Functional characterization of ORCTL2 – an organic cation transporter expressed in the renal proximal tubules

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Abstract Chromosome 11p15.5 harbors a gene or genes involved in Beckwith-Wiedemann syndrome that confer(s) susceptibility to Wilms' tumor, rhabdomyosarcoma, and hepatoblastoma. We have previously identified a transcript at 11p15.5 which encodes a putative membrane transport protein, designated organic cation transporter-like 2 (ORCTL2), that shares homology with tetracycline resistance proteins and bacterial multidrug resistance proteins. In this report, we have investigated the transport properties of ORCTL2 and show that this protein can confer resistance to chloroquine and quinidine when overexpressed in bacteria. Immunohistochemistry analyses performed with anti-ORCTL2 polyclonal antibodies on human renal sections indicate that ORCTL2 is localized on the apical membrane surface of the proximal tubules. These results suggest that ORCTL2 may play a role in the transport of chloroquine and quinidine related compounds in the kidney.

Key words: Beckwith-Wiedemann syndrome; Organic cation transporter; Tetracycline/H^+ antiporter; Organic cation transporter like-2; 11p15.5

1. Introduction

Beckwith-Wiedemann syndrome (BWS) is a congenital overgrowth syndrome, with a birth incidence of 1 in 13700, and characterized by macroglossia, exomphalos, visceromegaly, hemihypertropy, gigantism, and a predisposition to early childhood cancers including Wilms' tumor, rhabdomyosarcoma, adrenocortical carcinoma, and hepatoblastoma [1,2]. The gene(s) responsible for BWS map to 11p15.5, as determined by linkage analysis of autosomal dominant pedigrees [3,4]. The complexity of the genetics of this disease is underscored by the description of distinct regions to which chromosomal breakpoints in BWS individuals have been mapped [5,6], as well as a maternal inheritance associated with familial cases suggesting a role for genomic imprinting. The identification of a number of imprinted genes at 11p15.5 has led to the postulation that disruption of imprinting (e.g. by chromosome translocation) could contribute to aberrant expression of a number of genes from this region [7–9].

In an effort to identify genes at 11p15.5 that may contribute to the etiology of BWS, we and others have reported on the isolation of a transcript which encodes for a putative membrane associated protein, called organic cation transporter-like 2 (ORCTL2) [10]. This gene has also been referred to as BWR1A [11] and IMPT1 [12]. The product of ORCTL2 shows homology to a family of proteins which have been studied as drug efflux pumps in bacteria, such as the multidrug resistance protein of *Bacillus subtilis* (BMR) and the tetracycline (tet) transporter of *Escherichia coli* [10]. The homology of ORCTL2 to bacterial polyspecific cation transporters, as well as its high expression level in the kidney (as well as liver and colon), suggested to us that it may play a role in cation transport in these organs.

Small organic molecules, including glucose, amino acids, and many drugs, are transported at the proximal tubules of the mammalian kidney. Polyspecific organic cation transporters located at the apical and basolateral surfaces of renal epithelial cells mediate kinetically distinct excretion of several endogenous and exogenous compounds [13]. The mechanisms of organic cation transport across the brush border and the basolateral membrane have generally been studied using the model compound tetraethylammonium and these studies have shown that organic cation transport across the apical cell membrane occurs by an electroneutral proton/organic cation exchange. Recently, several of the responsible organic cation transporters have been cloned. A polyspecific transport protein, OCT1 [14], encodes a 1.8 kb mRNA and has the properties of a basolateral membrane organic cation transporter. OCT1 is involved in the transport of organic cations from the blood into epithelial cells, the first step in cation excretion. When expressed in *Xenopus laevis* oocytes, OCT1 mediates the electrogenic (NaCl)-independent and pH-independent uptake of a variety of endogenous cations such as choline, spermine, spermidine and of cationic drugs such as quinidine, quinine, and d-tubocurarine.

Schwienbacher et al. [11] have recently described two mutations associated with this gene – one in a breast cancer cell line predicted to cause premature termination of translation and a missense mutation in a rhabdomyosarcoma cell line, implying a potential role for ORCTL2 in tumor progression. Given the complex genetics of BWS, an understanding of the functional properties of genes from 11p15.5 could help in providing insight into the role played by some of these in contributing to the variety of phenotypes associated with this disorder. In this report, we investigate the functional properties of ORCTL2 and report on its distribution within the adult kidney. Localization of ORCTL2 suggests a role in transport of cation metabolites along the brush border of the epithelial cells of the proximal renal tubules.

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2. Materials and methods

2.1. Materials

Quinidine, chloroquine, rhodamine 6G, tetraphenylarsonium chloride, puromycin, tetracycline and protease inhibitors were all purchased from Sigma. Molecular biology supplies were purchased from indicated manufacturers.

2.2. Recombinant plasmids

The bacterial expression vector pT7-5 ORCTL2 used in bacterial survival assays was generated from pT7-5 LacY, which has been previously described [15]. pT7-5 LacY was digested with *Bam*HI and *Hin*dIII to remove the LacY gene while not disrupting the promoter/operator elements of the lac operon. PCR amplification was performed on ORCTL2 using primers D7-BamHI ATG (5′-CGGGATCCATGCAGGGAGCTCGGACT-3′) and D7-3′H(AS) (5′-CGGGATCCCGTCACCGGACTTTGTCCTT-3′). Because D7-BamHI ATG contains a *Bam*HI site immediately upstream of the ATG codon (underlined in the sequence), this manipulation placed the ORCTL2 ATG codon in the same context and position (relative to the Shine-Dalgarno sequence) as it was for LacY. The PCR product was digested with *Bam*HI and *Pst*I and was ligated to a 3′ truncated ORCTL2 cDNA insert which had been generated by digesting ORCTL2 with *Pst*I and *Hin*dIII. Subsequently, a three way ligation was performed to create pT7-5 ORCTL2, which places the ORCTL2 cDNA under IPTG inducibility.

For transient transfections studies in COS-7 cells, wild-type ORCTL2 and hemagglutinin tagged (HA) ORCTL2 cDNA was cloned into the eukaryotic expression vector, pcDNA3. The plasmid, pcDNA3/ORCTL2, was created by placing the *Bam*HI/*Eco*RI ORCTL2 cDNA (nucleotides −120 to +1333) into *Bam*HI/*Eco*RI digested pcDNA3 using T4 DNA ligase. pcDNA3/(HA)ORCTL2 contains an HA tag introduced between the native ATG codon and the second codon of ORCTL2 and was created by replacing a small portion of the ORCTL2 amino-terminal domain with oligonucleotides designed to incorporate the HA epitope sequences. Oligonucleotides used for these experiments were D7 5′HA (s) (5′-CGGGGTACCCCCCAGGATGTACCCCTACGACGTCCCCGACTACGCCTCCCTGCAGGGAGCT-3′) and D7 5′HA (as): (5′-CCCTGCAGGGAGGCGTAGTCGGGGACGTCGTAGGGGTACATCCTGGGGGGTACCCCGAGCT-3′) which, once annealed, were ligated into a *Sac*I site at position +12 of the ORCTL2 cDNA. The HA tagged cDNA was then digested with *Kpn*I (present at position −5) and *Eco*RI, the insert gel-purified, and ligated into pcDNA3 digested with the same restriction enzymes. pcDNA3/ORCTL2 (HA) contains nucleotides −120 to +1272 of the ORCTL2 cDNA with an HA tag juxtaposed next to the stop codon. The 3′ HA tag was introduced into ORCTL2 by PCR amplification using oligonucleotide primers: D7 3′HA (5′-GGGGTACCCCTCACAGGGAGGCGTAGTCGGGGACGTCGTAGGGGTACCGGACTTTGTCCTTCC-3′) and D7-1 (5′-GTCACCGACAGCATGCTG-3′) which amplified a 240 bp fragment of DNA between nucleotides +1042 and +1272 of the ORCTL2 cDNA, introducing an HA tag and a *Bam*HI site at the 3′ end of ORCTL2. This PCR fragment was then digested with *Sph*I (located at nucleotide +1178 of ORCTL2) and *Bam*HI and ligated into pcDNA3/ORCTL2 which had been digested with the same enzymes. All PCR amplification products were sequenced by the chain termination method to ensure the absence of unwanted mutations. The plasmid, pT7-5 Mdr1, has been previously described and contains the murine multidrug resistance protein (P-glycoprotein) under control of the lac operon [16].

2.3. Generation of ORCTL2-specific antibodies

ORCTL2-specific antibodies were generated by injecting New Zealand White rabbits with a 10 amino acid peptide, NH_3-LLVLWRKPMPQRKDKVR-COOH, corresponding to the last 10 carboxy-terminal amino acids of ORCTL2. Initial injections were performed with 350 μg of peptide in Freund's complete adjuvant and subsequent boosts were performed with 200 μg of peptide in Freund's incomplete adjuvant every 3 weeks. Peptide-specific antibodies were purified from the antisera using an affinity matrix generated by covalently linking the immunogen on an azlactone activated support (UltraLink Immobilized Carboxy, Pierce). Following incubation of serum with the coupled peptide, the column was washed, and peptide-specific antibodies were eluted using 4 M urea and dialyzed overnight against PBS.

2.4. Bacterial resistance assay

The bacterial resistance assay was performed using UTL2, a UV irradiated mutant of *E. coli* UT5600 (OmpT$^-$) that is sensitive to many cytotoxic drugs [16]. Resistance of UTL2 *E. coli* cells harboring pT7-5 LacY, pT7-5 Mdr1 of pT7-5 OCTR against various compounds was assayed in liquid medium. Cells harboring the appropriate plasmids were grown at 30°C in Luria broth (LB) supplemented with 100 μg ampicillin per ml. Overnight cultures were diluted 1:50 and grown to an $OD_{600} = 0.6$. Cells were then diluted and aliquoted (50 μl) into 96 well microplates containing 50 μl of the indicated concentrations of drugs and 0.1 mM IPTG. Plates were incubated in a 30°C shaker, and the OD_{600} was measured every 4 h for 16–20 h using a microplate reader. In experiments with chloroquine and quinidine, LB medium was supplemented with 60 mM Bis-Tris-propane to maintain the pH at 7.4.

2.5. Cell culture, transfections, and membrane preparations

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco BRL), penicillin (50 units/ml) and streptomycin (50 μg/ml) (Gibco BRL). For transient transfections, cells were seeded at a density of $2–5 \times 10^5$ cells per 100 mm plate one day prior to transfection. Cells were transfected using the calcium phosphate precipitation method [17]. For individual transfections, 12.5 μg of plasmid DNA was used and cells were harvested 48 h later.

After transient transfections, cells were harvested with a rubber policeman in cold PBS (phosphate buffered saline) containing 20 mM sodium citrate but lacking magnesium and calcium. Cells were collected in 15 ml conical tubes (Falcon) by centrifugation at $\sim 800 \times g$ for 5 min, resuspended in Tris-Mg buffer (10 mM Tris [pH 7.0], 5 mM $MgCl_2$) supplemented with the protease inhibitors leupeptin (1 μg/ml), aprotinin (2 μg/ml), and antipain (1 μg/ml). Cells were then subjected to Dounce (type A) homogenization and nuclei were eliminated by centrifugation in a clinical centrifuge at $\sim 1000 \times g$ for 5 min. Cellular membranes from the supernatant were collected by ultracentrifugation in an SW40 rotor (Beckman) for 30 min at 20 000 rpm. The membrane pellet was resuspended in TNE buffer (100 mM NaCl, 10 mM Tris [pH 7.0], 10 mM EDTA) containing 30% glycerol and protease inhibitors. These preparations were stored at −80°C until ready to use.

2.6. Immunofluorescence of renal sections using anti-ORCTL2 antibodies

Tissue from fetal kidneys (age 22 weeks) was stored at −70°C without further fixation. Sections (7–10 μm thickness) from tissues were cut with a cryostat, transferred onto glass slides, and fixed overnight at −20°C in methanol with 0.5% EGTA. Endogenous tissue was preblocked with PBS/methanol/H_2O_2 (59.5%/40%/0.5%) for 20 min at room temperature. After two washing steps in H_2O and one rinse in PBS, the section was buffered for 30 min in PBS/normal goat serum (10:1) at room temperature in a humid chamber. After this, the affinity purified antibody against ORCTL2 was applied to different sections in different dilutions ranging from 1:10 to 1:1000 and incubated in a humidified chamber overnight at 4°C. The tissue sections were washed three times with PBS and then overlaid with biotinylated anti-rabbit antibody diluted in PBS (1:100) for 30 min. This incubation was again followed by washing sections in PBS, after which the peroxidase reaction was performed for 20 min using the ABC Immunostain solution and visualized with a diaminobenzidine tetrahydrochloride solution (1 mg/ml) containing 0.01% H_2O_2 for 30 s to 4 min (Santa Cruz ABC Immunostain Systems). The staining reaction was stopped by immersing slides into distilled water, followed by a short counterstaining in hematoxylin. Tissue sections were dehydrated by rinsing in a series of washes with increasing ethanol concentrations (70–100%), washing in xylene, and mounting with Permount medium.

3. Results and discussion

Analysis of genes within a 1 Mbp PAC contig at human chromosome 11 band p15.5 identified an expressed sequence tag (EST) showing homology to organic cation transporters

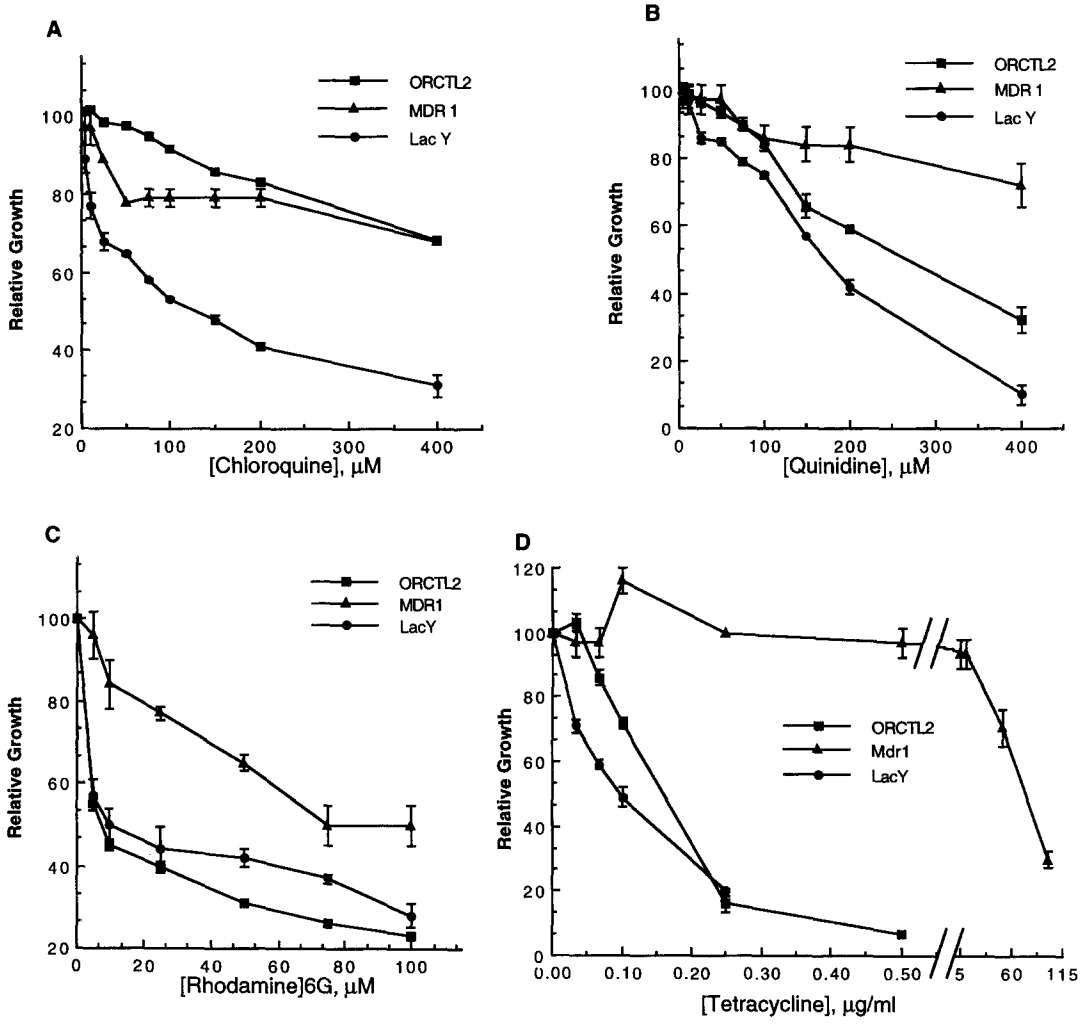

Fig. 1. Resistance of *E. coli* UTL-2 to chloroquine (A), quinidine (B), rhodamine 6G (C) and tetracycline (D). *E. coli* UTL-2 harboring either pT7-5 LacY, pT7-5 ORCTL2, or pT7-5 Mdr1 was grown in the presence of increasing concentrations of drug and the growth rates were monitored by measuring the OD_{600}, as indicated in Section 2. Relative growth is plotted as a function of drug concentration. Experiments were done in duplicate with three different bacterial colonies each time.

[10]. The gene corresponding to this EST was subsequently named ORCTL2 (organic cation transporter-like 2), BWR1A [11], or IMPT1 [13]. ORCTL2 shows highest homology with the *E. coli tetA* class E tetracycline resistance protein and the multidrug resistance protein 2 of *B. subtilis* [10], consistent with the idea that ORCTL2 is a membrane pump involved in cellular detoxification.

3.1. Transport properties of ORCTL2

Tetracycline resistance in bacteria is conferred by three biochemically distinct mechanisms: (i) energy-dependent active efflux of tetracycline, (ii) ribosome protection, and (iii) tetracycline modification. Tetracycline resistance genes have been grouped into 12 classes based on their failure to cross-hybridize under stringent conditions [18,19]. In Gram-negative bacteria, the outer membrane exhibits a strong permeability barrier to many lipophilic compounds and the primary mechanism of tetracycline resistance is based on active efflux of the drug [20]. These tetracycline extrusion molecules, such as tetA, belong to the large major facilitator family of membrane translocases [21,22] and mediate resistance by acting as a tetracycline/H^+ antiporters, exporting a tetracycline-divalent cation complex in exchange for a proton.

To elucidate a functional role of ORCTL2 as a transport protein, we employed a recently described bacterial-based growth assay [16]. Generally, wild-type *E. coli* cells are insensitive to most cancer and organic cation-like cytotoxic compounds because of the outer membrane permeability barrier. However, *E. coli* UTL2, a UV irradiation derived mutant of UT5600 (ompT$^-$), have a permeable outer membrane and are sensitive to the growth inhibitory effects of rhodamine 6G, daunomycin, puromycin, quinidine, and chloroquine [16]. The drug sensitivity profile of UTL2, transfected with pT7-5 LacY, a prokaryotic expression vector driving synthesis of LacY, is unchanged (Fig. 1). Chloroquine, quinidine, and rhodamine 6G are inhibitory to growth of this strain (Fig. 1A–C). In addition, this strain is also sensitive to tetracycline (Fig. 1D). As previously reported [16], when the murine P-glycoprotein gene is expressed in UTL2, the bacteria are protected against the cytotoxic effects of chloroquine, quinidine, and rhodamine 6G (Fig. 1A–C). We also find that P-glycoprotein can provide resistance to tetracycline as well (Fig.

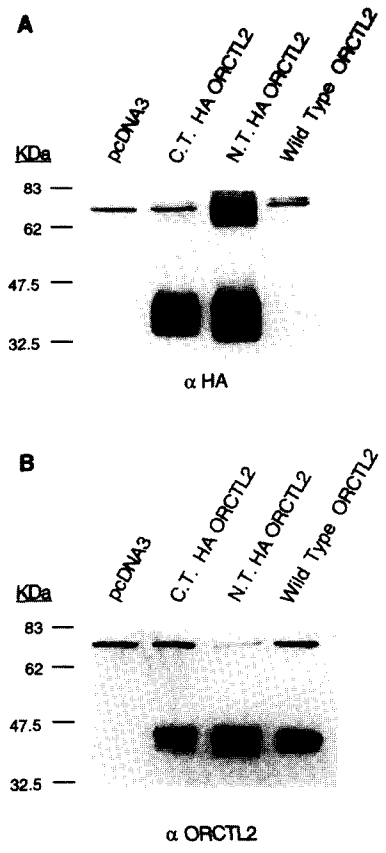

Fig. 2. Western blot analysis of ORCTL2 and HA (hemagglutinin) tagged ORCTL2 in membrane preparations of transfected COS-7 cells. Membrane fractions were prepared from carboxy-terminal HA tagged ORCTL2 (C.T.-HA ORCTL2), amino-terminal HA tagged (N.T.-HA ORCTL2), wild-type ORCTL2, and pcDNA3 alone transfected COS-7 cells as described in Section 2. 40 μg of protein was fractionated on a 10% SDS-PAGE. Proteins were transferred to Immobilon-P (Millipore) and probed with (A) 12CA5, an anti-HA epitope monoclonal antibody or (B) affinity purified anti-ORCTL2 polyclonal antibodies. The antibody-antigen complex was detected using the ECL chemiluminescense (NEN) detection system.

1D). When ORCTL2 was assayed in this heterologous expression system, robust resistance to chloroquine was observed (Fig. 1A) and weak resistance to quinidine was noticed (Fig. 1B), but no protective effect against either rhodamine 6G (Fig. 1C) or tetracycline (Fig. 1D) was observed. Also, ORCTL2 could not protect against either tetraphenylarsonium (TPA) or puromycin (data not shown), two other drugs which mdr1 has been shown to confer resistance to [16].

We attribute the ability of ORCTL2 to confer resistance to chloroquine (and partially to quinidine) as being due to the predicted transport properties of this molecule. It is somewhat surprising that ORCTL2 cannot confer resistance to tetracycline, considering the homology of ORCTL2 and the *E. coli* tetracycline resistance proteins. However, this may reflect the fact that primary protein sequence homology cannot be accurately used to define transport specificity. A case in point is the sequence comparison of QacA/B, two very similar proteins associated with resistance to antiseptics and disinfectants; Mmr, a resistance gene for the antibiotic methylenomycin A; and Tet(K) and Tet(L), the tetracycline efflux protein of Gram-negative bacteria. In this case, Tet(L) and Tet(K) show a lower degree of homology to the tetracycline resistance proteins of Gram-negative bacteria than do QacA/B and Mmr, yet they transport tetracycline and QacA/B and Mmr do not [23]. Our UTL2 growth experiments are suggestive of a transport function for ORCTL2 in handling chloroquine and/or quinidine related compounds.

3.2. Localization of ORCTL2 in the kidney

To determine the tissue and cellular distribution of ORCTL2, we raised polyclonal antibodies to a peptide derived from the carboxy-terminal sequence of ORCTL2. The ability of our antibodies to specifically recognize ORCTL2 was assessed by Western blot analysis (Fig. 2). Transfections in COS-7 cells were performed with either the empty expression cassette, pcDNA3, or pcDNA3 driving the synthesis of: (i) carboxy-terminal (C.T.) HA tagged ORCTL2, (ii) amino-terminal (N.T.) HA tagged ORCTL2, or (iii) wild-type ORCTL2. Membrane preparations from transfected cells were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and processed for Western blotting. Probing with anti-HA antibodies demonstrated that extracts from both N.T.-HA tagged and C.T.-HA tagged ORCTL2 transfected cells contained a novel protein migrating with a molecular mass of ~40 kDa (Fig. 2A). Probing with our anti-peptide antibody detected a protein of similar molecular mass in N.T.-HA tagged and C.T.-HA tagged ORCTL2 transfected cells, as well as in cells transfected with pcDNA/ORCTL2 (Fig. 2B). In these experiments, ORCTL2 migrates with an apparent molecular mass of ~40 kDa, although the predicted molecular mass is 48.8 kDa [10]. The reason(s) for this discrepancy is not understood, but it is clear that since the amino-terminal and carboxy-terminal HA tagged protein are synthesized, this difference in size is not due to alternative translation initiation at an internal ATG codon.

Immunohistochemical analysis of ORCTL2 was performed on sections of human fetal kidneys. Background staining was minimal when the specific anti-ORCTL2 antibody was omitted from the section (Fig. 3A). A survey of the renal peripheral and central structures clearly shows ORCTL2 staining at some glomeruli and the proximal parts of the tubules (Fig. 3B). The more differentiated glomeruli (found deeper in the cortex) display a lower degree of staining than their less differentiated counterparts, possibly reflecting a developmentally regulated phenomenon. ORCTL2 does not appear to be significantly expressed in the distal tubules (Fig. 3B). A higher magnification reveals that ORCTL2 is also present at the transition between the glomerulus and the tubules (Fig. 3C,E). This expression site suggests that ORCTL2 may be involved in transporting compounds into or from the primary filtrate. These experiments also revealed that ORCTL2 is localized to the apical membrane surface of the epithelial cells of the proximal renal tubules (Fig. 3D).

The renal proximal tubules are a major site of excretion of many xenobiotics and endogenous compounds, either directly or after metabolic transformation. These compound are transported mainly by organic cation or organic anion transport systems and recently, several such transporters have been cloned and shown to be expressed in the kidney. One such protein, considered to represent a new class of cation-specific transporters, is OCT1 [14]. OCT1 and a related protein OCT2 are both expressed in the proximal tubules of the kidney and can transport two prototypical organic cations, tetraethylammonium (TEA) and *N*-methylnicotinamide (NMN). Another OCT1-related transporter, OAT-1, is expressed almost exclu-

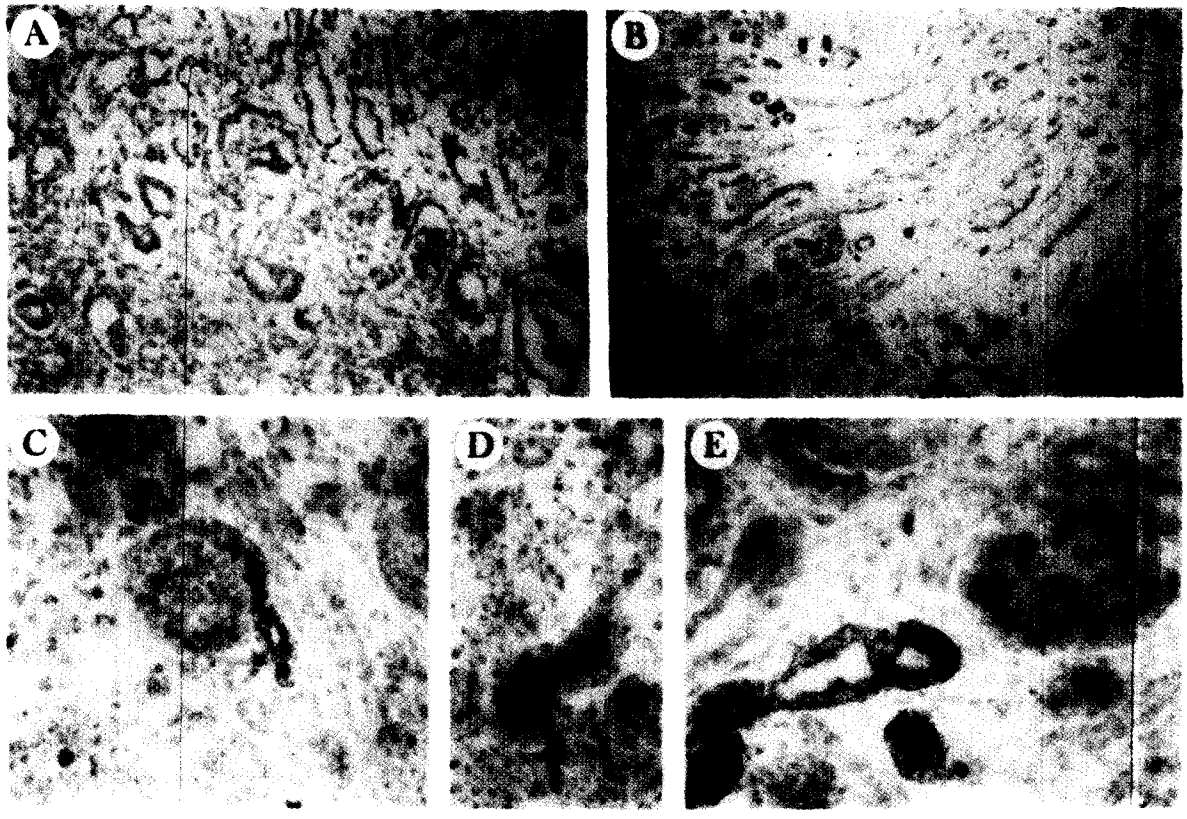

Fig. 3. Immunohistochemical detection of ORCTL2 in human fetal kidney sections (22 weeks old) using affinity purified anti-ORCTL2 polyclonal antibodies. A: Negative control staining (no anti-ORCTL2 antibody added). B: Survey of the kidney showing staining of ORCTL2 in peripheral and central renal structures. C: Staining of ORCTL2 at the transition of the glomerulus into the tubules. D: Staining of ORCTL2 to the apical membrane surface of the proximal tubules. E: Staining of ORCTL2 at the glomerular loop and within the proximal tubules.

sively in the kidney, and has been shown to transport *para*-aminohippurate in a Na^+-independent manner, and may be an organic anion/decarboxylate exchanger at the basolateral membrane of the proximal tubules [24]. There are a number of organic anion transporters that have been cloned and, given that some are also expressed in the kidney, they may have a role in renal transport. These include MRP1 (multidrug resistance-associated protein 1) and its homologues MRP2, MRP3, MRP4, and MRP5 [25]. MRP2, or cMOAT, is expressed in the apical membrane surface of the renal proximal tubules and is capable of conferring the multidrug resistance phenotype to some cell lines [14,25]. The multidrug-specific P-glycoprotein (MDR1) is also expressed in the apical membrane surface of the renal proximal tubules [26].

ORCTL2 represents a new class of mammalian transporters located at the renal proximal tubules, that may transport organic cations based on a proton efflux antiport mechanism similar to the mechanism used by the tetracycline efflux proteins. Given that this gene resides in the Beckwith-Wiedemann syndrome critical region (BWSCR) at 11p15.5, it is interesting to speculate on a possible role for ORCTL2 in the etiology of this disease and of associated malignancies. Schwienbacher et al. [11] have identified two cell lines with mutations in ORCTL2 – a breast cancer cell line (BT549) with an insertion causing premature termination and a rhabdomyosarcoma cell line (TE125-T) with a missense mutation at one ORCTL2 (converting a His residue to an Arg) allele. Clearly additional work is needed to imply a significant role for ORCTL2 in the progression of cancer but should one of the compounds transported by ORCTL2 prove to be a genotoxic agent, then one could envisage a model whereby inactivating ORCTL2 mutations lead to increased intracellular concentrations of the genotoxic agent, resulting in increased frequency of genetic lesions.

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