Isolation and partial characterization of a chloride channel gene which is expressed in kidney and is a candidate for Dent's disease (an X-linked hereditary nephrolithiasis)

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Dent's disease, an X-linked renal tubular disorder, is a form of Fanconi syndrome which is characterized by proteinuria, hypercalciuria, nephrocalcinosis, kidney stones and renal failure. Previous studies localised the gene responsible to Xp11.22, within a microdeletion involving the hypervariable locus DXS255. Further analysis using new probes which flank this locus indicate that the deletion is less than 515 kb. A 185 kb YAC containing DXS255 was used to screen a cDNA library from adult kidney in order to isolate coding sequences falling within the deleted region which may be implicated in the disease aetiology. We identified two clones which are evolutionarily conserved, and detect a 9.5 kb transcript which is expressed predominantly in the kidney. Sequence analysis of 780 bp of ORF from the clones suggests that the identified gene, termed hCIC-K2, encodes a new member of the CIC family of voltage-gated chloride channels. Genomic fragments detected by the cDNA clones are completely absent in patients who have an associated microdeletion. On the basis of the expression pattern, proposed function and deletion mapping, hCIC-K2 is a strong candidate for Dent's disease.

INTRODUCTION

Dent's disease (McKusick OMIM 310468) is a renal tubular disorder which is associated with low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis (kidney stones) and eventual renal failure (1−3). The primary defect responsible for this disorder has not been established. Previous analysis of two unrelated British families with twenty X-linked polymorphic markers localised the disease gene to chromosomal band Xp11.22 (4). A similar disorder affecting the renal proximal tubules, referred to as X-linked recessive nephrolithiasis, XRN (OMIM 310468) also maps to proximal Xp (5,6). However this differs from Dent's disease in that patients lack urinary acidification defects, rickets and osteomalacia (5,7,8). Recent linkage analysis has provided preliminary evidence that XRN maps to Xp11.4−p11.23 suggesting that there may be loci for two distinct diseases associated with nephrolithiasis on the short arm of the human X chromosome (9).

Although no cytogenetic abnormalities have been described for patients with Dent's disease, the affected members of one family (designated 12/89) have a microdeletion spanning the hypervariable locus DXS255 within Xp11.22 (4). The deletion has been shown to map within a 4 cm interval flanked on the proximal side by the locus DXS988 and on the distal side by the genes TFE3 and synaptophysin (SYP). The phenotype associated with this deletion is similar to that observed in non-deleted individuals with Dent's disease. In particular, patients are not mentally retarded nor do they show evidence of other clinical disorders, suggesting that the deletion is not responsible for a contiguous gene syndrome (4). As a single, major locus appears to be implicated, isolation of transcripts from the region involved in this microdeletion should provide a basis for the identification of candidate genes for the disease, particularly should they prove to be kidney-specific.

An approach which has been successful in the identification of transcripts from defined chromosomal regions involves the use of YACs or YAC fragments as hybridization probes to screen cDNA libraries constructed from a relevant source (10,11). We have constructed a YAC contig of approximately 2 Mb which spans the Xp11.23−p11.22 region (12). Included in this contig are YACs which contain the locus DXS255, and which must therefore overlap with at least part of the microdeletion associated with Dent’s disease. We have used a 185 kb DXS255 YAC to screen a renal cDNA library and have identified clones which are derived from a gene mapping within the deletion. The pattern of expression of this gene is highly specific to the kidney and the predicted amino acid sequence of the gene product shows a high degree of homology to previously isolated members of a family of voltage-gated chloride channel proteins. It therefore represents a strong candidate locus for the disorder.

RESULTS

Deletion analysis of family 12/89

Dent's disease is associated with a microdeletion spanning DXS255 in family 12/89 (4). In constructing a YAC contig around this locus we have used cloning of left/right ends of YAC

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Figure 1. Deletion analysis of patients with Dent's disease. (a) Sub-section of Xp11.23–p11.22 YAC contig spanning DXS255. Status of markers with respect to deletion in family 12/89 is indicated (+ = present, – = deleted). YAC sizes and reference numbers are shown. YWXD6129 was isolated from St. Louis library (30); all other YACs are from ICRF library (31). L(YAC name) and R(YAC name) denote new markers isolated from YACs by left and right end cloning respectively. Shaded part of a YAC indicates an autosome co-ligation. (b) PCR/hybridization results demonstrating that L(F1001) and L(G0201), which map on either side of DXS255, are outside the associated microdeletion of family 12/89, while L(6129) is within it. I: PCR of DNA from patients with Dent's disease using STSs for L(F1001) and L(6129) (see Methods for conditions). Lanes: W, water control; 1, normal male control; 2, normal female control; 3, male patient II.3 from family 12/89; 4, male patient III.1 from family 2/92; 4, 5, male patient case 2 (3); L(F1001) PCR (top) gave a product of the expected size in the patient from family 12/89 (lane 3), establishing this as the nearest undeleted marker distal to DXS255. L(6129) PCR product is absent in lane 3, indicating that this marker maps within the deletion. Both PCR assays amplified products of the expected size from normal male/female template, and from the other two unrelated patients. Products were absent from the water controls. II: Hybridization of L(G0201) probe to EcoRI digests of DNA from patients with Dent's disease, and normal controls. Lanes 1–5 correspond to the same individuals as in I. The cognate band of approx 30 kb is present in all lanes, indicating that L(G0201) is the nearest undeleted marker on the proximal side of DXS255.

inserts to generate new markers for the region (see Methodology). Further analysis of the microdeletion using these markers demonstrates that it is confined to the interval between L(F1001) and L(G0201), and spanning L(6129) (Fig. 1). This region is cloned in two overlapping YACs, yWXD6129 and ICRFy900C0191, of sizes 185 kb and 365 kb respectively, establishing a maximum distance of 550 kb between L(F1001) and L(G0201). The cognate 30 kb EcoRI fragment detected by hybridisation with L(G0201) (Fig. 1b) and the 4.1 kb EcoRI fragment detected by L(F1001) (data not shown) are both retained in the deletion patient. It therefore follows that the deletion must be less than ~515 kb. The YAC mapping data are supported by genomic pulsed-field maps which have shown the distance between DXS255 and DXS146 to be in the range of 230–900 kb (13).

Characterization of a 185 kb YAC containing DXS255

Using a PCR assay for DXS255 based on sequence information from a PstI–BglII fragment at the 5' end of the locus, we isolated a 185 kb YAC from the St.Louis YAC library (clone yWXD6129). Restriction mapping of the YAC with partial digests of the rare-cutting enzymes BstHII, Sall, SrlII, SfiI, EagI, MluI, Nael, NraI and NolI, localised DXS255 to a 40 kb BstHII fragment (Fig. 2). Two clones were isolated, one corresponding to the left end of the human insert of the YAC (L(6129)), the other mapping within 20 kb of the right end (L(F1001)). Both
were shown to be X-specific by analysis of a somatic hybrid panel (data not shown). In addition, the probes are present within independent YACs of the Xp11.22 YAC contig (12; Fig. 1). These observations suggest that the YAC insert is unlikely to be co-ligated, and enabled its orientation relative to the X chromosome to be determined (Fig. 2).

Screening of renal cDNA library

Mapping of the microdeletion associated with Dent’s disease (Fig. 1) indicates that the YAC, yWXD6129, must include a substantial proportion of the deleted region and may therefore contain at least part of a gene responsible for the disorder. The YAC was separated from the background of yeast chromosomes by preparative pulsed-field gel electrophoresis, purified and hybridized, following suppression of repetitive elements, to a human cDNA library made from adult kidney (Clontech). Two cDNA clones were isolated (designated RL.3 and RL.6) which mapped back to yWXD6129 (Fig. 3). RL.3 and RL.6 both contain inserts of 1.4 kb in size. Sequence overlap between the two was suggested by the observation that they cross-hybridized to each other. Both detect an X-specific 4.4 kb EcoRI fragment in digests of human genomic DNA and of yWXD6129 (Fig. 3a). Hybridization to partial digests, and sequence overlap with the marker L(6129), showed that both clones map to a 5.3 kb region at the extreme left end of the YAC insert, between the cloning site and a SalI site (Figs. 2 and 3b). This places them between 40 and 80 kb from DXS255 on its centromeric side, and suggests that they are within the microdeletion.

Deletion analysis with isolated cDNAs

RL.3 was used to probe a panel of EcoRI DNA digests from Dent’s disease patients and normal individuals. The 4.4 kb fragment detected by RL.3 in male and female normal controls was found to be completely absent in the affected male with the microdeletion (individual II.3 from family 12/89 in reference 4) confirming that the deletion encompasses at least part of the gene identified here (Fig. 4). No altered bands were seen in two other unrelated male patients with Dent’s disease.

Characterization of cDNA clones

On hybridization to genomic digests of distantly related species (a ‘zoo-blot’), RL.3 and RL.6 show a high degree of conservation, detecting homologous fragments in primates, marsupials, rodents, reptiles and birds (data not shown). RL.3 was used to probe a multiple tissue Northern blot (Fig. 5) and found to detect a mRNA of approximately 9.5 kb, expressed at high levels in kidney and at much lower levels in placenta and skeletal muscle.

Complete sequencing of RL.3 and RL.6 shows that they have a region of overlap of 878 bp. Fickett’s analysis (14) predicts that a putative open reading frame is present at the 5’ end of RL.3 extending from 1 to 780 bp in reading frame +1 and terminating with a TAG (Fig. 6). This would encode a C-terminal region of 260 amino acids. The putative protein is likely to be larger with the initiation codon further 5’ in an, as yet, unisolated part of the mRNA. In addition, sequence overlap was observed between RL.6 and the YAC probe L(6129), indicating that the gene is oriented with the 5’ end towards the telomere (Fig. 2).
The predicted protein sequence of the 780 bp ORF shows significant homology to all members of the most recently identified family of ion channels, the voltage-gated chloride channel (CIC) proteins (15). One of these, the CIC-K1 gene, is expressed highly in the kidney medulla of rats (16). Particularly high homology was found to the human CIC-N4 gene (Fig. 7), which is the most recent family member to be isolated (17). Hydrophobicity plots of the predicted amino acid sequence encoded by the RL.3 ORF identify two putative transmembrane domains which are homologous to domains 11 and 12 of CIC-N4 (Fig. 7).

DISCUSSION

By using a positional cloning strategy (18) we have identified a gene from Xp11.22 whose transcripts are expressed predominantly in kidney and which is deleted, at least in part, in affected members of a pedigree (12/89) segregating Dent’s disease. Analysis with flanking markers has established that the microdeletion must map within a 515 kb interval. Hybridization screening of kidney cDNA libraries with a 185 kb YAC, falling substantially within the deletion, was found to be an effective method for the isolation of sequences corresponding to potential candidate genes for the disorder.

Sequence analysis of 2 kb so far isolated from the transcript has identified an open reading frame of 780 bp, which is likely to be the 3' end of a gene. Further evidence supporting this as the main ORF of the transcript is the observation that its predicted protein sequence has significant similarity to all members of the CIC gene family. Neither the poly-A tail, nor the 5' end of the putative ORF, have been identified within the cDNAs analysed thus far; however, this is unsurprising given that the kidney

![Figure 4. Deletion mapping of RL.3 in patients with Dent’s disease. Lanes: 1, normal male; 2, normal female; 3, male patient II.3 from family 12/89 (4); 4, male patient III.1 from family 2/92 (4); 5, male patient case 2 (3). The 4.4 kb EcoRI fragment detected by RL.3 is absent in the patient from family 12/89 (lane 3) (top). Equal loading of lanes is demonstrated by a control hybridization with DXS7 (bottom).](image)

![Figure 5. Northern blot of poly-A-enriched mRNA from various human tissues probed with RL.3 (top). Subsequent probing with human β-actin revealed that the loading of the skeletal muscle track was significantly higher than in others (bottom). The relative expression of the 9.5 kb transcript detected by RL.3 in this tissue may therefore be even weaker than indicated. Positions of RNA molecular weight markers are indicated.](image)
specific mRNA detected in Northern blots is 9.5 kb. Other members of the CIC family have transcripts of a comparable size; for example the CIC-N4 mRNA is 7.5 kb, which encodes a protein of only 760 amino acids (17). Rescreening of renal cDNA libraries with RL.3 and RL.6 is in progress to isolate clones covering a larger part of the gene.

Sequence comparison indicates that the gene isolated here, which we propose to designate hCIC-K2, is a new addition to the family of voltage-gated chloride channels. These transmembrane proteins play an important role in various cellular functions such as regulation of cell volume, control of excitability and transepithelial transport (15). Following the isolation of the first of these, CIC-0, from Torpedo marmorata (15), sequence homology-based strategies successfully identified three additional genes in the rat: CIC-1, CIC-2 (both predominantly expressed in skeletal muscle) and CIC-K1 (predominantly expressed in kidney). All of these displayed a voltage-gated chloride channel activity in electrophysiological studies using Xenopus oocytes (16,19,20). Most recently, a novel gene called CIC-N4 (expressed in muscle, brain and heart) was identified in the human Xp22.3 region by positional cloning and found to be the most divergent member of the family (17). The amino acid sequences of the two putative transmembrane domains within the RL.3 clone of CIC-K2 are identical to D11 and D12 of CIC-N4 (Fig. 7), but show less homology to the corresponding domains of the other CIC genes, suggesting that CIC-K2/CIC-N4 may form a subset within the gene family.

Previous studies have indicated that a variety of chloride channels exist in kidney, but with the exception of CIC-K1, little is known of their precise nature at the molecular level (16,21). Many functions of the proximal tubule, including uptake of luminal protein, are dependent on the acidification of its endosomal compartment and electrophysiological studies have shown endocytic voltage-gated chloride channels to be crucial for such acidification (21). It is not unreasonable, therefore, to suppose that loss of hCIC-K2 activity may result in abnormalities of proximal tubular functioning, as observed in patients with Dent’s disease. Furthermore, studies of this gene in patients with XRN may help to evaluate the hypothesis that this disease is due to an independent locus on Xp.

Until now, CIC-1 was the only gene of this family which had been implicated in the aetiology of a human disorder. Disruption of CIC-1 is found in mice with autosomal recessive myotonia (22), whilst mutations in the human homologue are associated

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### Figure 6. Complete nucleotide and predicted amino acid sequence of the 1.4 kb cDNA clone RL.3. The initiation codon of the open reading frame lies 5' to this clone in an uncollected part of the transcript. The stop codon is indicated by an @. The two predicted hydrophobic domains are underlined.

### Figure 7. Comparison of protein sequence of hCIC-K2 with other members of the chloride channel family in regions of highest homology. Uppercase letters indicate identity with hCIC-K2. Letters in bold represent amino acids which are identical in all five peptide chains. The putative transmembrane domains D11 and D12 are underlined. CIC-N4, chloride channel encoded by human Xp22.3 (17); CIC-K1, rat kidney chloride channel (16); CIC-1, human muscle chloride channel (23); CIC-0, chloride channel from Torpedo marmorata (15).
with two different types of myotonia (23–25). Given the proposed role of hCIC-K2 as a kidney-specific chloride channel, the observation that it maps within an associated microdeletion of less than 515 kb, and the phenotypic similarity between deleted and non-deleted patients, there is strong evidence supporting this as a candidate gene for Dent's disease.

**METHODOLOGY**

**General procedures**

Conventional gel electrophoresis was in 0.8% (w/v) agarose (Sigma Type I-A)/1× TBE gels at 1.5 V/cm. Gels were transferred to Hybond-N+ (Amerham) by alkaline blotting, and filters neutralized in 0.2 M Tris–Cl (pH 7.5), 2 × SSC. Plasmids were prepared by standard methods (26), and inserts purified by the GeneClean (Bio 101) procedure. Labelling was by random priming with [α-32P]dCTP (27). Hybridization was at 65°C in Church Buffer (28), and washing to a final stringency of 40 mM sodium phosphate, pH 7.2/0.1% SDS, at the same temperature. Filters were exposed to Kodak XAR film with Kodak intensifying screens at 70°C, and stripped by washing in 2 M Tris–Cl, pH 7.5/1 mM EDTA/1% SDS at 65°C.

**PCR screening of YAC libraries with DXS255**

Primers for DXS255 were designed, using sequence of a Parl–BglII fragment neighbouring the VNTR (29). Primer sequences were 5'-GCTGTTGCCCCACGGT-3' and 5'-GGCCCACTGCGATTTGAAA-3'. The expected 472 bp product was amplified from human template using the following conditions: 200 μM dNTPs (Amerham); 10 mM Tris–HCl; 50 mM KCl; 1.5 mM MgCl2; 1.0 μM each primer; Taq polymerase (Boehringer Mannheim). Cycling parameters were as follows: 94°C, 5 min; 94°C, 30 s; 53°C, 30 s; 72°C, 30 s; 5 cycles. Screening PCR pools of the St. Louis YAC library (30) using the above conditions identified clone yWXD6129.

**PCR screening of YAC libraries with L6129**

Primers sequences used were 5'-GACTCTTTGAGGAAGCTACAG-3' and 5'-ATCATTGACTCCAGC-3', to give a 1.39 bp product. Conditions were identical to those used above, but cycling parameters were: 94°C, 5 min; 94°C, 30 s; 50°C, 30 s; 75°C, 18 s; 36 cycles. Screening PCR pools of ICRF YAC library (31) identified clone ICRFy900C191.

**PCR assay for L(F1001)**

Primers sequences used were 5'-TGCTCCTTCTCCTAGGC-3' and 5'-GGTTTGTGCTTTG-3', to give a 1.06 kb product on amplification of human genomic template. Conditions were identical to those used above, but cycling parameters were: 94°C, 5 min; 94°C, 30 s; 52°C, 30 s; 75°C, 18 s; 36 cycles.

**Preparation and analysis of YAC DNA**

Agarose plugs containing YAC DNA were prepared by standard methods (32). YAC DNA was digested with a range of amounts of restriction enzyme (0.1, 0.3, 1, 5 and 15 units) for 1 h in the appropriate buffer. Digests were separated by pulsed field gel electrophoresis in a 'Walther' apparatus (33) using the following parameters: 1.5% (w/v) agarose (Sigma, Type I-A)/1× TAE, 16°C, 3.6 V/cm; 13 s switch time; 31 h run time. Gels were exposed to UV light for 4 min prior to blotting. Blots were probed sequentially with left and right YAC vector arm, and M276 (which recognises DXS255).

**Plasmid end reductase of YACs**

DNA from YACs which had been digested to completion with NdeI was purified by the GeneClean procedure, and ligated under conditions which favour circularization of fragments. Es.coli 'Top 10' cells (In-Virogen) were transformed by electroporation with the circularized DNA, and ampicillin-resistant transformants selected.

**Right end cloning of YACs by Inverse-PCR (34)**

DNA from YAC plucks which had been digested to completion with HaeIII was purified by the GeneClean procedure, and ligated under conditions which favour circularization. The ligated mix was used as a template for inverse PCR using primers specific for the right arm of pYAC4. Primer sequences: 5'-AATTCGACGACGATCTCAAA-3' and 5'-TTCAAGCTATGCGCCGGA-3'. Cycling parameters: 94°C, 5 min; 94°C, 1 min; 55°C, 1 min; 75°C, 2 min; 35 cycles. The product was digested with EcoR1 and HaelIII and cloned into pUC9 using standard techniques.

**Purification of yWXD6129**

The 185 kb YAC was separated from the yeast background by running undigested DNA from twelve concentrated agarose plugs on a pulsed field gel using the following parameters: 1.5% (w/v) agarose (Sigma Type I-A)/0.5× TAE, 16°C, 3.6 V/cm; 13 s switch time; 30 h run time. The gel was visualized under UV, the YAC fragment excised and purified by the GeneClean procedure.

**Screening of cDNA libraries**

A cDNA library from adult renal tissue (Clontech) was used. 4× 10^6 recombinants were plated on four 22×22 cm plates, and two replica plaque lifts on Hybond-N+ (Amerham) were prepared from each plate according to manufacturers instructions. Purified YAC DNA was labelled for 6 h at 37°C with 200 μCi [α-32P]dCTP. Pre-reassociation of probe was carried out in 0.125 M sodium phosphate, pH7.2, containing 1 μg/ml denatured sonicated human placental DNA for 3 h at 65°C, corresponding to a C0.8 value of 180. Filters were prehybridized overnight in Church buffer in the presence of 100 μg/ml sonicated human placental DNA. Hybridization was carried out overnight in fresh buffer lacking placental DNA. Filters were washed as described above, but to a final stringency of 20 mM phosphate. Phage DNA was isolated using Wizard Lambda MiniPreps (Promega). Phage inserts were subcloned into pUC9 using standard techniques.

**Northern analysis**

A multiple tissue Northern blot containing poly-A-enriched RNA was obtained from Clontech. Hybridization and washing was according to manufacturers instructions (Clontech).

**DNA sequencing**

Clones were sequenced on both strands by the dideoxy method (35) with Sequenase version 2.0 (USB), using universal and reverse primers, as well as primers derived from the obtained sequence, dTTP was used to resolve compressions (USB).

**Cell-lines, genomic DNA samples and probes**

Genomic DNA was prepared by standard methods (26) from patients affected with Dent’s disease (3,4) and from the mouse–human X-only hybrid Thyl-X (36). The zoo-blot was made using DNA from the Oxford Genetics Laboratory collection. Human β-actin was obtained from Clontech.

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**REFERENCES**


