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

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Frédéric Sidoux-Walter‡, Nicole Lucien‡, Bernadette Olivès‡, Renée Gobin‡, Germain Rousselet‡, Erik-Jan Kamsteeg‡, Pierre Ripoche‡, Peter M. T. Deen‡, Jean-Pierre Cartron‡, and Pascal Bailly‡

From ‡INSERM U76, Institut National de la Transfusion Sanguine, 6 rue Alexandre Cabanel, 75015 Paris, France, the §Département de Biologie Cellulaire et Moléculaire, CEA Saclay, 91191 Gif-sur-Yvette Cedex, France, and the ¶Départment of Cell Physiology, University of Nijmegen, 6500 HB, Nijmegen, The Netherlands

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 HUT11U urea transporter, except for a Lys→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 into 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 JKnull individuals provided a rational explanation for the lack of Kidd/urea transporter protein at the red cell surface (13). Thus in JKnull 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 JKnull individuals who do not suffer from a clinical syndrome explaining the reduced capacity to concentrate urine of these individuals (15). No erythrocyte hemolysis has been reported in JKnull 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

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† To whom correspondence should be addressed: INSERM U76, Institut National de la Transfusion Sanguine, 6 rue Alexandre Cabanel, 75015 Paris, France. Tel.: 33-1-44-49-30-00; Fax: 33-1-43-06-50-19; E-mail: cartron@infobiogen.fr.

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

In mammals, urea is the chief end product of nitrogen catabolism and is produced in the liver by the urea cycle during the conversion for 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 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 (pCMBS) (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).

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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 leucocyte 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 sequence was 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 RNA 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. L07265) was PCR-amplified from a Jk1 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 semi-nested PCR amplification was carried out under stringent conditions (94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, for 30 cycles) using 200 ng of leucocyte DNA from 126 independent donors. The first amplification was done between SP-A (sense primer, 5′-gcagTTGTTGAAATCTATACCAG-3′) and AS-B (antisense primer, position 797–762) with 40 cycles and 200 ng of purified genomic DNA from 126 independent donors. The second amplification was done between primers SP-A and AS-C (antisense primer, position 728–705). The amplified fragments were subjected to the following conditions: 30 cycles, 90 s at 94 °C, 30 s at 55 °C, and 40 s at 72 °C. The second PCR was repeated for 10 cycles.

**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 gel, the ASP-1 probe (2.0 × 106 cpm) was hybridized overnight at 50 °C with 25 μg of total RNA isolated from transfected cRNA (cRNA from the pCR2.1 vector) using 2 ng of antisense transcripts (cRNA) and 2 ng of cRNA from the pUT2-A2 vector (Roche Molecular Biochemicals) with [3H]UTP (800 Ci/mmol) in 5 M MgCl2, pH 7.5, and microinjected with 50 nl of water or cRNA (350–40 ng/oocyte) in 50 nl. The microinjected oocytes were then kept at 18 °C for 2 or 3 days in Barth solution (200 mM 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) (23).

**Oocyte Flux Measurements**—After injection of 40 ng cRNA/oocyte, urea transport activity was measured by [3H]urea uptake as described previously (6), or under conditions as indicated in the text. In all these experiments, the Barth incubation solution contained [3H]urea (145 μM) and 5 μM [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 [3H]urea at each time point, corrected for the optically determined oocyte surface area.

Oocyte water permeability (Pw) 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 Pw (cm/s) was calculated from the initial osmotic cell volume increase between t = 0 and t = 90 s by the relation 1 = V0d/WV/dt/[(S0−Sr)/2] = V0d/WV/dt/[(S0−Sr)/2], where S0 is the oocyte surface area and Sr the molar volume of water (18 cm3/mole) (25).

In order to determine the influence of small solutes on the water movement across injected oocyte plasma membranes, oocytes were preincubated in 40 mosM Barth solution containing 2.0 M urea, 80 mosM formamide, 80 mosM acetamide, 80 mosM propionamide, 80 mosM ethylene glycol, 80 mosM glyproline, 80 mosM glycerol, or 80 mosM mero-erythritol, as described previously (17). Oocyte swelling was performed for 240 s. Osmolarity was checked with a Roehling osmometer just before the experiments.

In water or urea transport inhibition experiments, oocytes were incubated in 0.5–200 μM pCMBS (Sigma), 0.5 mosM phloretin (Sigma), or 0.3 mosM 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 fractioned in a Balzers 300 apparatus at −150 °C under 10–1 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 electron microscope at 80 kV. Representative series of images of P-face fringes were enlarged at 30,000× 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 Eckardy et al. (27).

**RESULTS**

**Identification of the Kidd/ Urea Transporter**—Using two primers located in the 5′- and 3′-untranslated regions of the
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* 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 the JK transcripts have a nucleotide sequence that encoded a polypeptide carrying only 2 Val-Gly motifs. Our extensive 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 BglII, 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 HaeIII-digested 4X174 and integrity of synthesized antisense RNA probe ASP-1 from HindIII-linearized recombinant plasmid are shown on the left.

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 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. 1. Sequence comparison of urea transporters. Top, schematic representation of HUT11 (Lys3Val-Gly) and HUT11A (Glu2Val-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‘-GTG GGA-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* allele is double underlined. Bottom, partial multiple alignment (CLUSTALW 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 protein sequence encoding the repeated dipeptides, and the PCR product was digested by BglII. Analysis on a 15% polyacrylamide gel revealed only the 36-bp fragment, which encodes the variable Val-Gly repeats (Fig. 2B) as determined independently (6), in clone HUT11.

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 BglII, 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 HaeIII-digested 4X174 and integrity of synthesized antisense RNA probe ASP-1 from HindIII-linearized recombinant plasmid are shown on the left.
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 ($P_{\text{urea}}$) 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 rapid uptake of urea with oocytes expressing HUT11A was significantly faster than with oocytes expressing HUT11, which was taken as a positive control (Fig. 3C). 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. 4A). However, as previously reported (6), the HUT11A-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 μm/s was strongly inhibited by pCMBS and phloretin, but not by HgCl$_2$ (Fig. 4B).

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. 5A, 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. 5B). The corresponding urea permeabilities calculated at 10 s were $124 \pm 10^{-6}$ 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
40 ng of cRNA per oocyte (Fig. 5A).

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. 6A, 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 as control. Urea uptake at 90 s and oocyte swelling ($P_w$) were determined as in Fig. 3A and 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 ± S.E.) are from 5 to 6 oocytes/point of at least three experiments.

**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. 7A). Although oocytes injected with 0.05, 0.1, and 40 ng of HUT11A cRNA/
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. 3C). 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. 3A). Dose-response analyses revealed that low HUT11A expression levels conferred urea, but not water, permeability on oocytes (Fig. 5A). 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. 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 P₆, whereas a similar increase in particles, occurring with injections between 0.1 and 40 ng of HUT11A cRNA, results in an increase in P₆ of about 150 μm/s (Fig. 5A); with an unchanged conformation, one would have

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2 E.-J. Kamsteeg and P. M. T. Deen, unpublished data.
electron microscopic analysis revealed a HUT11A particle clearly. HUT11A or, alternatively, the stability of the protein. Further Gly motifs might be critical for the functional properties of molecules expressed in oocyte membrane on injection of 0.1 ng. Whether this may be due to some differences in the physiological characteristics from the red cell urea transporter could not be inhibited by pCMBS or phloretin, whereas with a density of 14,000–32,000 copies 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/μm², 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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