the complete *P5CS* open reading frame (ORF) in overlapping segments. We generated first strand cDNA with primers DV2055 (antisense, 5′-CCAGAAGCATCCAGGTACACTT-3′), complementary to sequence in the 3′ untranslated region and DV1417 (antisense, 5′-GTGGATGTGGTCAATGGCATCCTGAACG-3′), complementary to bp 2040–2067 in the ORF (where +1 is the A of the initiation methionine). We then amplified first strand cDNA with primers DV4218 (sense, 5′-GTGGTGAGGAAGATACTTTGG-3′) and DV4169 (antisense, 5′-ATGCATAATTCCAGGTCCCC-3′) to generate a 2064 bp fragment (–40 to +2024) and DV4154 (sense, 5′-GAAGTTGAAGATCTTTGCCGC-3′) and DV4043 (antisense, 5′-TCAGTTGGTGTTTCTCTGAGG-3′) to generate a 767 bp fragment (+1621 to +2388). Cycling conditions were an initial denaturation step of 7 min at 95°C followed by 30 cycles of 1 min at 94°C, 1 min at 58°C and 2 min at 72°C. We gel-purified the PCR products, sequenced them directly and cloned them into a TA cloning vector (Invitrogen).

To confirm R84Q in genomic DNA, we amplified 40 ng of genomic DNA with exonic primers DV4136 (sense, 5′-CCAAGAGAATCGTGGTGAAG-3′) and DV4224 (antisense, 5′-CTCAACAATAGATGCCAAGC-3′) to generate a 91 bp fragment (+209 to +300) containing the mutation (251G→A). Cycling conditions were 7 min at 95°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. We sequenced the PCR product directly and used the same product for digestion with *Sty*I resulting in a 43 and a 48 bp fragment. Using this digest assay we screened 94 North African (Algerian, Moroccan and Tunisian) and 100 North American chromosomes for R84Q.

To examine RNA expression of the *P5CS* constructs in CHO-K1 cells we used 10 μg of total RNA treated with DNase (Ambion, Austin, TX) to generate first strand cDNA as described above with primer DV1417. We then amplified the cDNA with human-specific primers DV4369 (sense, 5′-GCTCCTCTGCTGAAACGTTTA-3′) and DV4368 (antisense, 5′-CTAGCCTGGTGACCTTATCA-3′) to generate a 543 bp fragment (+1258 to +1801). Cycling conditions were 7 min at 95°C followed by 27 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C.

All PCR reactions (50 μl) contained primers (100 ng of each), 1× standard PCR buffer (Gibco BRL, Gaithersburg, MD), dNTPs (200 μM) and *Taq* polymerase (2.5 U; Gibco BRL).

Construction of wild-type and R84Q human P5CS expression vectors

We subcloned the full-length human *P5CS*.short cDNA (–57 to +2973) (7) into pcDNA3 (Invitrogen) at the *Eco*RI cloning site. To introduce the R84Q mutation we harvested a 687 bp *Xcm*I fragment containing R84Q and the 6 bp of the long isoform from cloned patient cDNA and subcloned this fragment into the p*HsP5CS*.short construct resulting in p*HsP5CS*-R84Q.long. We then digested with *Esp*I, which cuts between R84Q and the region of the 6 bp insert, and *Pvu*I and ligated the two fragments of p*HsP5CS*-R84Q.long with the corresponding fragments of p*HsP5CS*.short resulting in p*HsP5CS*.long and p*HsP5CS*-R84Q.short. Then, as the CHO-K1 cells are relatively resistant to G418, we switched all the constructs into pZeoSV (Invitrogen) at the *Eco*RI cloning site. We sequenced p*HsP5CS*.short, the patient TA clone and all the ligations in both directions to verify that the sequences were as expected.

Transfections and growth study of CHO-K1 cells

We transfected p*HsP5CS*.short, p*HsP5CS*.long, p*HsP5CS*-R84Q.short, p*HsP5CS*-R84Q.long or pZeoSV into a subclone (CHO-K1-NC5) of the proline auxotrophic cell line CHO-K1 (ATCC CCL61) by electroporation as described (7,25). We selected for stable transformants in the minimal essential medium (MEM) with 10% fetal calf serum (FCS) and Zeocin (300 mg/l). For the transient transfection we used 2×10^7 cells and 30 µg of plasmid DNA; we harvested the cells after 48 h. For the growth study we selected stable transformants expressing the *P5CS* allele at the RNA level and inoculated each dish with 5000 cells. We grew them either on MEM with 10% FCS (Pro^+ medium) or on MEM with 10% dialyzed FCS lacking proline (Pro^- medium) and counted the cells after 96 h. All media contained Zeocin (300 mg/l).

P5CS enzyme assay and immunoblotting

We assayed P5CS activity radioisotopically as described by Wakabayashi *et al.* (9). We verified that P5CS activity is dependent on the presence of ATP, $MgCl_2$ and NADPH and increases linearly with added protein at concentrations between 100 and 1000 µg/ml (7).

To produce an antiserum against human P5CS we selected a peptide corresponding to the hydrophilic stretch of the C-terminal last 15 amino acids (DV16: KYLHENL-PIPQRNTN) of human P5CS. We conjugated DV16 to Keyhole Limpet hemocyanin and used it to inoculate rabbits and produce antiserum (Covance, Denver, PA). We conducted immunoblotting experiments according to standard protocols (26). We used a 1:500–1:1000 dilution of anti-DV16 antiserum to detect P5CS protein and an ECL kit to project signals. Preparation of protein samples for enzyme assay and immunoblotting was as described (9).

ACKNOWLEDGEMENTS

We thank Sandy Muscelli for assistance with the manuscript preparation. M.R.B. was supported by a grant from the Swiss National Foundation and the M. and W. Lichtenstein Stiftung. D.V. is an Investigator in the Howard Hughes Medical Institute.

REFERENCES

1. Phang, J.M., Yeh, G.C. and Scriver, C.R. (1995) Disorders of proline and hydroxyproline metabolism. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw Hill, New York, NY. pp. 1125–1146.
2. Valle, D. and Simell, O. (1995) The hyperornithinemias. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw Hill, New York, NY. pp. 1147–1185.
3. Windmueller, H.G. and Spaeth, A.E. (1981) Source and fate of circulating citrulline. *Am. J. Physiol.*, **241**, E473–E480.
4. Wu, G. (1998) Intestinal mucosal amino acid catabolism. *J. Nutr.*, **128**, 1249–1252.
5. Wang, T., Lawler, A.M., Steel, G., Sipila, I., Milam, A.H. and Valle, D. (1995) Mice lacking ornithine-δ-aminotransferase have paradoxical neonatal hypoornithinaemia and retinal degeneration. *Nature Genet.*, **11**, 185–190.
6. Cleary, M.A., Sivakumar, P., Olpin, S., Wraith, J.E., Walter, J.H., Till, J., Morris, A.A. and Besley, G.T. (1999) Ornithine aminotransferase deficiency: difficulties in diagnosis in the neonatal period. *J. Inherit. Metab. Dis.*, **22**, 69.
7. Hu, C.-A., Lin, W.-W., Obie, C. and Valle, D. (1999) Molecular enzymology of mammalian Δ^1-pyrroline-5-carboxylate synthase. *J. Biol. Chem.*, **274**, 6754–6762.
8. Kamoun, P., Aral, B. and Saudubray, J.-M. (1998) A new inherited metabolic disease: Δ^1 pyrroline 5-carboxylate synthetase deficiency. *Bull. Acad. Natl Med.*, **182**, 131–139.
9. Wakabayashi, Y., Yamada, E., Hasegawa, T. and Yamada, R. (1991) Enzymological evidence for the indispensability of small intestine in the synthesis of arginine from glutamate. I. Pyrroline-5-carboxylate synthase. *Arch. Biochem. Biophys.*, **291**, 1–8.
10. Brody, L.C., Mitchell, G.A., Obie, C., Michaud, J., Steel, G., Fontaine, G., Robert, M.-F., Kaiser-Kupfer, M.I. and Valle, D. (1992) Ornithine-δ-aminotransferase mutations causing gyrate atrophy: allelic heterogeneity and functional consequences. *J. Biol. Chem.*, **267**, 3302–3307.
11. Valle, D., Downing, S., Harris, S. and Phang, J. (1973) Proline biosynthesis: multiple defects in Chinese hamster ovary cells. *Biochem. Biophys. Res. Commun.*, **53**, 1130–1136.
12. Kao, F.-T. and Puck, T.T. (1967) Genetics of somatic mammalian cells. IV. Properties of Chinese hamster cell mutants with respect to the requirement for proline. *Genetics*, **55**, 513–524.
13. Smith, R.J. and Phang, J.M. (1978) Proline metabolism in cartilage: the importance of proline biosynthesis. *Metabolism*, **27**, 685.
14. Adams, E. and Frank, L. (1980) Metabolism of proline and the hydroxyprolines. *Annu. Rev. Biochem.*, **49**, 1005.
15. Byers, P.H. (1995) Disorders of collagen biosynthesis and structure. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill, New York, NY. pp. 4029–4077.
16. Rosenthal, W.N., Blitzer, M. and Insler, M.S. (1986) Aqueous amino acid levels in Fuchs' corneal dystrophy. *Am. J. Ophthalmol.*, **102**, 570–574.
17. Wuu, J.-A., Wen, L.-Y., Chuang, T.-Y. and Chang, G.-G. (1988) Amino acid concentrations in serum and aqueous humor from subjects with extreme myopia or senile cataract. *Clin. Chem.*, **34**, 1610–1613.
18. Hejtmancik, J.F., Kaiser-Kupfer, M.I. and Piatigorsky, J. (1995) Molecular biology and inherited disorders of the eye lens. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill, New York, NY. pp. 4325–4349.
19. Kannabiran, C., Basti, S., Balasubramanian, D., Majumdar, K., Rao, G.N., Kaiser-Kupfer, M.I. and Hejtmancik, J.F. (1998) Autosomal dominant zonular cataract with sutural opacities: result of a splice site mutation. *Invest. Ophthalmol. Vis. Sci.*, **39**, S418.
20. Litt, M., Carrero-Valenzuela, R., LaMorticella, D.M., Schultz, D.W., Mitchell, T.N., Kramer, P. and Maumenee, I.H. (1997) Autosomal dominant cerulean cataract is associated with a chain termination mutation in the human beta-crystallin gene *CRYBB2*. *Hum. Mol. Genet.*, **6**, 665–671.
21. Shiels, A., Mackay, D., Ionides, A., Berry, V., Moore, A. and Bhattacharya, S. (1998) A missense mutation in the human connexin50 gene (*GJA8*) underlies autosomal dominant 'zonular pulverulent' cataract, on chromosome 1q. *Am. J. Hum. Genet.*, **62**, 526–532.
22. Phang, J.M. (1985) The regulatory functions of proline and pyrroline-5-carboxylic acid. *Curr. Top Cell. Regul.*, **25**, 91–132.
23. Fleming, G.A., Steel, G., Valle, D., Granger, A. and Phang, J.M. (1986) The aqueous humor of rabbit contains high concentrations of pyrroline-5-carboxylase. *Metabolism*, **35**, 933.

24. Shafqat, S., Velaz-Faircloth, M., Henzi, V.A., Whitney, K.D., Yang-Feng, T.L., Seldin, M.F. and Fremeau, R.T.J. (1995) Human brain-specific L-proline transporter: molecular cloning, functional expression and chromosomal localization of the gene in human and mouse genomes. *Mol. Pharmacol.*, **48**, 219–229.
25. Melkonyan, H., Sorg, C. and Klempt, M. (1996) Electroporation efficiency in mammalian cells is increased by dimethyl sulfoxide (DMSO). *Nucleic Acids Res.*, **24**, 4356–4357.
26. Harlow, E. and Lane, D. (1999) *Using Antibodies: A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY.