

At Physiological Expression Levels the Kidd Blood Group/Urea Transporter Protein Is Not a Water Channel*

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The Kidd (JK) blood group locus encodes a urea transporter that is expressed on human red cells and on endothelial cells of the vasa recta in the kidney. Here, we report the identification in human erythroblasts of a novel cDNA, designated HUT11A, which encodes a protein identical to the previously reported erythroid HUT11 urea transporter, except for a Lys⁴⁴ → Glu substitution and a Val-Gly dipeptide deletion after proline 227, which leads to a polypeptide of 389 residues versus 391 in HUT11. Genomic typing by polymerase chain reaction and transcript analysis by ribonuclease protection assay demonstrated that HUT11A encodes the true Kidd blood group/urea transporter protein, which carries only 2 Val-Gly motifs. Upon expression at high levels in *Xenopus* oocytes, the physiological Kidd/urea transporter HUT11A conferred a rapid transfer of urea (which was insensitive to *p*-chloromercuribenzenesulfonate or phloretin), a high water permeability, and a selective uptake of small solutes including amides and diols, but not glycerol and meso-erythritol. However, at plasma membrane expression levels close to the level observed in the red cell membrane, HUT11A-mediated water transport and small solutes uptake were absent and the urea transport was poorly inhibited by *p*-chloromercuribenzenesulfonate, but strongly inhibited by phloretin. These findings show that, at physiological expression levels, the HUT11A transporter confers urea permeability but not water permeability, and that the observed water permeability is a feature of the red cell urea transporter when expressed at unphysiological high levels.

facilitated urea transporters have been molecularly characterized in different animal species: (i) transporters encoded by the UT-A gene, only present in the kidney, and (ii) more ubiquitous transporters encoded by the UT-B gene, present in red cells, kidney, and brain (3–5).

The first human erythroid urea transporter (HUT11) was identified by homology cloning and was later shown to be encoded by the Kidd (*JK*)¹ blood group locus (6, 7). The HUT11 cDNA encodes a membrane glycoprotein of 391 amino acids, which facilitates urea transport. Expression studies in *Xenopus* oocytes have shown that HUT11 urea transport was inhibitable by phloretin and *para*-chloromercuribenzenesulfonate (pC-MBS) (6), as expected for the transporter of human erythrocytes (8). Immunohistochemical and *in situ* hybridization studies have shown that HUT11 is also expressed on endothelial cells of the vasa recta in the inner and outer medulla of the kidney, but not on the epithelial cells of the renal tubules, interstitial cells, and glomerular cells (9). This distribution of expression of HUT11 fully accounted for studies in which a physiological urea transport has been described (10), as well as for the model of experimental hydronephrotic rat kidneys showing a strong staining of the urea transporter on preserved intact vasculature despite a complete loss of the tubular epithelium (11). These findings suggested that, in the renal circulation, the Kidd/urea transporter is involved in countercurrent exchange between the ascending and descending vasa recta to prevent loss of urea from the medulla and to enhance the cortico-papillary osmolality gradient, which is critical in the urinary concentrating mechanism (12).

Recently, we have reported that the *JK* gene, which encodes the human urea transporter, is composed of 11 exons extending over 30 kilobase pairs of DNA (13), and we have identified the molecular basis of the *JK*^{*A}/*JK*^{*B} polymorphisms (14). We also found that different splice site mutations in two unrelated *Jk*_{null} individuals provided a rational explanation for the lack of Kidd/urea transporter protein at the red cell surface (13). Thus in *Jk*_{null} individuals, the absence of Kidd/urea transporter protein on red cells and probably on endothelial cells of the vasa recta should alter the urea recycling mechanism, thus explaining the reduced capacity to concentrate urine of these individuals (15). No erythrocyte hemolysis has been reported in *Jk*_{null} individuals who do not suffer from a clinical syndrome except for the urine concentrating defect.

Interestingly, another urea transporter has been characterized, which is only expressed in human kidney (16, 17). This

In mammals, urea is the chief end product of nitrogen catabolism and is produced in the liver by the urea cycle during the conversion of arginine to ornithine. Additionally, urea is a key component in the urinary concentrating mechanism, in which it is essential for renal water retention and prevention of dehydration. In this later process, urea transporters in red cells and the kidney have been shown to play a pivotal role (reviewed in Refs. 1 and 2). In the last 5 years, two types of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y19039.

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¹ The abbreviations used are: *JK*, Kidd locus; P-face, protoplasmic face of the membrane; IMP, intramembrane particle; RT, reverse transcription; PCR, polymerase chain reaction; pCMB, *para*-chloromercuribenzenesulfonate; nt, nucleotide(s); bp, base pair(s).

transporter, which is called HUT2 (hUT-A2), is 62% identical to HUT11 (hUT-B2). Both human urea transporters are encoded by genes localized on chromosome 18q12, suggesting that they evolved from duplication of a common ancestor.

Here, we report the isolation of a cDNA clone encoding a polypeptide called HUT11A, which is slightly different from HUT11. Genomic and transcript analysis demonstrated that HUT11A, and not HUT11, is the physiological product of the Kidd blood group locus. Additionally, functional studies in *Xenopus* oocytes showed that HUT11A is only a urea transporter at physiological expression levels, but a water and small solute transport activity at high, unphysiological, expression levels.

MATERIALS AND METHODS

Blood Samples and Reagents—Blood samples from individuals of common Jk phenotypes were collected from anticoagulated blood and used for total reticulocyte RNA and leukocyte genomic DNA extraction. Restriction endonucleases and modifying enzymes were from New England Biolabs (Hertfordshire, United Kingdom (UK)). Radiolabeled nucleotides and [¹⁴C]urea (1.96 GBq/mmol) were from Amersham Pharmacia Biotech (Bucks, UK), and [³H]raffinose (188.7 GBq/mmol) was from NEN Life Science Products. The Expand High Fidelity PCR system from Roche Molecular Biochemicals (Mannheim, Germany) was used for PCR amplification. Nucleotide sequences were determined on both strands by the dideoxy chain termination method (Sanger) with ThermoSequenase fluorescently labeled primer cycle sequencing kit from Amersham Pharmacia Biotech using 5'-(Cy5) primers (Genset, Paris, France).

Reverse Transcription-PCR of the Jk cDNA and cDNA Constructs—Full-length cDNA encoding the human Kidd blood group/urea transporter protein was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using total reticulocyte RNAs extracted by the acid-phenol-ammonium method (18) from blood samples using a sense primer (position -21 to -1) and an antisense primer (position 1234-1211) as described previously (11). AQP-1 cDNA (accession no. L07268) was PCR-amplified from a λgt11 human bone marrow cDNA library (CLONTECH) using sense (position -15 to -1) and antisense (position 829 to 849) primers. All cDNAs were subcloned into the EcoRV-digested pT7TS plasmid (kindly provided by P. Krieg, Institute for Cell and Molecular Biology, Austin, TX) and sequenced on both strands, using an automated Alf-Express sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). For primer designation, nucleotide +1 was taken as the first nucleotide of the HUT11 initiation codon (5).

PCR Genomic Typing of the Sequence Encoding the Val-Gly Repeats—To determine whether the genomic DNA encoded for a transporter with 2 or 3 Val-Gly dipeptide repeats, a hemi-nested PCR amplification was carried out under stringent conditions (94 °C for 30 s, 62 °C for 30 s, 72 °C for 10 s, for 30 cycles) using 200 ng of leukocyte DNA from 126 independent donors. The first amplification was done between SP-A (sense primer, 5'-gcagTTGTTGAAATCTATACCAG-3', exonic position 664-682) and AS-B (antisense primer, position 797-774) and the second amplification between the primers SP-A and AS-C (antisense primer, position 728-705), under the same experimental conditions. The PCR products were digested with 5 units of BglIII restriction enzyme and electrophoresed on a 15% (w/v) polyacrylamide gel stained with ethidium bromide.

Ribonuclease Protection Assays—Using a described experimental strategy (19), an antisense RNA probe (ASP-1) encompassing 406 nucleotides between positions 426 and 831 of the HUT11 cDNA was constructed, which encodes 3 Val-Gly dipeptide repeats. For this, the corresponding cDNA fragment was amplified between primers SP (sense primer, position 426-447) and AS (antisense primer, position 831-811) and cloned into the pCR2.1 vector (Invitrogen, SA). From HindIII-linearized recombinant plasmid, the antisense-RNA probe ASP-1 was *in vitro* synthesized using the Riboprobe Core System from Promega (Madison, WI) in the presence of [^α-³²P]UTP (800 Ci/mmol, NEN Life Science Products). After purification on a 5% (w/v) acrylamide, 8 M urea gel, the ASP-1 probe (2.0 × 10⁵ cpm) was hybridized overnight at 50 °C with 25 ng of *in vitro* synthesized sense transcripts (cRNAs) encoding the 2Val-Gly or 3Val-Gly motifs (mCAP mRNA capping kit; Stratagene, La Jolla, CA) as controls, 2 μg of human bone marrow poly(A⁺) RNAs (CLONTECH), and 20 μg of total reticulocyte RNAs from two unrelated individuals, and then digested with a RNase A/T1 mixture, according to the manufacturer's instructions (RPAII; Ambion, Austin, TX). Subsequently, the protected RNA fragments were separated on a 5% (w/v) denaturing polyacrylamide gel (8 M urea) and

exposed to a Biomax MS film with intensifying screens at -80 °C.

Plasmid Preparation and Oocyte Expression—The pT7TS-cDNA constructs were linearized with *Sma*I restriction enzyme, and capped sense RNAs were synthesized using T7 RNA polymerase from the mCAP mRNA capping kit (Stratagene). Expression studies were carried out by microinjection of cRNAs in collagenase-treated *Xenopus laevis* oocytes as described previously (20). Briefly, stage V and VI oocytes from mature female *X. laevis* (CNRS, Nantes, France) were removed, defolliculated with 1.4 units/ml collagenase type A (Roche Molecular Biochemicals) in OR2 solution (82.5 mM NaCl, 2 mM KCl, 5 mM Hepes, 1 mM MgCl₂, pH 7.5), and microinjected with 50 nl of water or cRNA solution (0.05-40 ng/oocyte in 50 nl). The microinjected oocytes were then kept at 18 °C for 2 or 3 days in Barth solution (200 mosM) containing 50 μg/ml Geneticin with daily changes of Barth solution until functional tests.

Immunocytochemistry—Three days after the microinjection, groups of 3-6 oocytes without chorionic membrane were embedded in paraffin as described (21). Sections (7 μm thick) were stained overnight at 4 °C with 10 μg/ml affinity-purified anti-N-terminal (residues 8-22) antibodies of the Kidd/urea transporter protein, described and used previously (7, 22), and visualized with fluorescein-conjugated goat anti-rabbit IgG (1:100 dilution) for 1 h at room temperature (23).

Oocyte Flux Measurements—After injection of 40 ng cRNA/oocyte, urea transport activity was measured by [¹⁴C]urea uptake as described previously (6), or under conditions as indicated in the text. In all these experiments, the Barth incubation solution contained 8 μCi/ml [¹⁴C]urea (145 μM) and 5 μCi/ml [³H]raffinose as a control of oocyte plasma membrane integrity. Incubation time was 90 s, except for time-course experiments where the incubation time varied from 0 to 60 min. After washing and solubilization, the samples were subjected to liquid scintillation in a counter, and urea permeability (*P*_{urea}) was calculated from the oocyte-associated amount of [¹⁴C]urea at each time point, corrected for the optically determined oocyte surface area.

Oocyte water permeability (*P*_w) was measured by a swelling assay 3 days after the injection (24). Oocyte swelling was performed at 18 °C after the transfer from Barth solution (200 mosM) to 40 mosM at *t* = 0. Permeability measurements were made by a microscopy technique using a Nikon Eclipse TE300 microscope (Nikon, Paris, France) (2× objective) coupled to a Biocom computer system of image integration (Biocom, Les Ulis, France). The osmotic water permeability *P*_f (cm/s) was calculated from the initial osmotic cell volume increase between *t* = 0 and *t* = 90 s by the relation $P_f = V_o d(V/V_o) / dt / [S v_w (\Delta osm)]$, where *S* is the oocyte surface area and *v*_w the molar volume of water (18 cm³/mol) (25). In order to determine the influence of small solutes on the water movement across injected oocyte plasma membranes, oocytes were placed at *t* = 0 in 40 mosM Barth solution adjusted to 200 mosM with the tested solute including formamide, acetamide, propionamide, ethylene glycol, propylene glycol, glycerol, or meso-erythritol, as described previously (17). Oocyte swelling was performed for 240 s. Osmolarity was checked with a Roebbling osmometer just before the experiments.

In water or urea transport inhibition experiments, oocytes were incubated in 0.5 mM pCMBS (Sigma), 0.5 mM phloretin (Sigma), or 0.3 mM HgCl₂ (Merck, Darmstadt, Germany) for 20, 10, and 5 min, respectively, before and during the assay at 18 °C.

Electron Microscopy—The same batches used to measure the urea permeability were prepared to determine the particle density in the P-face plasma membrane of oocytes. Before fixation, the oocytes were rapidly emptied of their cytoplasm by aspiration with a pipette (diameter >100 μm). H₂O- and cRNA-injected oocytes were fixed in 2.5% glutaraldehyde in Barth solution for 2 or 3 h at 18 °C, then washed in Barth solution. Emptied oocytes were incubated in Barth solution supplemented with 30% glycerol for 1 h at room temperature and placed between two copper sample holders and then frozen in melting freon. Samples were fractured in a Balzers 300 apparatus at -150 °C under 10⁻⁷ torr vacuum. Fractured surfaces were coated with platinum at 45 °C and carbon at 90 °C under the conditions described by the manufacturer. Replicas were cleaned in bleach, washed in distilled water, and observed in a Philips EM400 microscope at 80 kV. Representative series of images of P-face fractures were enlarged at 95,000× final magnification in order to determine the number of intramembrane particles (IMP) in known areas as described by Zampighi *et al.* (26). Some images were amplified by 800,000 to estimate the size of the particles according to Eskandary *et al.* (27).

RESULTS

Identification of the Kidd/Urea Transporter—Using two primers located in the 5'- and 3'-untranslated regions of the

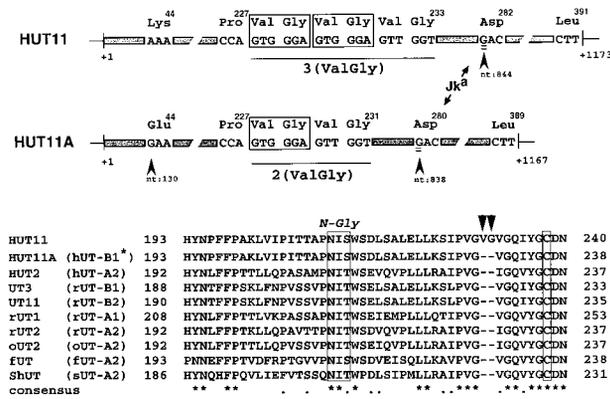


FIG. 1. Sequence comparison of urea transporters. *Top*, schematic representation of HUT11 (Lys/3Val-Gly) and HUT11A (Glu/2Val-Gly) coding sequences (solid bar), which differ by a single base substitution, A130G (vertical arrowhead) changing Lys to Glu at position 44, and by a deletion of one 5'-GTGGGA-3' hexanucleotide (boxed), resulting in the absence of one dipeptide Val-Gly after proline 227. The dipeptide deletion leads to a polypeptide chain of 389 residues in HUT11A, versus 391 in HUT11. The G nucleotide typical of the JK^*A allele is double underlined. *Bottom*, partial multiple alignment (CLUSTAL W program; Ref. 28) of urea transporters currently characterized, including those described recently (29, 30). The asterisk (*) indicates a possible designation of urea transporters in a recently proposed nomenclature (3). Accordingly, hUT-B1 would refer to the physiological transporter HUT11A (this study) and not to HUT11 (as previously thought). Protein sequences are in the one-letter code. The conserved cysteine and potential N-glycosylation sites are boxed. Conserved residues and conservative types are indicated as asterisks and as dots. The coding sequence of HUT11A has been deposited in the EMBL data base under the accession number Y19039.

previously reported cDNA clone HUT11 (11) in a RT-PCR reaction, a new cDNA clone called HUT11A was isolated from human reticulocyte RNAs. Comparison of HUT11 and HUT11A cDNA sequences, showed that both clones derived from a JK^*A allele (G838) but differed in two ways: (i) an A130G transition resulting in a Lys⁴⁴ → Glu substitution, and (ii) a hexanucleotide (GTG GGA) deletion in HUT11A that leads to a Val-Gly dipeptide deletion after the Pro-227 (Fig. 1). Because of this deletion, HUT11A encoded a polypeptide chain of 389 amino acids versus 391 for HUT11. Sequence alignments of several urea transporters indicated that only HUT11 contained an additional Val-Gly dipeptide (Fig. 1), which is located within the third external loop and is in close proximity of the N-glycosylation site (Asn-211).

Since the initially reported HUT11 clone was derived from a human bone marrow cDNA library (CLONTECH, catalog HL 1058b, lot 1911), constructed from RNAs isolated from a single adult individual, we developed a hemi-nested PCR assay on genomic DNA to determine whether the repeat of 2 or 3 Val-Gly motifs is a common polymorphism in the human population. Accordingly, a hemi-nested PCR was carried out on the genomic DNA from 126 unrelated blood donors of different Jk phenotypes to amplify a 63/69-bp fragment encompassing the sequence encoding the repeated dipeptides, and the PCR product was digested by *Bgl*II. Analysis on a 15% polyacrylamide gel revealed only the 36-bp fragment, which encodes the variant with two Val-Gly dipeptide repeats (Fig. 2A). The 42-bp fragment encoding a 3 Val-Gly repeat was obtained only in the HUT11 cDNA control. These data show that the presence of 2 or 3 Val-Gly motifs is not allelic and that the *JK* locus only encodes 2 Val-Gly motifs. Allele-specific PCR analysis also indicated that the Lys⁴⁴ → Glu polymorphism was not allelic to the *JK* locus (data not shown).

To further confirm these results, Kidd/urea transporter transcripts (bone marrow poly(A⁺) RNA and two total RNA reticulocyte preparations) were analyzed by ribonuclease protection

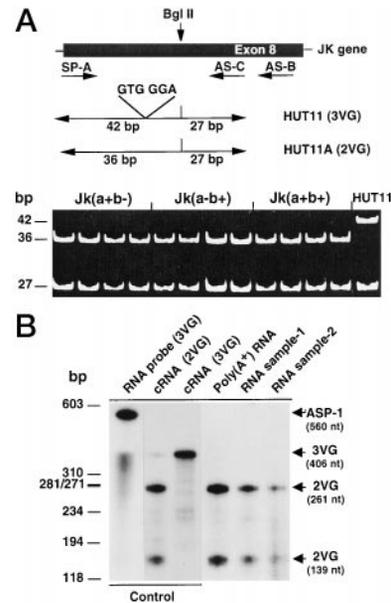


FIG. 2. Analysis of genomic DNA and transcripts encoding Val-Gly repeats. *A*, DNA genotyping by PCR-RFLP. The 63/69-bp fragment in exon 8, encompassing the region encoding the repeated Val-Gly (VG) dipeptide, was amplified by hemi-nested PCR using SP-A, AS-B, and AS-C primers from 126 unrelated individuals of common Jk phenotypes and the HUT11 cDNA (encoding 3 Val-Gly) as control. The final products were digested by *Bgl*II, analyzed on 15% polyacrylamide gel, and stained with ethidium bromide. The results from four typical unrelated individuals of each of the three common phenotypes (Jk(a+b-), Jk(a-b+) and Jk(a+b+)) are shown. Fragment sizes (bp) are given on the left. *B*, transcript analysis by ribonuclease protection assay. Using the antisense RNA probe ASP-1 (encoding a 3 Val-Gly repeat), control sense cRNA (synthesized *in vitro*) encoding 3 or 2 Val-Gly repeats (25 ng) have protected fragments of 406 and 261 nt plus 139 nt, respectively. Poly(A⁺) (2 μg) and total reticulocyte RNAs (20 μg) from unrelated individuals have protected fragments of 261 and 139 nt, confirming the presence of 2 Val-Gly dipeptides in the JK transcripts. Size marker from *Hae*III-digested ϕ X147 and integrity of synthesized antisense RNA probe ASP-1 from *Hind*III-linearized recombinant plasmid are shown on the left.

assay using a labeled antisense RNA probe (ASP-1) encoding 3 Val-Gly repeats (Fig. 2B). With cRNA encoding the 3Val-Gly, a protected RNA fragment of 406 bp was obtained. Due to the non-complementary 6-nucleotide stretch, two protected fragments of 261 and 139 bp were obtained with a cRNA encoding the 2Val-Gly repeat as a template. With poly(A⁺) RNA as well as total RNAs from unrelated individuals, only hybridization signals of 261 and 139 nt were detected, which indicated that the JK transcripts have a nucleotide sequence that encoded a polypeptide carrying only 2 Val-Gly motifs. Our extensive analysis of genomic DNAs and RNAs from several individuals thus indicated that the physiological urea transporter was encoded by the sequence found in clone HUT11A and not, as initially reported (6), in clone HUT11.

Transport Studies—To analyze the functional features of HUT11 (Lys⁴⁴/3Val-Gly) and HUT11A (Glu⁴⁴/2Val-Gly), 40 ng of cRNAs encoding these proteins were injected into *Xenopus* oocytes. Immunocytochemical analysis using affinity-purified anti-N-terminal antibodies (7, 22) revealed that both proteins were expressed at the plasma membrane of oocytes, which rendered these cells suitable for functional analysis (Fig. 3A). Nevertheless, for the same amount of cRNA injected, the HUT11A signal at the oocyte plasma membrane was clearly stronger than the HUT11 signal. Water-injected controls were not stained, showing the specificity of the antibody labeling (Fig. 3A).

Next, the time course of urea uptake by HUT11 and HUT11A expressing *Xenopus* oocytes was explored (Fig. 3B). HUT11 and

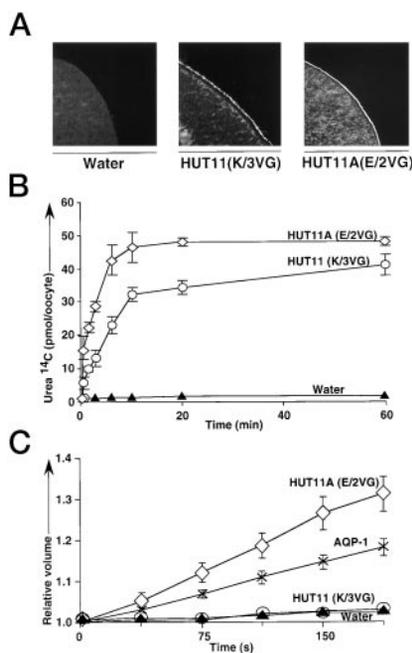


FIG. 3. Immunocytochemical analysis and characterization of urea transport and water permeability of oocytes injected with cRNAs encoding HUT11 and HUT11A proteins. *A*, *Xenopus* oocytes were injected with 40 ng of cRNA encoding HUT11 or HUT11A. Water-injected oocytes were used as negative control. After 3 days, oocytes were fixed and sections of injected oocytes were stained with an affinity-purified antibody against the N terminus of the Kidd/urea transporter protein and visualized with fluorescein isothiocyanate-conjugated anti-rabbit IgG as described under "Materials and Methods" and then imaged using a Nikon Eclipse TE300 microscope (Nikon, Paris, France) (20 \times objective). Images were recorded with epifluorescence illumination and treated with a Biocom computer system of image integration (Biocom, Les Ulis, France). *B*, time course of urea uptake in *Xenopus* oocytes injected with 50 nl of water (\blacktriangle) as control, 40 ng of HUT11 cRNA (\circ), or 40 ng of HUT11A cRNA (\diamond). Urea uptake was determined as a function of time as described previously (3). Shown are data (mean \pm S.E.) from 5 to 6 oocytes/point of at least three experiments. *C*, time course of oocyte swelling at 18 $^{\circ}$ C in response to a 5-fold dilution of extracellular Barth solution. Oocytes (5–6 oocytes/point) were injected with water (\blacktriangle) as control, HUT11 cRNA (\circ), HUT11A cRNA (\diamond), or AQP-1 cRNA (\times); 40 ng/oocyte in 50 nl as in *A*. Data (mean \pm S.E.) correspond to one experiment representative of at least three.

HUT11A expressing oocytes showed a rapid initial urea uptake that was 15 and 45 times higher than in water-injected oocyte controls, respectively. The urea permeabilities (P_{urea}) of HUT11- and HUT11A-injected oocytes, determined at 90 s, were, respectively, $15.7 \pm 0.82 \times 10^{-6}$ cm/s ($n = 56$) and $46.6 \pm 1.86 \times 10^{-6}$ cm/s ($n = 72$) versus $1.02 \pm 0.11 \times 10^{-6}$ cm/s ($n = 68$) for water-injected oocytes ($p < 0.001$). Consequently, the plateau corresponding to the equilibration of urea was more rapidly reached with oocytes expressing HUT11A than with HUT11. These data suggest that both HUT11 and HUT11A function as efficient urea transporters. In all experiments, the raffinose permeability was not increased, which indicate that the plasma membrane integrity was intact (data not shown).

Examination of the [14 C]urea uptake also indicated that, for a given level of cRNA injected (40 ng), the urea transport activity was greater for HUT11A-injected oocytes than for HUT11-injected oocytes (Fig. 3*B*). This could result from either a faster turnover of HUT11A with comparable levels of expression or a higher level of HUT11A with a comparable turnover. The latter hypothesis was most likely since immunostaining of the same oocytes clearly indicated a higher expression level of HUT11A as compared with HUT11 (Fig. 3*A*).

To test whether HUT11A and HUT11 also confer water permeability, oocytes expressing these proteins were tested in

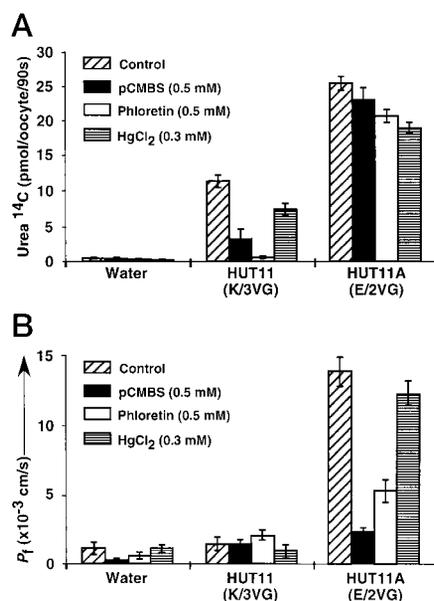


FIG. 4. Effect of inhibitors on urea transport and water permeability of oocytes expressing HUT11 or HUT11A. *A*, effect of 0.5 mM pCMBS, 0.5 mM phloretin, and 0.3 mM HgCl $_2$ on urea transport by HUT11 and HUT11A cRNA-injected oocytes. For each injected oocyte, 40 ng of cRNA were used and at least 5–6 oocytes/point, were preincubated in pCMBS-, phloretin-, or HgCl $_2$ -containing medium for 20, 10, and 5 min, respectively, before the experiment. Urea uptake was for 90 s, and data are mean \pm S.E. from 5 to 6 oocytes/point. Experiments were repeated three times. *B*, effect of pCMBS, phloretin, and HgCl $_2$ on water permeability (P_f) of oocytes expressing HUT11 and HUT11A. Oocytes were injected as in *A* and then incubated with pCMBS (0.5 mM), phloretin (0.5 mM), and HgCl $_2$ (0.3 mM) before and during permeability measurements. P_f values are means \pm S.E. from 5 to 6 oocytes/point.

a standard swelling assay. Surprisingly, oocytes expressing HUT11A swelled significantly faster than oocytes expressing AQP1, which was taken as a positive control (Fig. 3*C*). As already reported (7), swelling of oocytes expressing HUT11 was not different from water-injected controls in these conditions.

Urea transport analysis in the presence of pharmacological inhibitors revealed that the HUT11A-mediated urea flux was poorly inhibited by pCMBS, phloretin, and HgCl $_2$ (Fig. 4*A*). However, as previously reported (6), the HUT11-mediated urea flux was strongly inhibited by pCMBS (74%) and phloretin (85%) but only slightly inhibited by HgCl $_2$ (30%). Incubation with inhibitors showed that the HUT11A-mediated osmotic water permeability (P_f) of about 150 μ m/s was strongly inhibited by pCMBS and phloretin, but not by HgCl $_2$ (Fig. 4*B*).

We speculated that the unusual properties of HUT11A as compared with HUT11 might have resulted from a difference in the expression level of the two transporters in oocytes (see above). To test this hypothesis, the urea and water transport and HUT11A plasma membrane expression levels were determined in oocytes injected with different amounts of HUT11A cRNA. As shown in Fig. 5*A*, the urea transport rate increased steadily with injections of between 0.005 and 0.05 ng of HUT11A cRNA and sustained at a plateau rate of about 20 pmol/90 s for oocytes injected with 0.05–40 ng of cRNA. Accordingly, the urea uptake between 10 and 360 s of oocytes injected with 0.05, 0.1, or 40 ng/oocyte HUT11A cRNA, was identical (Fig. 5*B*). The corresponding urea permeabilities calculated at 10 s were $124 \pm 11 \times 10^{-6}$ cm/s ($n = 20$), $119 \pm 12 \times 10^{-6}$ cm/s ($n = 20$), and $133 \pm 7 \times 10^{-6}$ cm/s ($n = 20$), respectively, versus $1.6 \pm 1 \times 10^{-6}$ cm/s ($n = 25$) for water-injected controls. In contrast, using oocytes from the same batch, a water permeability was detected only when at least 0.5 ng of HUT11A cRNA was injected and steadily increased up to

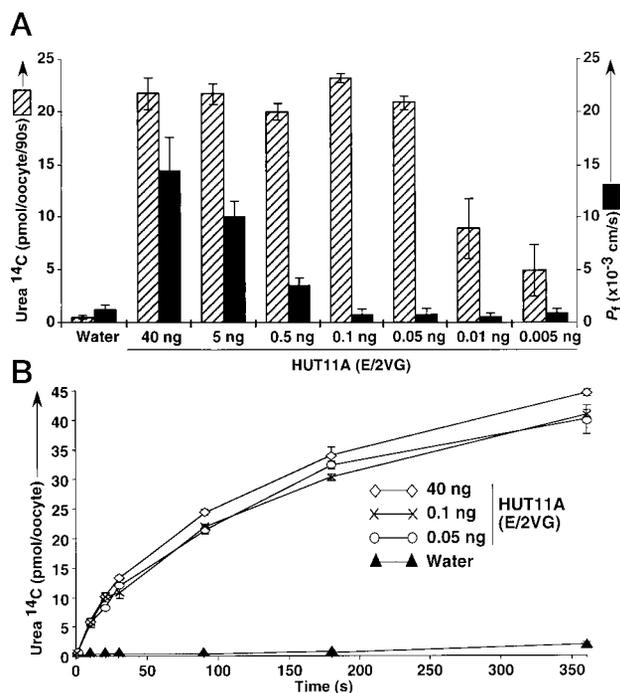


FIG. 5. Dose response of urea transport and water permeability in oocytes expressing HUT11A. *A*, oocytes (5–6 oocytes/point) were injected with increasing amounts of HUT11A cRNA or 50 nl of water as control. Urea uptake at 90 s and oocyte swelling (P_f) were determined as in Fig. 3*A* and *B*. *B*, Time course of urea uptake in *Xenopus* oocytes injected with 0.05, 0.1 or 40 ng of HUT11A cRNA or with 50 nl of water as control. Urea uptake was determined at 10, 20, 30, 90, 180, and 360 s. Data (mean \pm S.E.) are from 5 to 6 oocytes/point of at least three experiments.

40 ng of cRNA per oocyte (Fig. 5*A*).

We next compared the effect of pharmacological inhibitors (pCMBS and phloretin, 1 mM) on the HUT11A-mediated urea transport in oocytes injected with small (0.1 ng) and large (40 ng) amounts of cRNA, since there was no difference in the urea permeability under these conditions (see Fig. 5). As shown from Fig. 6*A*, the urea flux mediated by oocytes injected with 0.1 ng of HUT11A cRNA was poorly inhibited by pCMBS (18%), but was strongly inhibited by phloretin (79%), thus partly exhibiting the properties of the urea transporter from human red cells (8). In contrast, when the amount of HUT11A cRNA injected was increased to 40 ng/oocyte, phloretin no longer inhibited the urea transport.

To analyze the influence of small solutes (non-electrolytes) on the water movement across oocytes injected with low and high levels of HUT11A cRNA, the initial rates of swelling, between $t = 0$ and $t = 240$ s, were measured in an iso-osmotic solution containing 160 mM small solutes adjusted to 200 mosM with diluted Barth solution (Fig. 6*B*). Oocytes injected with 20 ng of HUT11A cRNA swelled significantly in a solution containing either amides (formamide, acetamide, and propionamide) or diols (ethylene glycol and propylene glycol), but not in a solution containing glycerol (triol) or meso-erythritol (tetraol). However, when less 0.1 ng of cRNA was injected per oocyte, there was no difference with the controls.

Membrane Expression of HUT11A in Oocyte Plasma Membranes—Since the transport studies suggested functional differences in *Xenopus* oocytes injected with small and large amounts of HUT11A cRNA, paraffin-embedded sections of oocyte membranes were immunostained with a well characterized, affinity-purified antibody directed against the N terminus of the Kidd/urea transporter protein (7, 22) (Fig. 7*A*). Although oocytes injected with 0.05, 0.1, and 40 ng of HUT11A cRNA/

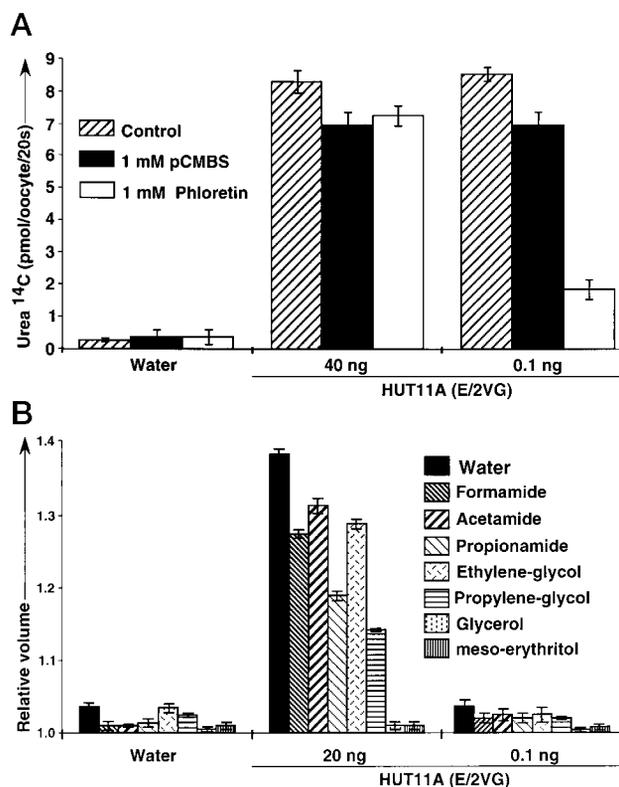


FIG. 6. Effect of inhibitors and permeability to different solutes at low and high levels of HUT11A-mRNA injection. *A*, effect of pCMBS and phloretin on urea transport of oocytes injected with small or large amounts of HUT11A cRNA. Oocytes injected with cRNAs (40 and 0.1 ng) or water were preincubated in 1 mM pCMBS or phloretin as in Fig. 4*A*. Urea uptake was for 20 s. Data are expressed as mean \pm S.E. from 6–8 oocytes/point from one representative experiment. *B*, volume increase (between $t = 0$ and $t = 240$ s) of oocytes injected with small or large amounts of HUT11A cRNA (20 and 0.1 ng) or water as control, and placed in 40 mosM Barth solution adjusted to 200 mosM with the solute. Two solute families were tested: the amides, including formamide, acetamide, and propionamide; and the polyols, including ethylene glycol, propylene glycol, glycerol, and meso-erythritol. Swelling of oocytes placed in 40 mosM Barth solution was also reported.

oocyte exhibited similar levels of urea transport (see Fig. 5*A*), immunocytochemical staining clearly revealed increasing levels of HUT11A expression on the plasma membrane (Fig. 7*A*). No staining was obtained with water-injected oocytes.

To determine whether the functional properties of HUT11A could be explained by the formation of large oligomers, oocytes from the same batches were examined by electron microscopy (Fig. 7*B*). No ultrastructure alteration of the oocyte plasma membrane were detected in these experiments. The density of IMP inserted in the P-face plasma membrane was measured, which was $289 \pm 28/\mu\text{m}^2$ for water injected oocytes (Fig. 7*C*). After subtraction of this endogenous background, the net minimal IMP density increased from $200 \pm 49/\mu\text{m}^2$ for oocytes injected with 0.05 ng to $458 \pm 88/\mu\text{m}^2$ for those injected with 40 ng of HUT11A cRNA (Fig. 7*C*). Apparently, an 800-fold increase in injected cRNA resulted only in a 2.3-fold increase in IMP density.

As a preliminary analysis of the membrane organization of the urea transporter, the diameter of P-face particles was determined in oocytes expressing HUT11A (40 ng of cRNA injected) and control oocytes. Control oocytes and oocytes expressing HUT11A appeared to express P particles with a mean diameter of 7 ± 0.5 and 6.5 ± 0.5 nm, respectively (Fig. 7*D*). Assuming that each membrane helix occupies $1.40 \pm 0.03 \text{ nm}^2$ (27) and a film thickness of 1.20 ± 0.2 nm, our cross-sectional area data predict a membrane protein containing 9 ± 3 helices.

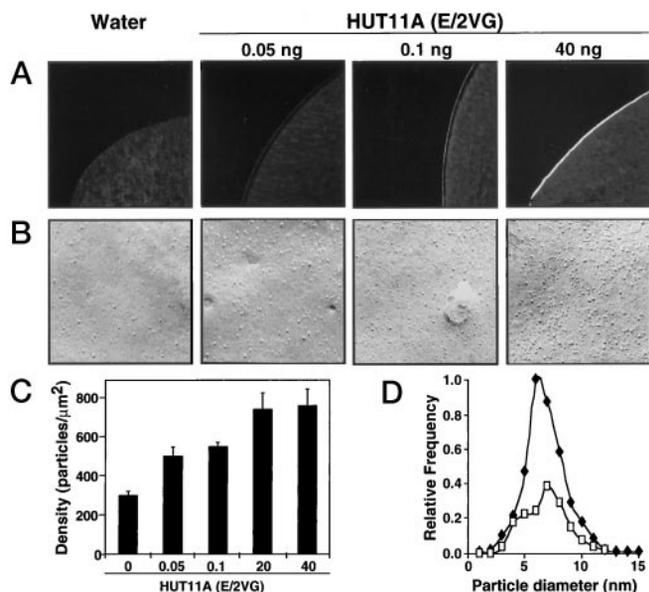


FIG. 7. Immunocytochemical analysis and freeze-fracture electron microscopy of plasma membrane of oocytes injected with HUT11A cRNA. Oocytes were injected with water as control, 0.05, 0.1, 0.1, 20, or 40 ng of cRNA encoding HUT11A. *A*, 3 days after injection, fixed oocyte sections were prepared and stained as described under "Materials and Methods," and the images were analyzed as described in Fig. 3*A*. *B*, P-faces obtained after freeze-fracture of the same batches of injected oocytes were replicated by unidirectional shadowing with platinum. 95,000 \times final magnification representative images for each set of conditions are shown. *C*, histogram of particle density (particles/ μm^2) from images ($n = 4-15$) of different P surfaces of two or three oocytes for each set of conditions. *D*, size distribution of P-face particles in oocytes expressing HUT11A (\blacklozenge) and water-injected oocyte control (\square). The particle densities were $289 \pm 28/\mu\text{m}^2$ ($n = 294$) and $747 \pm 88/\mu\text{m}^2$ ($n = 2035$) for water-injected controls and 40 ng of cRNA injection, respectively. HUT11A particles had a mean diameter of 6.5 ± 0.5 nm close to the IMP density of the water-injected oocyte control.

If the hydrophobicity profile, predicting 10 transmembrane domains, is assumed to be correct, these data suggest that the HUT11A urea transporter functions as a monomer.

DISCUSSION

HUT11A, and Not HUT11, Is the Physiological Urea Transporter—In the present study, we used RT-PCR amplification of human reticulocyte RNAs to identify a new cDNA clone, called HUT11A, encoding a polypeptide nearly identical to the Jk blood group/urea transporter characterized previously as the predicted product of the HUT11 clone (6, 7). However, HUT11A and HUT11 encode two distinct polypeptides of 389 and 391 residues, respectively, which differ by a Val-Gly dipeptide motif after Pro-227. The two polypeptides differ also by a Lys⁴⁴ \rightarrow Glu polymorphism, unrelated to the *JK**A*/JK*B* alleles.

PCR analysis of genomic DNA from 126 unrelated donors and ribonuclease protection assay have unambiguously shown that the *JK* gene and the *JK* transcript encode a protein carrying 2 instead of 3 Val-Gly motifs. Therefore, the previously reported HUT11 clone (6) may result either from a rare *JK* allele encoding 3 Val-Gly motifs or from a cloning artifact in the cDNA library used.

HUT11A, but Not HUT11, Confers Water Permeability on Oocytes—When we compared the functional properties of the HUT11A and HUT11 transporters, we found that *Xenopus* oocytes injected with 40 ng of cRNAs encoding HUT11A or HUT11 both expressed proteins at the plasma membrane of oocytes and exhibited a detectable urea transport activity (Fig. 3, *A* and *B*). However, the facilitated urea transport mediated by HUT11, but not by HUT11A, was highly sensitive to pCMBS

and phloretin. In addition, oocytes expressing HUT11A appeared to be highly permeable to water, whereas oocytes expressing HUT11 had the same water permeability as the water-injected controls (Fig. 3*C*). In fact, HUT11A-expressing oocytes swelled significantly faster than AQP1-expressing oocytes. These findings are in line with the results of Yang and Verkman (31), who reported an increase in water permeability of *Xenopus* oocytes expressing the rat homologue of HUT11A called UT3.

In HUT11A-expressing oocytes, urea transport was not inhibited by mercurials, whereas water transport could be inhibited by pCMBS but not by HgCl₂ (Fig. 4, *A* and *B*). In addition, the HUT11A urea transport could not be inhibited by phloretin, whereas the water permeability could. In this respect, it was unclear whether the human urea transporter was the functional equivalent of the rat UT3 protein, because one report indicated that HgCl₂ did not inhibit UT3-mediated urea and water transport (31), whereas others (32, 33) showed inhibition of UT3 urea transport by pCMBS. Moreover, these reports showed a strong inhibition of UT3 urea transport, whereas our data showed no inhibition of HUT11A urea transport by phloretin. At this stage, these results indicated that, although our genomic analyses proved that HUT11A was the physiological urea transporter, HUT11, but not HUT11A, showed the inhibitory features found for the native red cell urea transporter (8).

HUT11A-mediated Water Permeability and Uptake of Small Solutes Are Caused by Overexpression—A possible explanation for the observed differences between HUT11 and HUT11A was the relatively high expression level of HUT11A in oocytes (Fig. 3*A*). Dose-response analyses revealed that low HUT11A expression levels conferred urea, but not water, permeability on oocytes (Fig. 5*A*). In combination with immunocytochemical analyses (Fig. 7, *A-C*), it can furthermore be concluded that an increase in HUT11A plasma membrane expression did result in an increase in water permeability, whereas the urea permeability was saturated. This may be due to a saturation of the oocyte cell machinery or to some intracellular degradation of the cRNA injected. The latter hypothesis is unlikely since Northern blot analysis showed that the cRNA injected in oocytes remained stable at least for 72 h (13).

These results indicate that above 0.1 ng of injections, the urea transport rate is not determined by the number of HUT11A transporters in the membrane, but by an unknown factor intrinsic to the oocytes. In line with this finding is that injection of oocytes with 3 or 10 ng of AQP2 cRNA does not result in increased water permeability, whereas the plasma membrane expression level is significantly increased.² Our results also explain the observed water permeability of UT3, as reported by Yang and Verkman. (31), because these authors injected 5 ng of the corresponding cRNA.

From the structure-function point of view, the water permeation of HUT11A is interesting. As AQP-1 and HUT11A do not share any sequence homology and as no electron crystallography data on the urea transporter are available, one explanation for this water transport activity might be that the HUT11A transporter takes another conformation at high density in the oocyte membrane allowing water transport to occur. This hypothesis is corroborated by the following data. First, the increase in HUT11A particles in the plasma membrane with injections of between 0 and 0.1 ng of HUT11A cRNA does not result in an increase in P_f , whereas a similar increase in particles, occurring with injections between 0.1 and 40 ng of HUT11A cRNA, results in an increase in P_f of about 150 $\mu\text{m/s}$ (Fig. 5*A*); with an unchanged conformation, one would have

² E.-J. Kamsteeg and P. M. T. Deen, unpublished data.

expected a measurable P_f of about 75 $\mu\text{m/s}$ for 0.1-ng injections. Second, with the injection of 40 ng of HUT11A cRNA, the urea transport could not be inhibited by pCMBS or phloretin, whereas with an injection of 0.1 ng of cRNA there was significant inhibition with phloretin (Fig. 6A). If the conformation of the urea transporter is indeed changed with high injections, the relevance of the single-channel water permeability for UT3 (1.4×10^{-14} cm^2/s) as determined by Yang and Verkman (31) is doubtful. Alternatively, the water permeability noted with HUT11A might be a result of some undefined oocyte perturbation caused by injection of large amounts of cRNA. It is striking in this regard that injection of 800-fold more HUT11A cRNA (0.05–40 ng) only resulted in a 2.3-fold net increase membrane particles (Fig. 7C). However, this alternative is unlikely because, when large amounts of cRNAs for HUT11, HUT2, or the anion exchanger Band-3 (AE1) were injected, no permeability to water and small solutes was detected (data not shown).

A finding in line with the water transport activity mediated at high levels of HUT11A expression is that small non-electrolytes including amides and diols pass through the oocyte membrane via the same unknown mechanism (Fig. 6B). However, the glycerol, meso-erythritol, and raffinose exclusion was size-selective. In any case, these data strongly suggest that, at high levels of HUT11A expression, there is a loss of transport specificity that is confirmed by the loss of phloretin sensitivity.

Since the HUT11 and HUT11A expression constructs differ only in the described coding sequence, the difference noted in expression of both proteins was likely to be caused by the amino acid differences between HUT11 and HUT11A. Substitution of Lys for Glu⁴⁴ in HUT11A followed by expression in oocytes revealed that this polymorphism did not modify the transport properties of HUT11A.³ Therefore, the presence of only 2 Val-Gly motifs might be critical for the functional properties of HUT11A or, alternatively, the stability of the protein. Further studies will be required to define the role of this motif more clearly.

The unusual properties of HUT11A seen at high density level in oocytes were not related to the formation of oligomers since electron microscopic analysis revealed a HUT11A particle diameter of about 6.5 ± 0.5 nm, which is consistent with a monomeric form of the Kidd/urea transporter (characterized by 9 ± 3 transmembrane helices). This result is in conflict with a size of 469 ± 36 kDa for the red cell urea transporter determined by radiation inactivation (34). As *N*-glycosylation of HUT11A glycoprotein in the red cells cannot clear up this size difference (7), further investigation will be required to address this issue.

Low Expression of HUT11A Confers Physiological Urea Transporter Characteristics—Upon injection of oocytes with low level (0.1 ng) of HUT11A cRNA, urea transport was facilitated and was slightly sensitive to pCMBS (18%) and strongly sensitive to phloretin (79%). No water or small solute transport occurred under these conditions. These data are all in line with the physiological characteristics from the red cell urea transporter (8), although we cannot explain why HUT11A-mediated urea transport was not strongly inhibited by pCMBS in a nonerythroid context. Whether this may be due to some difference in the membrane properties of these cells require further studies.

From our data, we calculate that the number of HUT11A molecules expressed in oocyte membrane on injection of 0.1 ng of cRNA is similar to that of the physiological urea transporter in red blood cells. Assuming a density of 14,000–32,000 copies of Jk/urea transporter per red cell (35, 36), the calculated

surface density is 100–200 molecules/ μm^2 , which corresponded to the particle density seen by electron microscopy in oocytes injected with 0.05–0.1 cRNA/oocyte (Fig. 7C). We also noted that the urea permeability of oocytes injected with 0.05 ng (1.24×10^{-4} cm/s measured at 10 s) is close to the urea permeability of the erythrocyte plasma membrane (2.70×10^{-4} cm/s ; Ref. 37). Thus, upon injection of oocytes with 0.05–0.1 ng of HUT11A cRNA, physiological densities of urea transporter are expressed, which confer no detectable water permeability or small solute uptake. According to these results, it is unlikely that HUT11A confers the residual red cell water permeability in Colton-null erythrocytes, which lack AQP1 (38). More likely, this permeability can be accounted for by AQP3, which is also expressed in red cells (39).

In summary, two important conclusions can be deduced from these studies: (i) the oocyte expression system must be used under carefully controlled conditions to preserve urea transport specificity, and (ii) HUT11A is the red cell urea transporter, which accounts for urea permeability but not water permeability or small solute uptake of normal erythrocytes in physiological conditions.

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REFERENCES

- Bankir, L. (1995) in *The Kidney* (Brenner, B. M., and Rector, F. C., Jr., eds) 5th Ed., pp. 571–606, Saunders Co., Philadelphia
- Gillin, A. G., and Sands, J. M. (1993) *Semin. Nephrol.* **13**, 146–154
- Sands, J. M., Timmer, R. T., and Gunn, R. B. (1997) *Am. J. Physiol.* **273**, F321–F339
- Hediger, M. A., Smith, C. P., You, G., Lee, W. S., Kanai, Y., Shayakul, C. (1996) *Kidney Int.* **49**, 1615–1623
- Berger, U. V., Tsukaguchi, H., and Hediger, M. A. (1998) *Anat. Embryol.* **197**, 405–414
- Olivès, B., Neau, P., Bailly, P., Hediger, M. A., Rousset, G., Cartron, J.-P., and Ripoche, P. (1994) *J. Biol. Chem.* **269**, 31649–31652
- Olivès, B., Mattei, M.-G., Huet, M., Neau, P., Martial, S., Cartron, J.-P., and Bailly, P. (1995) *J. Biol. Chem.* **270**, 15607–15610
- Mayrand, R. R., and Levitt, C. (1983) *J. Gen. Physiol.* **81**, 221–237
- Xu, Y., Olivès, B., Bailly, P., Fischer, E., Ripoche, P., Ronco, P., Cartron, J.-P., and Rondeau, E. (1997) *Kidney Int.* **51**, 138–146
- Pallone, T. L. (1994) *Am. J. Physiol.* **267**, R260–R267
- Promeneur, D., Rousset, G., Bankir, L., Bailly, P., Cartron, J.-P., Ripoche, P., and Trinh-Trang-Tran, M. M. (1996) *J. Am. Soc. Nephrol.* **7**, 852–860
- Macey, R. I., and Yousef, L. W. (1988) *Am. J. Physiol.* **254**, C669–C674
- Lucien, N., Sidoux-Walter, F., Olivès, B., Moulds, J., Le Pennec, P.-Y., Cartron, J.-P., and Bailly, P. (1998) *J. Biol. Chem.* **273**, 12973–12980
- Olivès, B., Merriman, M., Bailly, P., Bain, S., Barnett, A., Todd, J., Cartron, J.-P., and Merriman, T. (1997) *Hum. Mol. Genet.* **6**, 1017–1020
- Sands, J. M., Gargus, J. J., Fröhlich, O., Gunn, R. B., and Kokko, J. P. (1992) *J. Am. Soc. Nephrol.* **2**, 1689–1696
- Olivès, B., Martial, S., Mattei, M.-G., Matassi, G., Rousset, G., Ripoche, P., Cartron, J.-P., and Bailly, P. (1996) *FEBS Lett.* **386**, 156–160
- Martial, S., Olivès, B., Abrami, L., Couriaud, C., Bailly, P., You, G., Hediger, M., Cartron, J.-P., Ripoche, P., and Rousset, G. (1996) *Am. J. Physiol.* **271**, F1264–F1268
- Izraeli, S., Pfeleiderer, C., and Lion, T. (1991) *Nucleic Acids Res.* **19**, 6051
- Maillet, P., Delaunay, J., and Baklouti, F. (1996) *Hum. Mutat.* **7**, 61–64
- Martial, S., Ripoche, P., and Ibarra, C. (1991) *Biochim. Biophys. Acta* **1090**, 86–90
- Mc Lean, I. W., and Nakane, P. K. (1974) *Histochem. Cytochem.* **22**, 1077–1083
- Xu, Y., Olivès, B., Bailly, P., Fischer, E., Ripoche, P., Ronco, P., Cartron, J.-P., and Rondeau, E. (1997) *Kidney Int.* **51**, 138–146
- Mulders, S. A., Knoers, N. V. A. M., van Lieburg, A. F., Monnens, L. A. H., Leumann, E., Wuhl, E., Schober, E., Rijss, J. P. L., von Os, C. H., and Deen, P. M. T. (1997) *J. Am. Soc. Nephrol.* **8**, 242–248
- Deen, P. M. T., Verdijk, M. A., Knoers, N. V. A. M., van Essen, A. J., Proesmans, W., Mallmann, R., Monnens, L. A. H., von Os, C. H., and van Oost B. A. (1994) *Science* **264**, 92–95
- Abrami, L., Tacnet, F., and Ripoche, P. (1995) *Eur. J. Physiol.* **430**, 447–458
- Zampighi, G. A., Kreman, M., Boorer, K. J., Lodd, D. D., Bezanilla, F., Chandy, G., Hall, J. E., and Wright, E. M. (1995) *J. Membr. Biol.* **148**, 65–78
- Eskandari, S., Wright, M. E., Kreman, M., Starace, M. D., and Zampighi, A. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11235–11240
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673–4680
- Verbavatz, J. M., Couriaud, C., Berthouaud, V., Gobin, P., Bailly, P., Ibarra, C., Silberstein M., Simon, M., Hediger, M., Ripoche, P., Rousset, G., and de Rouffignac, C. J. (1996) *Am. Soc. Nephrol.* **7**, 1274 (abstr.)

³ F. Sidoux-Walter, J.-P. Cartron, and P. Bailly, unpublished data.

30. Smith, C. G., and Wright, P. (1999) *Am. J. Physiol.* **276**, R622–R626
31. Yang, B., and Verkman, A. S. (1998) *J. Biol. Chem.* **273**, 9369–9372
32. Tsukaguchi, H., Shayakul, C., Berger, U. V., Tokui, T., Brown, D., and Hediger, M. A. (1997) *J. Clin. Invest.* **99**, 1506–1515
33. Couriaud, C., Ripoche, P., and Rousselet, G. (1996) *Biochim. Biophys. Acta* **1309**, 197–199
34. Dix, J. A., Ausiello, D. A., Jung, C. Y., and Verkman, A. S. (1985) *Biochim. Biophys. Acta* **821**, 243–252
35. Mannuzzu, L. M., Moronne, M. M., and Macey, R. I. (1993) *J. Membr. Biol.* **133**, 85–97
36. Masouredis, S. P., Sudora, E., Mahan, L., and Victoria, E. J. (1980) *Blood* **56**, 969–971
37. Brahm, J. (1983) *J. Gen. Physiol.* **82**, 1–23
38. Mathai, J. C., Mori, S., Smith, B. L., Preston, G. M., Mohandas, N., Collins, M., van Zijl, P. C. M., Zeidel, M. L., and Agre, P. (1996) *J. Biol. Chem.* **271**, 1309–1313
39. Roudier, N., Verbavatz, J. M., Maurel, C., Ripoche, P., and Tacnet, F. (1998) *J. Biol. Chem.* **273**, 8407–8412