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Characterisation of renal chloride channel, *CLCN5*, mutations in hypercalciuric nephrolithiasis (kidney stones) disorders

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Mutations of the renal-specific chloride channel (*CLCN5*) gene, which is located on chromosome Xp11.22, are associated with hypercalciuric nephrolithiasis (kidney stones) in the Northern European and Japanese populations. *CLCN5* encodes a 746 amino acid channel (CLC-5) that has ~12 transmembrane domains, and heterologous expression of wild-type CLC-5 in *Xenopus* oocytes has yielded outwardly rectifying chloride currents that were markedly reduced or abolished by these mutations. In order to assess further the structural and functional relationships of this recently cloned chloride channel, additional *CLCN5* mutations have been identified in five unrelated families with this disorder. Three of these mutations were missense (G57V, G512R and E527D), one was a nonsense (R648Stop) and one was an insertion (30:H insertion). In addition, two of the mutations (30:H insertion and E527D) were demonstrated to be *de novo*, and the G57V and E527D mutations were identified in families of Afro-American and Indian origin, respectively. The G57V and 30:H insertion mutations represent the first *CLCN5* mutations to be identified in the N-terminus region, and the R648Stop mutation, which has been observed previously in an unrelated family, suggests that this codon may be particularly prone to mutations. Heterologous expression of the mutations resulted in a marked reduction or abolition of the chloride currents, thereby establishing their functional importance. These results help to elucidate further the structure–function relationships of this renal chloride channel.

INTRODUCTION

Four disorders of hereditary hypercalciuric nephrolithiasis (kidney stones), that have been referred to as Dent's disease, X-linked recessive nephrolithiasis (XRN), X-linked recessive hypophosphataemic rickets (XLRH) and the idiopathic low molecular weight proteinuria of Japanese children (JILP), have been reported to be due to mutations of the X-linked renal-specific chloride channel, *CLCN5* (1–8). All of these four diseases have features in common, and they represent renal tubular disorders that are characterised by low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis and renal failure. In addition, other renal proximal tubular defects, which include aminoaciduria, phosphaturia, glycosuria, kaliureseis, uricosuria and an acquired impairment of urinary acidification, may also occur (1–3,5,6). However, there are differences between these disorders; for example, rickets has been a particular feature of Dent's disease and XLRH, but not XRN and JILP, and severe renal failure has been a feature of Dent's disease and XRN (1–6). Dent's disease, XRN and XLRH were mapped to Xp11.22 (2,4,5), and a microdeletion in one patient with Dent's disease facilitated the isolation and characterisation of a renal chloride channel gene, *CLCN5* (2,9,10). DNA sequence analysis detected different *CLCN5* mutations in patients with the four hypercalciuric nephrolithiasis disorders, thereby establishing its causal role in these diseases (7,8). The common genetic aetiology of *CLCN5* mutations and the phenotypic similarities between all these syndromes indicates that they are variants of one disorder, which we propose to refer to as Dent's disease.

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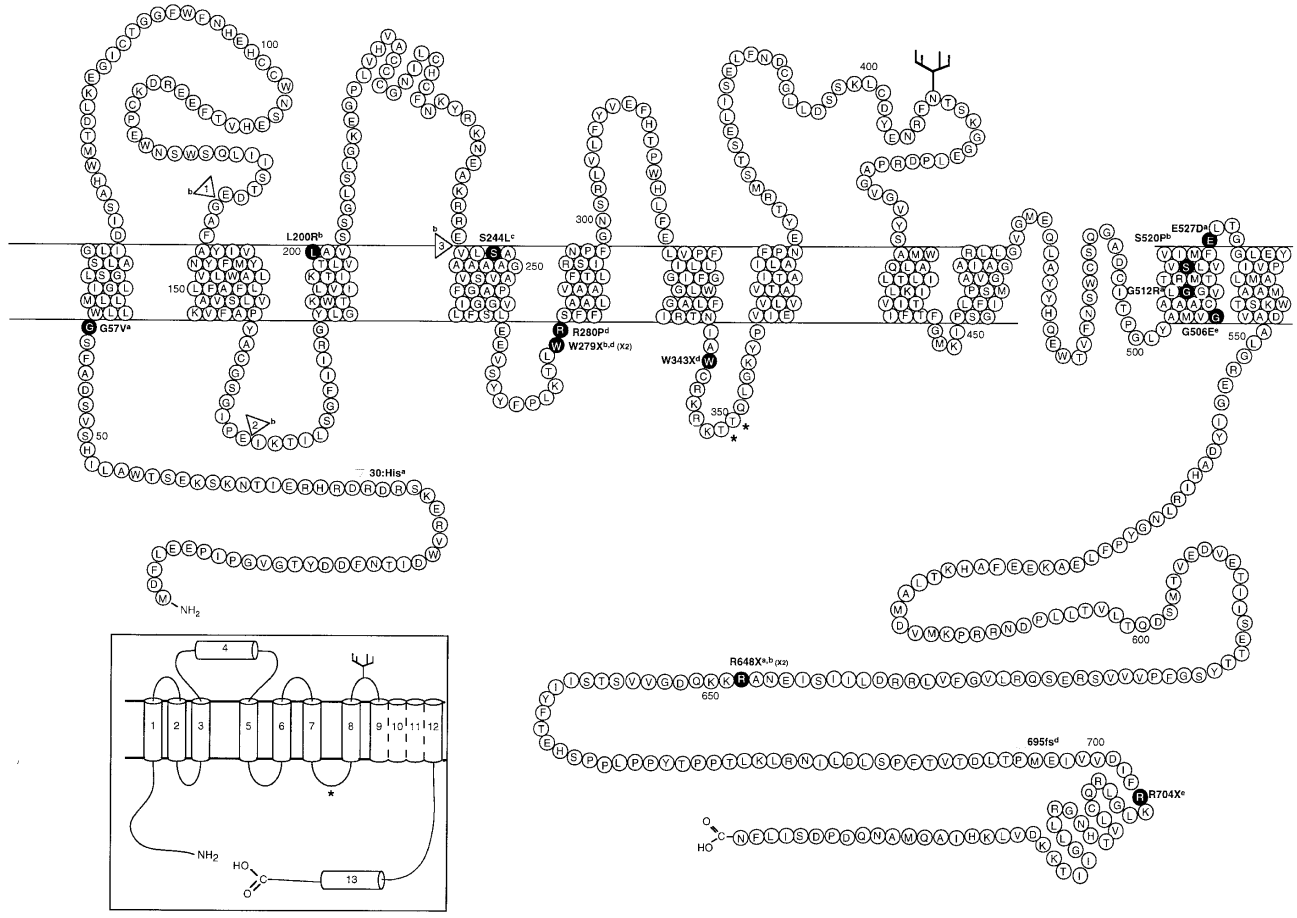

Figure 1. Schematic representation of the *CLCN5* mutations within the framework of the predicted topology of CLC-5 (7), which consists of 746 amino acids (9,10). The correct topology of the CLC-5 putative transmembrane domains (D1–D12) is based upon a model (7,11) illustrated in the inset. The consensus phosphorylation and glycosylation sites are indicated by asterisks and branch sites, respectively. The five mutations (30:Hins, G57V, G512R, E527D and R648X) detected by the present study ([a]) are indicated together with those previously reported (7,8) in families with Dent's disease[b], XLRH[c], JILP[d] and XRN[e]. Of the total 20 disease-associated *CLCN5* genetic abnormalities, four (20%) have been identified in D11, which is encoded by exons 9 and 10, and the W279X and R648X mutations have been observed twice in unrelated families.

The human *CLCN5* gene has a 2238 bp coding sequence, which consists of 12 exons that span 25–30 kb of genomic DNA, and encodes a 746 amino acid protein (9,10). *CLCN5* belongs to the family of mammalian voltage-gated chloride channel genes (*CLCN1–CLCN7*, and *CLCKa* and *CLCKb*, respectively) that have ~12 transmembrane domains (9,11,12) (Fig. 1). These chloride channels have an important role in the control of membrane excitability, transepithelial transport and possibly cell volume (11,13). Heterologous expression of wild-type *CLCN5* in *Xenopus* oocytes has revealed that the channel, CLC-5, conducts chloride currents that are outwardly rectifying and time-independent, and similar expression of disease-associated CLC-5 mutants demonstrated markedly reduced or absent currents (7,8,14). However, a correlation between the mutations, their *in vitro* functional consequences and the phenotypic variations could not be established. We have pursued further investigations to define the structural and functional relationships of this novel renal chloride channel and its role in hypercalciuric nephrolithiasis, by characterising additional *CLCN5* mutations in patients with familial and non-familial forms of this disorder.

RESULTS

Five families with Dent's disease (Table 1) were identified from the UK, USA, Italy (15) and the Indian subcontinent (Table 2), and 22 members (nine affected males, seven affected females, two unaffected males and four unaffected females) were studied. DNA sequence analysis of the entire 2238 bp coding region of the *CLCN5* gene from one affected member of each of the five families revealed the presence of five different mutations (Table 2) which consisted of three missense (G57V, G512R and E527D), one nonsense (R648X) and one in-frame insertion at codon 30 (30:Hins). The G57V mutation resulted from a G to T transversion (G**G**C→G**T**C), the G512R mutation resulted from a G to C transversion (**G**GT→**C**GT), the E527D mutation (Fig. 2) resulted from an A to T transversion (GA**A**→GA**T**), the R648X mutation from a C to T transition (**C**GA→**T**GA) and the in-frame insertion (30:H) resulted from an insertion of ACC at codons 30 and 31 (Fig. 3). The G57V, G512R, E527D and R648X mutations resulted in alterations of *Msp*I, *Hph*I, *Sau*3A1 and *Taq*I restriction enzyme sites, respectively, that facilitated their

confirmation (Fig. 2). Interestingly, the R648X (**C**GA→**T**GA) mutation has been observed previously (7) in an unrelated (confirmed by microsatellite polymorphism analysis, data not shown) British family with Dent's disease, and this may represent a possible mutational hot-spot (Fig. 1). The ACC in-frame insertion (30:H) was not associated with an alteration in a restriction enzyme site, and this mutation was confirmed by sequence-specific oligonucleotide (SSO) hybridisation analysis (Fig. 3). The G57V, G512R and R648X mutations were demonstrated to co-segregate with the disease in each of families 8/95, 13.3/95 and 3/95, respectively (Table 2). However, the 30:H insertional and E527D mutations were demonstrated to arise *de novo* (Figs 2 and 3), and interestingly the E527D mutation was found to co-segregate with the disease in subsequent generations. The absence of each of these five DNA sequence abnormalities in 110 alleles from 69 unrelated normal individuals (28 males, 41 females) established that these abnormalities were mutations and not functionally neutral polymorphisms which would be expected to occur in >1% of the population.

The two missense mutations, G57V and G512R, and the nonsense mutation R648X predict structurally significant alterations to CLC-5 and are thus likely to be of importance in the aetiology of the disease. The nonsense mutation R648X predicts a truncated CLC-5 channel that lacks the 100 amino acids from the C-terminal loop (Fig. 1), and the missense mutations G57V and G512R predict a disruption of the helix in the first transmembrane domain, D1, and a disruption of the charge distribution within D11, respectively (Table 2). The effects upon CLC-5 function of such nonsense and missense mutations, which have been previously assessed (7,8), are likely to be a loss of function. The effects of the missense mutation E527D, in which there is a minor change involving a loss of a methyl group from the glutamate (E) to convert it to an aspartate (D) (Fig. 1), are difficult to predict, although it is important to note that E527 is one of the most conserved amino acids and is present in all the known CLCs, including those from plants, yeast, *Escherichia coli*, cyanobacteria, fish and mammals (11,13). Similarly, the insertion of the positively charged histidine (H) at codon 30 in the N-terminus region, amidst the peptide sequence between codons 24 and 34 (REKSRDRDRHR) that is already rich in the positively charged amino acids arginine (R) and histidine (H), is also difficult to predict; interestingly, this sequence also has positively and negatively charged amino acids in alternating positions and if these were arranged in a β-pleated sheet with one side positive and the other negative, then insertion of any extra amino acid would result in a disruption of the charge distribution (Table 2). The functional effects of the missense and insertion CLC-5 mutants were therefore assessed by heterologous expression in *Xenopus* oocytes and measurements of chloride currents as previously described (7,8). The results demonstrated that the insertional (30H) and missense (G57V) mutations markedly reduced the chloride channels to ~30 and 50% of the wild-type CLC-5 respectively, whilst the missense mutations G512R and E527D abolished the chloride currents (Fig. 4).

Table 1. Clinical and biochemical abnormalities in nine patients from five families with Dent's disease

	Family[a] 2/95	8/95		13.3/95	7.3/95			3/95	
Individual	II.1[p]	II.1[p]	II.2	II.1[p]	II.1[p]	III.1	III.2	II.1[p]	II.2
Age (years) at diagnosis	2	5	4	3	19	4	3	3	0.5
Sex	M	M	M	M	M	F	F	M	M
Urinary abnormalities									
Low molecular weight proteinuria	+	+	+	+	+	–	+	+	+
Hypercalciuria	+	+	+	+	–	+	+	+	+
Renal failure[b]	–	–	–	+	++	–	–	+	+
Rickets[c]	–	–	–	–	+	–	–	+	+
Nephrocalcinosis[d]	–	+	+	+	+	–	–	+	+

+ = present, – = absent.
[a]Family designation is shown, and each individual is indicated by a generation (I, II or III) and sibship number (1 or 2) (Figs 2 and 3); [p]indicates the proband.
[b]Renal failure: + = moderate (<97 ml/min/1.73m^2) (ref. 8); ++ = end stage, requiring dialysis and transplantation.
[c]Rickets detected by raised serum alkaline phosphatase activity or radiologically.
[d]Nephrocalcinosis detected by ultrasonography or radiology.

Table 2. *CLCN5* mutations detected in families with Dent's disease

Family (origin)	Base change	Codon/amino acid change	Restriction enzyme change/SSO	Predicted effect
2/95 (British/Caucasian)	ACC in-frame insertion	30:H insertion	SSO	Disruption of charge distribution?
8/95 (USA, African)	G**G**C→G**T**C	Gly57Val	*Msp*I	Disruption of helix in D1
13.3/95 (USA, Caucasian)	**G**GT→**C**GT	Gly512Arg	*Hph*I	Disruption of charge distribution within D11
7.3/95 (Indian)	GA**A**→GA**T**	Glu527Asp	*Sau*3AI	?
3/95 (Italian)	**C**GA→**T**GA	Arg648Stop	*Taq*I	Loss of 100 amino acids from cytoplasmic domain

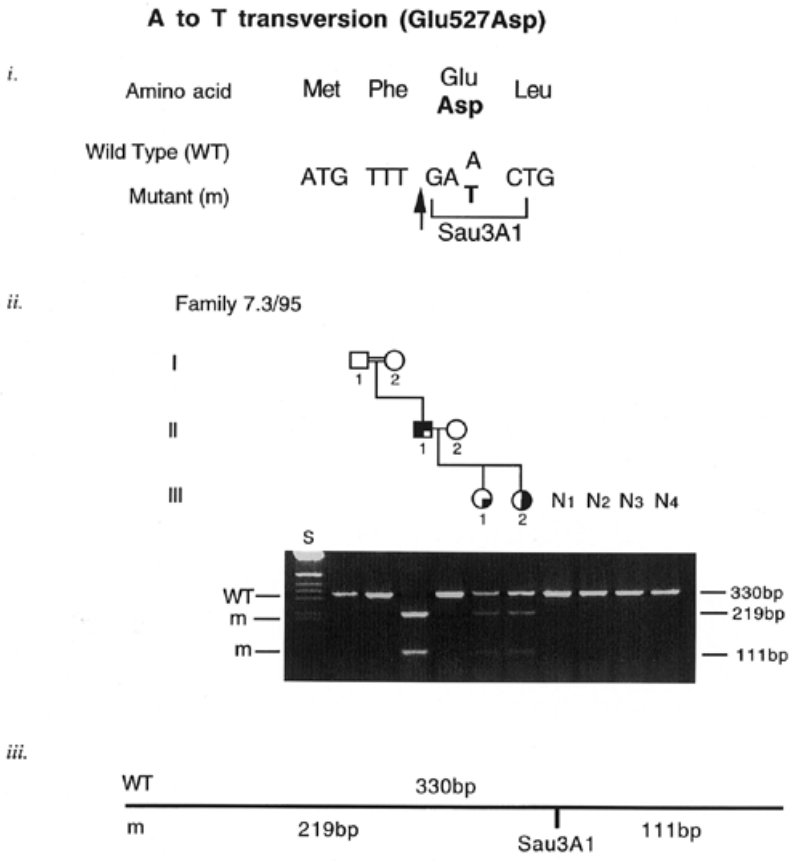

Figure 2. Detection of *de novo* missense mutation in exon 10 in family 7.3/95, affected with Dent's disease, by restriction enzyme analysis. DNA sequence analysis of individual II.1, who was of Indian origin, revealed an A to T transversion at codon 527 (panel i), thus altering the wild-type (WT) sequence, GA**A**, encoding a glutamate (E), to the mutant (m) sequence, GA**T**, encoding an aspartate (D). This missense mutation (E527D) also resulted in the gain of a *Sau*3A1 restriction enzyme site (/GATC). PCR amplification and *Sau*3A1 digestion (panel ii) would result in two products of 219 and 111 bp from the mutant sequence but only one product of 330 bp from the normal sequence, as is illustrated in the restriction map in panel iii. Co-segregation of this E527D mutation with the disease was demonstrated in individual II.1 and his affected daughters (III.1 and III.2), panel ii. However, the E527D mutation was absent in the consanguineous parents of individual II.1, thereby demonstrating the *de novo* nature of the mutation. In addition, the absence of this E527D mutation in 110 alleles from 69 (28 males, 41 females) normal individuals, N_1–N_4 shown (panel ii), indicates that it is not a common DNA sequence polymorphism. Individuals are represented as: unaffected male (open square) and female (open circle); affected with low molecular weight proteinuria (filled upper right quadrant); hypercalciuria (filled lower right quadrant); nephrocalcinosis and nephrolithiasis (filled lower left quadrant); and end stage renal failure (filled upper left quadrant). The standard size marker (S) in the form of the 1 kb ladder is indicated. Similar restriction enzyme analysis was used to confirm and demonstrate the mutations G57V, R648X and G512R (Fig. 1) of families 8/95, 13.3/95 and 3/95 (Table 2), respectively.

DISCUSSION

Our results, which have identified five mutations of the *CLCN5* gene in five additional families with Dent's disease, expand the spectrum of such mutations (Fig. 1) that are associated with this renal tubulopathy. In addition, our results are the first to demonstrate the occurrence of *de novo CLCN5* mutations (Figs 2 and 3) and their inheritance in a subsequent generation (Fig. 2). Furthermore, our findings reveal that *CLCN5* mutations are not confined to any specific population but that they may occur in individuals of Northern European, Japanese, Indian or Afro-American origin (Table 2). The *CLCN5* mutations of our present and previous reports (7,8) reveal that the mutations are scattered through the different regions of CLC-5 (Fig. 1), and a correlation between the mutations and the severity of the phenotypes could not be established (8).

In order to assess the effect of mutations on CLC-5 function *in vitro*, we measured macroscopic currents in *Xenopus* oocytes expressing these mutants. The currents elicited by wild-type CLC-5 in *Xenopus* oocytes (and also in transfected CHO cells, Günther and Jentsch, unpublished) are strongly outwardly rectifying and can be detected only in a very positive, seemingly unphysiological voltage range. These observations led us to speculate (7,14) that to function in the physiological range, CLC-5 may require another as yet unknown subunit or regulatory mechanism that was absent in our expression systems but was present *in situ* in kidney cells. However, despite these limitations, our present and previous studies (7,8) clearly demonstrate that the *Xenopus* oocyte expression system is useful for assessing the functional consequences of *CLCN5* mutations. Indeed, all the *CLCN5* mutations either markedly reduced or abolished the chloride currents (Fig. 4), which represent CLC-5 currents expressed in the plasma membrane. These currents may be reduced by several possible mechanisms, which include a decrease in the steady-state levels of the protein, a diminished trafficking to the plasma membrane, a reduced open probability of the channel or a reduction in single channel conductance. However, immunoprecipitation of mutant CLC-5 proteins synthesized in *Xenopus* oocytes revealed no differences in

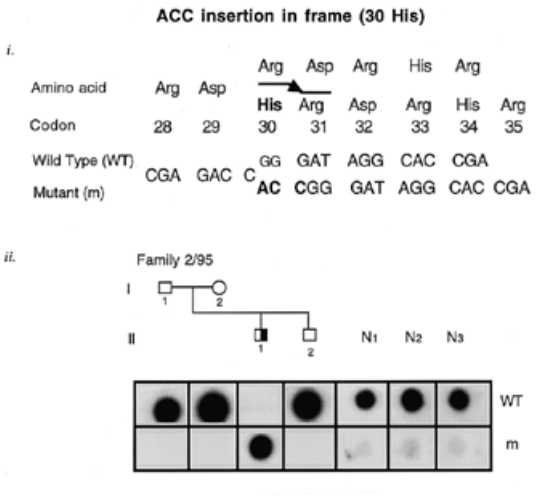

Figure 3. Insertional mutation in exon 2 of the *CLCN5* gene in family 2/95 affected with Dent's disease. DNA sequence analysis of individual II.1 revealed an in-frame ACC insertion involving codon 30 (panel i), thus altering the wild-type (WT) sequence CGG, which encodes an arginine (R) to the mutant sequence CAC, which encodes a histidine (H). The codon numbering in panel i refers to that of the mutant (m) sequence. The mutation (30:H insertion) was in-frame and resulted in a mutant 747, instead of the WT 746, amino acid CLC-5 channel, in which the mutant R31 to N747 corresponded to the R30 to N746 (Fig. 1) sequence of the WT channel. This insertional mutation was confirmed and demonstrated to be a *de novo* mutation by SSO hybridisation analysis (panel ii). Thus, the unaffected parents (I.1 and I.2) were found to have only the WT sequence, as was the unaffected brother (II.2). In addition, the absence of this insertional mutation in 110 alleles from 69 (28 males, 41 females) normal individuals, N_1–N_3 shown (panel ii), indicates that it is not a common DNA sequence polymorphism. The pedigree symbols indicating the phenotype of each individual are as described in Figure 2.

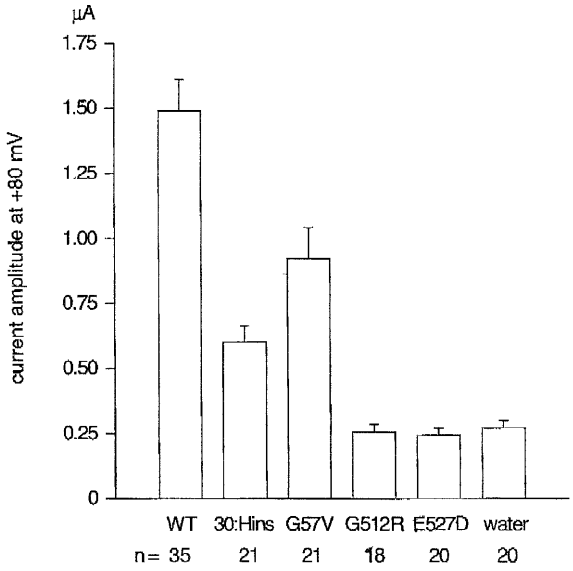

Figure 4. Electrophysiological analysis of *Xenopus* oocytes expressing human wild-type (WT) CLC-5 and the CLC-5 mutants 30:Hins , G57V, G512R and E527D. The averaged (mean ± SEM, *n* = number of oocytes) whole cell currents at +80 mV in *Xenopus* oocytes injected with the WT CLC-5 (1.50 ± 0.12 μA) and the CLC-5 mutants 30:H ins (0.60 ± 0.06 μA), G57V (0.92 ± 0.12 μA), G512R (0.26 ± 0.03 μA), E527D (0.24 ± 0.03 μA) and water control (0.27 ± 0.03 μA) are shown. The WT CLC-5 demonstrated a strongly outwardly rectifying current that was abolished by the G512R and E527D mutations. The G512R mutation occurs in the highly conserved (>95% identity between CLC-3, CLC-4 and CLC-5) region of the putative D11 (Fig. 1), and the E527D mutation involves a residue that is conserved in all the CLCs (11,13). However, the 30:H ins and G57V mutations which are located in the less conserved (>65% identity between CLC-3, CLC-4 and CLC-5) amino-terminus region did not abolish the Cl^- currents but did markedly reduce them to 52.9 ± 10% and 34.3 ± 5%, respectively, when compared with the wild-type.

steady-state expression (data not shown), and we have not yet been able to measure single CLC-5 channels, which may be due to a low single channel conductance as observed with other CLC channels (16), or to exclude any of the other possibilities. Despite these limitations, it is of importance to note that the decrease in the observed surface currents, which may be the consequence of altered protein stability, defective targeting or a loss of channel function proper, is related to the predicted disease-causing character of these *CLCN5* mutations. Thus, the nonsense mutation (R648X), which leads to a C-terminal truncation of the protein, leads to a loss of CLC-5 function as shown previously (7). The two missense mutations (G57V and G512R) affect amino acids which are likely to be structurally important. The G512R mutation (Fig. 1), which is located in a putative transmembrane domain, resulted in currents indistinguishable from negative controls, although the G57V mutation, at the cytoplasmic beginning of domain D1, resulted in a 50% reduction in the currents (Fig. 4), indicating that it was better tolerated. The profound effects of the disease-causing conservative mutation, E527D, which lead to a total loss of function in the electrophysiological assay, are surprising at first sight. However, this glutamate is one of the most conserved residues in CLC proteins and is found at equivalent positions in plants, yeast, *E.coli* and cyanobacteria (11,13). The insertional mutation, (30:H ins) (Fig. 3), which involved the cytoplasmic amino-terminal region of CLC-5, is of particular interest in understanding the structure–function relationships of CLC proteins, as to date only one other disease-associated missense mutation involving this region has been reported; this was observed in the homologous muscle chloride channel CLC-1 in which the mutation R105C was associated with myotonia (17). The His insertion, at codon 30, disrupts a peculiar motif of alternating positively and negatively charged residues that are conserved in CLC-3, CLC-4 and CLC-5, thereby indicating the functional importance of this region. Thus, our mutational analysis of *CLCN5* disease-related abnormalities has helped to reveal potentially important functional regions of this renal chloride channel.

MATERIALS AND METHODS

Patients and families

The families of five probands (Table 1), who suffered from multiple proximal renal tubular defects in association with nephrocalcinosis, nephrolithiasis and renal failure, were investigated after obtaining informed consent. The proximal tubular abnormalities included: a low molecular weight proteinuria, which consisted of β2 microglobulin (normal <0.05 mg/mmol creatinine), α_1 microglobulin (normal <0.7 mg/mmol creatinine) and retinol-binding protein (normal <0.02 mg/mmol creatinine) (1); an increased urinary excretion of calcium (calcium:creatinine ratio >0.71 mmol/mmol), phosphate, potassium, glucose, urate and amino acids; and an acquired impairment of urinary acidification (1–3,5,15). Venous blood samples were obtained from 16 affected and six unaffected members of the five families, which were from

different populations (Table 2), and used for mutational analysis of the *CLCN5* gene (7,8).

DNA analysis

Leukocyte DNA was extracted and used with *CLCN5*-specific primers for PCR amplification utilising conditions previously described (7,8). The PCR products were gel purified and the DNA sequence determined by *Taq* polymerase cycle sequencing using fluorochrome-labelled di-deoxy terminators, and resolved on a semi-automated detection system (ABI 373A sequence, Applied Biosystems) (7,8,18,19). In addition, DNA sequence abnormalities were confirmed either by restriction endonuclease analysis of genomic PCR products obtained by the use of the appropriate primers or by SSO hybridisation analysis (7,18,19); for the SSO hybridisation analysis, the wild-type oligonucleotide (19mer) was TCTCGAGACCGGGATAGGC and the mutant oligonucleotide (19mer) was TCGAGACC**ACC**GGGATAGG (Fig. 3). The DNA sequence abnormalities were demonstrated to co-segregate with the disorder and to be absent as common polymorphisms in the DNA obtained from 69 unrelated normal individuals (28 males, 41 females). Microsatellite polymorphism analysis at *DXS988* and at *D11S533*, and Southern blot hybridisation analysis were performed as described previously (2,20).

Functional expression in *Xenopus* oocytes

After engineering an *Nco*I site at the initiator methionine, a cDNA encoding human CLC-5 was inserted into the *Nco*I site of the expression vector pTLN (21), which contains *Xenopus* globin untranslated sequences, to boost expression in the oocyte. Mutations were introduced by recombinant PCR and verified by sequencing. Capped cRNA was prepared by *in vitro* transcription, using SP6 RNA polymerase, from the linearised construct. About 10–20 ng of cDNA was injected into *Xenopus* oocytes, which previously had been manually defolliculated and briefly collagenased (22). After incubating for 3 days at 17°C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM HEPES pH 7.6], oocyte conductance was measured at room temperature by a standard two-electrode voltage-clamp technique (7) using a Turbo-TEC-05.amplifier (NPI, Tamm Germany) and pCLAMP software (Axon Instruments, Inc); recordings were performed in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES pH 7.4). The currents were measured at the membrane potential of +80 mV and were normalised to the wild-type amplitude for each mutant. Four different batches of cRNAs and eight different batches of oocytes for the wild-type and mutant constructs were used, and similar results were obtained. The results were expressed as mean values ± SEM.

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