Iminoglycinuria and hyperglycinuria are discrete human phenotypes resulting from complex mutations in proline and glycine transporters

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Iminoglycinuria (IG) is an autosomal recessive abnormality of renal transport of glycine and the imino acids proline and hydroxyproline, but the specific genetic defect(s) have not been determined. Similarly, although the related disorder hyperglycinuria (HG) without iminociduria has been attributed to heterozygosity of a putative defective glycine, proline, and hydroxyproline transporter, confirming the underlying genetic defect(s) has been difficult. Here we applied a candidate gene sequencing approach in 7 families first identified through newborn IG screening programs. Both inheritance and functional studies identified the gene encoding the proton amino acid transporter SLC36A2 (PAT2) as the major gene responsible for IG in these families, and its inheritance was consistent with a classical semidominant pattern in which 2 inherited nonfunctional alleles conferred the IG phenotype, while 1 nonfunctional allele was sufficient to confer the HG phenotype. Mutations in SLC36A2 that retained residual transport activity resulted in the IG phenotype when combined with mutations in the gene encoding the imino acid transporter SLC6A20 (IMINO). Additional mutations were identified in the genes encoding the putative glycine transporter SLC6A18 (XT2) and the neutral amino acid transporter SLC6A19 (B0AT1) in families with either IG or HG, suggesting that mutations in the genes encoding these transporters may also contribute to these phenotypes. In summary, although recognized as apparently Mendelian disorders, IG and HG exhibit complex molecular explanations depending on a major gene and accompanying modifier genes.

Introduction
Many metabolic diseases, such as Hartnup disorder (1) and phenylketonuria (2), have been assumed to fit a classical Mendelian autosomal model of inheritance. In classical Mendelian diseases such as hemochromatosis (3), reduced penetrance is often invoked to explain the failure to develop disease despite inheritance of 2 abnormal alleles. Iminoglycinuria (IG; OMIM 242600) in some families also exhibits a more complex inheritance pattern, including reduced penetrance (4). Both parents from the family in which IG was first identified exhibited a normal urinary phenotype (5), whereas later studies described families in which the parents exhibited hyperglycinuria (HG; OMIM 138500) without iminociduria (6–8). Consequently, IG was thought to arise when 2 defective alleles of a common transporter for glycine, proline, and hydroxyproline are present (9), whereas heterozygosity of the putative defective transporter would manifest as HG. Observed exceptions to this pattern led to speculation regarding “incompletely recessive” forms or the involvement of alleles with different molecular outcomes (4, 10). However, in the absence of an explanation of its molecular pathogenesis, the genetic complexity of IG has remained obscure since its first description more than 50 years ago.

Although generally classified as benign inborn errors of amino acid transport (11), IG and HG have been associated with hypertension, glycosuria (12), nephrolithiasis (OMIM 138500; known as IG type II) (13, 14), mental retardation (15), atypical gyrate atrophy (16), deafness (8), and blindness (17, 18). In the absence of controlled prospective studies of IG and HG cohorts, some of these associations may represent ascertainment bias. Based on detailed biochemical studies in 7 families, Lasley and Scriver (19) suggested that 3 genes account for imino acid and glycine reabsorption in the kidney proximal tubule, namely a common transporter for both types of amino acids and a specific transporter each for glycine and imino acids. This model of 1 common and 2 specific transporters is well supported by transport studies in intact tubules, brush-border membrane vesicles, and cell lines from a wide variety of organisms (20).

In the absence of sufficient family material to undertake traditional linkage analysis, we adopted a candidate gene sequencing approach targeting recently identified epithelial proline and glycine transporters to dissect the molecular pathogenesis of IG and HG. Comparison of earlier physiological data with the properties of cloned proline and glycine transporters suggests that all major

Nonstandard abbreviations used: cRNA, complementary RNA; HG, hyperglycinuria; IG, iminoglycinuria.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. doi:10.1172/JCI36625.

The Journal of Clinical Investigation http://www.jci.org
mediators of proline and glycine transport have been identified at the molecular level (20) in recent years. Two common transporters have been cloned that cotransport proline, hydroxyproline, and glycine together with protons, namely SLC36A1 (PAT1) and SLC36A2 (PAT2) (21). A major feature distinguishing these transporters is their substrate affinity: SLC36A1 is a low-affinity transporter, whereas SLC36A2 is a high-affinity transporter. SLC36A1 has been characterized as the intestinal imino acid and glycine transporter and suggested as a candidate for IG (22, 23). More recently, 2 groups including ours identified SLC6A20 (IMINO) as a specific imino acid transporter (24, 25). Studies in the SLC6A18 nullizygous mouse suggest that this gene encodes a high-affinity renal epithelial transporter specific for glycine (26); however, attempts to functionally express SLC6A18 have not been successful (27, 28). Physiological and genetic data also demonstrate that the general neutral amino acid transporter SLC6A19, which is mutated in Hartnup disease (28–30), contributes significantly to imino acid and glycine transport in kidney and intestine (31, 32).

In this report, we describe 2 novel SLC36A2 mutations, one allele of which apparently acts in a semidominant manner, while a second allele appears to be acting recessively. Our results strongly suggest that the SLC36A2 mutations together with polymorphisms in the modifiers SLC6A20, SLC6A18, and SLC6A19 constitute the genetic basis for these intriguing human phenotypes.

**Results**

Seven families, including French-Canadians (pedigrees 1–3) and Australians (pedigrees 4–7), each containing an index case initially identified from newborn urinary screening programs for IG (33, 34), were recruited for this study (Figure 1). Most probands who had IG diagnosed as infants (subjects 1.3, 1.4, 2.3, 2.4, 3.3, 4.4, and 7.6) were reconfirmed in this study, whereas subjects 5.3 and 6.4 persisted with only HG. The term HG included any individual exhibiting elevated urinary glycine levels on at least one occasion. All urinary amino acid quantitations from the 7 pedigrees are shown in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI36625DS1). The phenotypic distinction between HG and IG was unambiguous: excess urinary proline and hydroxyproline (always accompanied by excess glycine) indicated IG, whereas isolated excess urinary glycine indicated HG. The variability in phenotype observed in these pedigrees, particularly between parents, suggested the possibility that genetic heterogeneity was a contributing factor.

Genomic DNA samples from all 7 IG and HG families were sequenced to identify mutations in known imino acid and glycine transporter candidates SLC36A1, SLC36A2, SLC6A20, SLC6A18, and SLC6A19, including coding regions and exon-intron boundaries. Mutations causing nonsense, missense, and splice site changes and polymorphisms of uncertain significance are summarized in Table 1. Mutations that cosegregated with the IG and HG phenotypes were detected in the genes encoding SLC36A2 (the common glycine and imino acid transporter), SLC6A20 (the specific imino acid transporter), and SLC6A18 (the specific glycine transporter). Despite biochemical evidence for the candidacy of SLC36A1, no mutations segregating with the urinary phenotypes were identified in that gene.

The main contribution to IG and HG arises from defects in the imino acid and glycine transporter SLC36A2 (Table 1), thereby proving the assumption that the common transporter is the major gene contributing to both IG and HG. A 260G→T mutation resulting in the substitution of a highly conserved glycine residue by valine (G87V) in the second transmembrane helix of SLC36A2 (Figure 2A and Figure 3A) was identified in 5 pedigrees. In pedigree 7 (of Coptic-Egyptian descent), a splice donor site mutation in the first intron of SLC36A2, IVS1+1G→A, was identified that was predicted (35, 36) to impair normal splicing. In addition, a

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**Figure 1**

Pedigrees with HG or IG. Filled symbols represent individuals with IG; half-filled symbols, those with HG. Open symbols represent normal family members. An arrow indicates the index case. Pedigrees 1–3 are French-Canadian; pedigrees 4–7 are Australian; pedigree 7 is of Coptic-Egyptian descent. The urine of subject 6.1 was not tested, and subject 5.1 was deceased.
common SNP, 596C→T (frequency, 0.075; Table 1), in SLC6A20 that causes a substitution of threonine by methionine (T199M) in the fifth transmembrane region (Figure 2B and Figure 3B) was identified in 6 pedigrees. In families 1–3, IG was only observed in the fifth transmembrane region (Figure 2B and Figure 3B) was a shift of the curve to the right. In the case of proline, the shift of the substrate concentration dependence (Figure 4, B and C) resulting in an increased K_v value, which is visible as a shift of the curve to the right. In the case of proline, the K_v value increased from 0.14 ± 0.01 mM to 0.39 ± 0.02 mM and in the case of glycine, from 0.49 ± 0.07 mM to 2.35 ± 0.11 mM. The maximum transport velocity (Figure 4, B and C) and surface expression (Figure 4, D–F) were preserved in the G87V mutant.

The data in Figure 5 confirm that this mutation resulted in the use of an alternative splice site 70 bp downstream of the WT donor splice site, which in turn led to truncation of the protein, thereby abrogating its expression (Supplemental Figure 1). SLC6A20 was almost completely inactivated by the SNP T199M (Figure 4A and Figure 6) due to a reduction of the maximum velocity from 282 ± 6 pmol/10 min to 35 ± 3 pmol/10 min per oocyte, which affected substrate dependence (Figure 6A), Na⁺ dependence (Figure 6B), and Cl⁻ dependence (Figure 6C). Similar to the SLC36A2 G87V mutant, surface expression was not altered (Figure 6, D–F).

A further level of complexity was observed in the putative glycine transporter SLC6A18. A number of SNPs were identified in all
families, but not in all individuals with IG or HG. These included known SNPs 957C→G (Y319X) and 1433T→C (L478P) (37) and novel mutations 235G→A (G79S) and 1486G→A (G496R). All missense mutations/SNPs occurred in transmembrane domain residues that are highly conserved between SLC6A18 orthologs and within the amino acid transporter branch (II) of the SLC6 family (38, 39) (Figure 2, C and D, and Figure 3C). Although SLC6A18 could not be functionally expressed in heterologous systems (27, 28), surface expression of mutants G79S and G496R in oocytes was abrogated (Supplemental Figure 2, B and E), truncated protein Y319X (Supplemental Figure 2C) was reduced, and the L478P protein was normal (Supplemental Figure 2D).

The partial inactivation of SLC36A2 by the G87V mutation explained why IG was only observed when a homozygous mutation was accompanied by haploinsufficiency of SLC6A20 in pedigrees 1–3 (Table 1). In the presence of normal gene sequences for SLC6A20, SLC36A1, and SLC6A18, the homozygous SLC36A2 G87V mutation detected in subject 4.4 did not explain the iminoaciduria and marked HG. Consequently, we identified a known polymorphism in SLC6A19 within the splice acceptor site of intron 7, IVS7–4G→A, that cosegregated with IG (Table 1) and was predicted by information theory–based analysis (35, 36) to affect splicing and branch site selection.

Altered function in imino acid and glycine transporters revealed by the genotype and phenotype of subjects with HG and IG is consistent with their expression in the nephron. SLC36A2, the common high-capacity proline and glycine transporter, was localized within the S1 segment of the most proximal part of the convoluted tubule adjacent to the glomerulus (Figure 7A). In contrast, the expression of SLC6A20 was restricted to S2 segments in the renal cortex and S3 straight tubules traversing into the medulla (Figure 7B), thus establishing a mutually exclusive renal tubular distribution of SLC36A2 and SLC6A20 (Figure 7C).

Discussion

In order to explain the urinary phenotypes of IG and HG, we have identified mutations in several amino acid transporters that are

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Figure 2

Mutations identified in IG and HG candidate genes SLC36A2, SLC6A20, and SLC6A18 encode highly conserved amino acid residues in transmembrane regions. Multiple amino acid sequence alignments were performed using ClustalW. Human RefSeq protein SLC36A2 (NP_861441.1) was aligned with SLC36A1 (NP_510968.2), SLC36A3 (NP_861439.2), and SLC36A4 (NP_689526.2). SLC6A20 (NP_064593.1) and SLC6A18 (NP_872438.1) were aligned with members of the SLC6 amino acid transporter (II) family (39): SLC6A15 (NP_877499.1); SLC6A16 (NP_054756.2); SLC6A17 (NP_001010898.1); and SLC6A19 (NP_001003841.1). Orthologs from other species were sourced from Ensembl release 47. Gray shading indicates residues that are conserved in the majority of sequences. Mutations identified in this study are indicated with arrows: (A) SLC36A2 G87V; mutation is in the second transmembrane helix; (B) SLC6A20 T199M; mutation is in the fifth transmembrane helix; (C) SLC6A18 G79S; mutation is in the second transmembrane helix; and (D) SLC6A18 L478P and G496R, both located in the tenth transmembrane helix.
known to be involved in the reabsorption of proline and glycine in the kidney (20). The major gene involved in IG and HG is the common imino acid and glycine transporter SLC36A2. We identified 2 mutant alleles, one of which completely (IVS1+1G→A) and the other partially (G87V) abolished its activity. The SNP T199M in SLC6A20 was observed in 6 of the 7 independent pedigrees and almost completely inactivated SLC6A20 in an in vitro model. In the putative glycine transporter SLC6A18, mutations G79S and G496R abolished surface expression, while the SNP Y319X introduced a stop codon. We have excluded the previously suggested candidate amino acid transporter SLC36A1 (22) and instead demonstrated a central causative role for SLC36A2 as well as SLC6A20. The contributions of SLC6A18 and SLC6A19 are discussed below.

The complex genetics of IG and HG is illuminated by examination of specific pedigrees presented in Table 1. Inheritance of 2 SLC36A2-null alleles (IVS1+1G→A) in individual 7.6 was the only example of the 7 cases of IG explained by a simple autosomal recessive model. In the remaining 6 cases of IG, reduced activity of SLC36A2 due to homozygous G87V mutations was present, but additional haploinsufficiency of the proline transporter SLC6A20 due to the T199M allele was observed in subjects 1.3, 1.4, 2.3, 2.4, and 3.3. The combination of single alleles of SLC36A2 G87V and SLC6A20 T199M (individuals 1.2, 2.2, and 3.1) was not sufficient to cause IG. Thus, the genetic model consistent with the observed pattern of inheritance associated with the SLC36A2 G87V mutation is classical semidominant inheritance, albeit with additional contributions from modifier loci. Heterozygosity of the null mutation SLC36A2 IVS1+1G→A resulted in HG, as seen in 7.1, 7.2, 7.4, and 7.5. Partial inactivation of SLC36A2 due to the G87V mutation alone also caused HG in all but 1 (subject 1.1) of the 8 heterozygotes. In 6 of the 7 SLC36A2 G87V heterozygotes with HG (subjects 1.2, 2.2, 3.1, 3.2, 4.2, and 5.3), additional inactivating mutations in SLC6A18 (G79S, Y319X) were found.

Neonates frequently exhibit “physiological” IG, which disappears after “maturaton” of kidney function (11, 40, 41). This developmental IG has been attributed to a delay in the expression of transporters (42), which we now propose to involve reduced function of the common transporter SLC36A2 combined with the specific imino acid transporter SLC6A20 and the putative glycine transporter SLC6A18. In the pedigrees we studied, most cases of IG detected at birth persisted to adulthood, but in 2 subjects (nos. 5.3 and 6.4), it resolved to HG or normality. The genotypes in subjects 5.3 and 6.4 would lead to partial loss of proline and glycine transport capacity that would result in IG at birth, but maturation of the remaining intact transporters would compensate in adulthood (Table 1).

Homozygosity of the SLC6A20 T199M SNP alone did not result in prolinuria or IG (see subject 6.2), suggesting that the bulk of proline reabsorption is carried out by SLC36A2, the major gene involved in IG. In contrast to the impaired glycine transport and

![Figure 3](http://www.jci.org)
hypertension observed in the SLC6A18 nullizygous mouse model (26), the presence of homozygous stop codons in SLC6A18 in subject 5.2 did not result in HG in the absence of other mutations. This result together with the high frequency of the Y319X allele in the normal population suggests a minor role for this transporter in glycine transport in the human kidney. Genotype/phenotype comparison in family 7, however, suggested that the SLC6A18 Y319X (and G496R) alleles are associated with increased urinary glycine levels (Table 1 and Supplemental Table 1). Our results are consistent with physiological data suggesting the presence of 4 different transporters for proline and glycine in the kidney, namely the common transporter SLC36A2, the specific proline transporter SLC6A20, the specific glycine transporter SLC6A18, and the general neutral amino acid transporter SLC6A19, which is affected in Hartnup disorder (20). At this stage, we cannot exclude the possibility that SLC6A18 is a nonfunctional transporter in humans and that another glycine-specific transporter might harbor mutations in IG or HG. However, because SLC6A20 is the major allele affected in the 2 disorders, the role of such a putative transporter would be a modifying one.

Neonatal screening studies report the frequency of IG to be around 1:10,000 (11, 41, 43–45), with one exception reporting a higher frequency of 1:1,200 (11). An accurate estimate of IG frequency in adult populations has not been made, but approximately 40% of neonatal IG disappears over the first year of life (11). This suggests an adult frequency of about 1:20,000, similar to that of other rare disorders. The SLC36A2 G87V allele was detected in all 3 French-Canadian pedigrees (nos. 1–3) and Australian pedigrees (nos. 4 and 5). The observed frequency of the SLC36A2 G87V allele, which did not cause IG in homozygotes, was 0.012. Under random mating, it is expected to occur in the homozygous state at a frequency of 0.00014 (1:6,900). This allele only caused IG together with haploinsufficiency of the SLC6A20 T199M allele mainly in European populations (frequency, 0.075). This variant of IG would thus occur with a combined frequency of 1.08 × 10⁻⁵ (1:92,600). The SLC36A2 IVS1+1G→A allele occurred at a frequency of 0.004. Homozygosity for this rare allele would be expected at a frequency of 1.6 × 10⁻⁵ (1:62,500).

This study suggests a model of imino acid and glycine reabsorption in the kidney (Figure 8) that permits prediction of the phenotype based on the genotype (Figure 9). The transporter for glycine and imino acids SLC36A2 is located in the earliest segment of the proximal tubule, as would be predicted for a high-capacity transporter (Figure 8). In the S2 and S3 segments of the proximal tubule, the low-capacity specific transporters for imino acids (SLC6A20) and glycine (SLC6A18) extract the residual amino acids. A reiterative process was used to estimate the contribution of each transporter to proline and glycine uptake in the kidney.
The optimized model suggests that the human kidney has a higher capacity for proline uptake than for glycine uptake. Thus, mutations affecting iminoglycine transporters will more readily cause glycinauria than prolinuria. If it is assumed that 100% reabsorption capacity is required to remove all proline and glycine, the model predicts a total capacity of 140% for proline uptake, comprising SLC36A2 (60%), SLC6A20 (40%), and SLC6A19 (40%). The total capacity for glycine transport would be 110%, comprising SLC36A2 (40%), SLC6A18 (10%), and SLC6A19 (60%). This model correctly replicates the phenotype in 95% of the cases in our cohort. Evidence has been presented that the paracellular pathway is difficult to estimate but could constitute part of the capacity that was attributed to SLC6A19.

The quantitative genotype/phenotype relationship of renal imino acid physiology (Figure 9) resolves a number of unexplained observations from previous cases. For example, pedigrees have been observed by our group (family 1) and Scriver and Hechtman (47), in which one parent with HG and the other with normal urine have a child with persistent IG. This can be explained by partially inactivating mutations not sufficient to cause HG alone and causing IG only in combination with more severe mutations or gene modifiers. In another well-recognized physiological variant, Greene et al. reported a case of IG with a normal maximal transport rate ($T_m$) for proline but a marked splay in the renal tubular titration curve for proline reabsorption, consistent with a mutation affecting the $K_m$ of proline transport (14). This human physiological measurement accurately describes our functional analyses of the SLC36A2 G87V mutation (Figure 2), which has the same maximum velocity but a change of affinity for proline. Thus, it is proposed that phenotypes can be predicted from the genotype with high fidelity in this and other complex disorders when the contribution of each gene to the phenotype is known.

In conclusion, the inactive or reduced function of SLC36A2 is the predominant determinant of the IG and HG phenotypes. Inactivating mutations in SLC6A20 and possibly SLC6A18 (or SLC6A19) account for additional features including the degree of urinary solute loss. Our model explains these related phenotypes by integrating observations from neonatal screening, developmental changes, and genetic and biochemical analyses of amino acid transporter genes and proteins. To our knowledge, this is the first explanation of an inherited disorder in terms of a major gene with its accompanying modifier genes for the reduced penetrance observed.

**Methods**

Informed written consent was received from the subjects.

Collection and analysis of urine. Morning urine samples from subjects were tested at the Quebec Mass Urinary Screening Programme for hereditary metabolic disorders. Quantitative urinary amino acid analysis was performed by ion exchange chromatography using the Biochrom 20 or 30 platform (Biochrom Labs Inc.) (33). Sulfonated cation resin was used with lithium citrate buffers, and norleucine was included as an internal standard. All amino acids were measured using ninhydrin detection at an absorbance of 570 nm, except for proline and hydroxyproline, measured at 440 nm. Similar methods and chemistry were used to quantitate urinary amino acids from the Australian pedigrees.

Sequencing of candidate genes. DNA was isolated from 4-ml saliva samples collected using the Oragene DNA Self-Collection Kit (DNA Genotek) (33). The genomic structures of SLC6A18, SLC6A19, SLC6A20, SLC36A1, and SLC36A2 were determined, and primers were designed across the exon-intron boundaries using Primer3 software (http://primer3.sourceforge.net) (Supplemental Table 2). PCR products were sequenced in forward and reverse directions. All chromatograms were viewed and contigs created.
using Sequencher 4.8 (Gene Codes Corp.). Orthologs of human SLC36A2, SLC6A20, and SLC6A18 proteins and other members in the amino acid transporter branch (II) of the SLC6 family were obtained from Ensembl release 47 (www.ensembl.org). All sequence alignments were created using the ClustalW algorithm in MacVector 9.5 (MacVector Inc.).

Allele frequencies. A panel of 590 normal Australian genomic DNAs and an additional panel of 95 genomic DNA samples from French-Canadian subjects (obtained from the University of Montreal, Montreal, Quebec, Canada) were screened to determine the allele frequency of novel mutations. SLC36A2 G87V was determined by an RFLP resulting from inac-

Figure 6
Transport activity of mutant SLC6A20. Oocytes were injected with WT or mutant cRNA encoding the imino acid transporter SLC6A20 (25 ng). After incubation for 4 days, [14C]proline uptake was determined in the presence of different concentrations of proline (A) and at a proline concentration of 50 μM in the presence of different Na+ concentrations (B) or different concentrations of Cl− (C). Each data point represents the mean ± SD transport activity of n = 10 oocytes. Surface expression of eGFP-transporter fusions of WT (D) and mutant T199M (E) SLC6A20 in Xenopus laevis oocytes in comparison to noninjected oocytes (F). Each experiment was repeated 3 times, with equivalent results.

Figure 7
Expression of SLC36A2 and SLC6A20 in adult human kidney. (A) Immunofluorescence of SLC36A2 (green) revealed colocalization (yellow) with the proximal tubule marker Lotus teragonolobus agglutinin (LTA; red) on the apical membrane of the S1 segment of proximal tubules. Arrows indicate SLC36A2 expression in the S1 segment of a proximal tubule close to the glomerulus (g). (B) Immunofluorescence of SLC6A20 (green) revealed colocalization (yellow) with LTA (red) on the apical membrane of the S2-S3 segment of proximal tubules. Arrow indicates SLC6A20 expression in the S3 segment of a proximal tubule. (C) Colocalization was not observed in sections stained for both SLC6A20 (green) and SLC6A20 (red). Negative controls for A, B, and C showed no staining (data not shown). Original magnification, ×40. Scale bars: 30 μm.
ivation of an ApaI restriction enzyme site and SLC36A2 IVS1+1G→A was determined by an RFLP resulting from inactivation of an HpyCH4IV restriction enzyme site. A custom Taqman SNP assay (Applied Biosystems) run on a Rotor-Gene RG3000 (Corbett Life Sciences) was used to measure the frequency of SLC6A18 G496R. Allele frequencies of known SNPs SLC6A18 Y319X, SLC6A20 T199M, and SLC6A19 IVS7–4G→A were confirmed by allele-specific PCR. Primers, products, and probes are detailed in Supplemental Table 3.

Molecular cloning and site-directed mutagenesis. Human SLC36A2, SLC6A20, and SLC6A18 were amplified by RT-PCR from human kidney RNA (BD Biosciences). For mutant transporter studies, pGEM-He-Juel (48) plasmids containing SLC36A2, SLC6A20, or SLC6A18 transporters were used as templates for site-directed mutagenesis using the QuikChange II Kit (Strata gene). All introduced mutations were confirmed by sequencing. Primers and products are detailed in Supplemental Table 3.

Minigene construction. The cDNAs for SLC36A2 and SLC6A20 were PCR amplified from human kidney cDNA (BD Biosciences) using DyNAzyme EXT (New England BioLabs Inc.) and cloned into pGEM-T-Easy (Promega). A 326-bp fragment containing the ATG start codon and exons 1 and 2 of SLC36A2 was PCR amplified with MluI ends from pGEM-T-SLC36A2 and cloned into pEF-BOS for addition of an N-terminal c-myc tag (Supplemental Table 2). Genomic DNA from subjects 7.3 (normal) and 7.6 (IG) were used as templates for amplification of 3.3-kb fragments containing exon 1, the entire first intron with WT IVS1+1G or mutant IVS1+1A alleles, and part of exon 2. These were ligated into pGEM-T-Easy and then subcloned into pEF-BOS with MluI overhangs. A 1,247-bp BamHI/XhoI-ended PCR product containing SLC36A2 exons 2–10 and with the stop codon removed was cloned into pcDNA3.1 (Invitrogen) adapted for C-terminal FLAG epitope tagging. Each minigene had an N-terminal c-myc tag and a C-terminal FLAG tag. SLC6A20 cDNA was cloned into pcDNA3.1 containing a c-myc epitope as a negative control for minigene expression.

Cell culture and immunofluorescence. MDCKII cells were maintained in DMEM supplemented with 10% (v/v) FCS, penicillin, and streptomycin. MDCKII cells were transfected with 2 μg pcDNA3.1 vectors containing SLC36A2 minigenes using 10 μl Lipofectamine 2000 (Invitrogen). After 48 hours, 500 μg/ml G418 was added for a period of 10 days. G418-resistant pools were transferred to 8-well chamber slides (BD Biosciences) coated in 5 μg/ml poly-L-lysine for 30 minutes. The cells were cultured for 3 more days, washed once in PBS, fixed in 50% methanol/50% ethanol (v/v) for 10 minutes at –30°C, air-dried, and rehydrated in PBS. After blocking for 1 hour at 37°C in PBS containing 20% (v/v) BlokHen II (AvesLab) and 0.1% (v/v) Triton X-100, mouse monoclonal anti-c-myc (clone 4A6; Upstate) or anti-FLAG (clone M2; Sigma-Aldrich) was added.
Figure 9
Prediction of the renal phenotype from the genotype. The results of in vivo urine analysis and in vitro transport experiments were combined to estimate the contribution of SLC6A19, SLC6A2, SLC6A20, and SLC36A2 to proline and glycine reabsorption in the kidney. Reabsorption capacity above 100% would manifest as a normal urinary phenotype. The urine phenotype is indicated below the subject as normal (NM), HG, or IG.

The following formula was used to estimate proline and glycine reabsorption: (activity of allele 1 + activity of allele 2)/2 + (activity of allele 1 + activity of allele 2)/2.

The following contributions to proline transport were used: SLC36A2 (60 AU); SLC6A18 (0 AU, not a substrate); SLC6A19 (40 AU); SLC6A20 (40 AU). The following contributions to glycine transport were used: SLC36A2 (40 AU); SLC6A18 (0 AU, not a substrate); SLC6A19 (40 AU); SLC6A20 (0 AU, not a substrate). The activity of individual alleles was calculated as a fraction of 1, reflecting the activity of the mutant compared with the WT when expressed in Xenopus laevis oocytes. The algorithm correctly replicates the urinary phenotype of 25 of the 28 individuals.
that the laser light does not penetrate far into the oocyte because of its egg yolk content. As a result, membrane proteins in the endoplasmic reticulum or Golgi apparatus are not visible; only proteins expressed at the surface are excited (50). The fluorescence intensity is therefore proportional to the surface expression.

Transporter model construction. A topology plot was generated for SLC6A2 using transmembrane helix prediction as calculated by the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). The model was confirmed using the TOPCONS website (http://topcons.cbr.su.se/). Homology models of SLC6A18 and SLC6A20 were derived from the bacterial homolog of the Na’/Cl’-dependent neurotransmitter LeuTα (51). Optimally aligned sequences were submitted to the Swiss-PDB server (52), and the structures generated were visualized using the PyMol program (DeLano Scientific).

Acknowledgments

We thank the families who consented to participate in this study. Financial support was provided by the Australian National Health and Medical Research Council (project grant 402730), the Australian Research Council (Discovery Project DP0877897), the Private Practice Fund at The Canberra Hospital, the Rebecca L. Cooper Foundation, and The Cell and Gene Trust. Assistance in clinical amino acid quantitation was provided by Robert Giguère of the Biochemical Genetics Service, University of Sherbrooke Health Centre, and Kevin Carpenter of the NSW Biochemical Genetics and Newborn Screening Services at The Children’s Hospital at Westmead. The identification of individuals with neonatal IG would not have been possible without the pioneering studies of Bridget Wilcken. We thank Robyn Soper of the Department of Anatomical Pathology, Royal Prince Alfred Hospital, for assistance; and Jean-Baptiste Rivière and Guy Rouleau of the Centre of Excellence in Neuromics, University of Montreal, for normal French-Canadian human genomic DNA samples.

Received for publication June 30, 2008, and accepted in revised form October 1, 2008.

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