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Kidd Blood Group and Urea Transport Function of Human Erythrocytes Are Carried by the Same Protein*

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The gene encoding the urea transporter of human erythrocytes (HUT11 clone) has been cloned recently (Olives, B., Neau, P., Bailly, P., Hediger, M. A., Rousselet, G., Cartron, J. P., and Ripoche, P. (1994) *J. Biol. Chem.* 269, 31649–31652). Now, this gene has been assigned to chromosome 18q12-q21 by *in situ* hybridization, as also found for the Kidd (Jk) blood group locus. In coupled transcription-translation assays, the HUT11 cDNA directed the synthesis of a 36-kDa protein which was immunoprecipitated by a human anti-Jk^3 antibody produced by immunized Jk(a−b−) donors whose red cells lack Kidd antigens. The anti-Jk^3 antibody also immunoprecipitated a protein material of 46–60 kDa from all red cell membranes, except those from Jk(a−b−) cells. After *N*-glycanase digestion the 46–60-kDa component was reduced to 36 kDa. A rabbit antibody raised against the predicted NH_2-terminal amino-acids of the HUT11 protein reacted on immunoblots with a 46–60-kDa component present in all human erythrocytes except those from Jk(a−b−) individuals. Jk(a−b−) red cells lack the Kidd/urea transport protein and have a selective defect of the urea transport capacity, but a normal water permeability and aquaporin-associated Colton blood group antigens. These findings indicate that the erythrocyte urea transporter is encoded by the Kidd locus and may have implications for the biology of urea transporters and their tissue-specific regulation.

Urea is rapidly transported across the red cell membrane via a facilitated diffusion pathway (1–3) which plays an important role when the blood traverses the kidney where the urea concentration varies greatly between the cortex and the medulla. Rapid urea transport helps to preserve the osmotic stability and deformability of the red cells and to stabilize osmotic gradients in the renal medulla (4, 5). In the kidney, there is a similar transport system which is critical in the urinary concentrating mechanism (6, 7). Although erythrocytes and renal cells share a urea transport mechanism with similar characteristics with respect to kinetics and effect of inhibitors, it is not clear whether both transporters are identical. Moreover, studies in different tissues suggest that several urea transporters are likely to exist since only some are susceptible to regulation by the anti-diuretic hormone (8).

The molecular identification of the urea transporter remained elusive until You *et al.* (9) successfully cloned the transporter (UT2 clone) by functional expression in *Xenopus* oocytes microinjected with cRNA from rabbit renal medulla. Based on these findings, a urea transporter was recently cloned from a human bone marrow cDNA library (HUT11 clone). HUT11 transcripts have been identified in human erythroid and renal tissues and expression studies in *Xenopus* oocytes demonstrated that the human protein mediated a facilitated urea transport which was inhibited by phloretin, *para*-chloromercuribenzene sulfonate, and urea analogs (10). The rabbit and human proteins share 62% sequence homology, have a similar predicted membrane topology, and exhibit an internal homology of the NH_2-terminal half and COOH-terminal half (11).

The possibility that the urea transport of human erythrocytes might be related to Kidd[1] blood group antigens was raised by the observation that red cells from Jk(a−b−) individuals which lack Kidd antigens exhibited an increased resistance to lysis in aqueous 2 M urea (12). This was confirmed by Fröhlich *et al.* (13) who demonstrated that these cells exhibit a defect in urea transport, whereas chloride, water, and ethylene glycol permeabilities were the same as control cells. The absence of Kidd antigens and deficiency in urea transport suggested that both phenotypes could be carried by a single polypeptide (13). Here, we used the HUT11 cDNA to demonstrate that the urea transporter of human erythrocytes is encoded by the Kidd locus.

MATERIALS AND METHODS

cDNA Clones and in Situ Hybridization—The cDNA encoding the rabbit (clone UT2) and human (clone HUT11) urea transporters were described elsewhere (9, 10). For *in situ* hybridization, a 1,100-base pair fragment of the HUT11 cDNA was ^{3}H-labeled by nick translation to a specific activity of 1.7×10^8 dpm/mg and hybridized (100 ng/ml) to human metaphase chromosomes as described (14). The specific site of hybridization was determined by Giemsa staining after R-banding and autoradiography (21 days).

Blood Samples and Antibodies—Red cell samples from Jk(a−b−) (B.S., Lon., and Lef.), Rh_{null} (Fric. and DAA) and Co(a−b−) (Sar.) individuals were collected from anticoagulated blood and used within 3 days or stored frozen in liquid nitrogen until used. The rabbit antibody against the human urea transporter was obtained by immunization with a synthetic peptide derived from the predicted protein sequence (residues 16–31) encoded by the cDNA clone HUT11 (10). The human anti-Jk^3 antiserum was obtained from an immunized Jk(a−b−) individual, and the polyclonal anti-CHIP28 was described earlier (15).

Immunochemical Analysis—Intact red cells were ^{125}I-labeled by IODO-GEN (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril, Pierce) as described (16). Labeled red cells (200 μl) were incubated with human anti-Jk^3 (20 μl) for 2 h at 37 °C, washed twice, and the membranes were

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[1] The abbreviations used are: Kidd, Jk; PAGE, polyacrylamide gel electrophoresis.

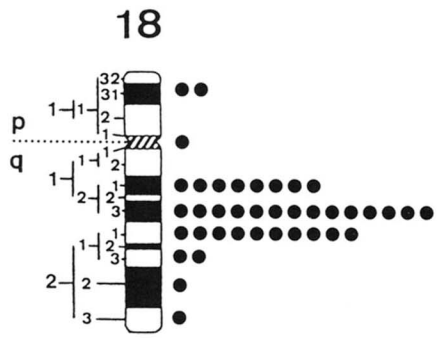

FIG. 1. **Distribution of silver grains on human chromosome 18 labeled by *in situ* hybridization with the HUT11-cDNA probe.** In the 100 metaphase cells examined, 182 silver grains associated with chromosomes, among which 21.4% were located on chromosome 18. Over 80% mapped to the 18q12-q21.1 region.

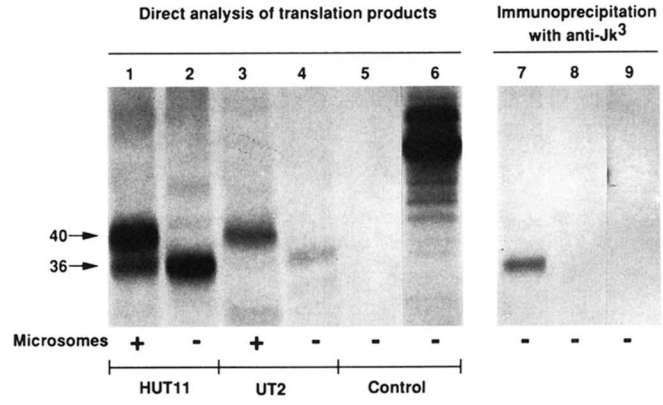

FIG. 2. **Expression of human and rabbit transporters in transcription-translation-coupled reticulocyte lysate system.** *Left*, autoradiogram of L-[^{35}S]methionine-labeled proteins analyzed by SDS-PAGE. *Lanes 1* and *2*, pT7TS-HUT11 with and without canine pancreatic microsomal membranes; *lanes 3* and *4*, pT7TS-UT2 vector with and without canine pancreatic microsomal membranes; *lane 5*, pT7TS alone; *lane 6*, plasmid with luciferase (control). *Right*, autoradiogram of ^{35}S-labeled proteins immunoprecipitated with the human anti-Jk^3 and analyzed by SDS-PAGE. *Lane 7*, pT7TS-HUT11; *lane 8*, pT7TS alone; *lane 9*, luciferase (control). *Arrows* on the *left* refer to products size (kDa).

prepared and solubilized in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl containing 5% (w/v) Triton X-100 and 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (Pefabloc, Boehringer Mannheim, Mannheim, Germany). The supernatant was incubated for 1 h at 4 °C with a rabbit anti-human IgG (Immunotech, Marseille, France) and the immunoprecipitates were washed on a sucrose gradient (16). For *N*-glycanase treatment, the labeled immunocomplexes from Jk(a+b+) red cells were digested during 16 h at 37 °C with 20 units/ml of peptidyl-*N*-glycosidase F from *Flavobacterium meningosepticum* (25,000 units/mg of protein, Boehringer Mannheim) in 20 mM sodium phosphate, pH 7.2, containing 1% (w/v) Triton X-100, 1% (w/v) SDS, and 1% β-mercaptoethanol. Control reaction was carried out in enzyme-free buffer in otherwise identical conditions. Untreated and *N*-glycanase-treated immunoprecipitates were resuspended in 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, 5% (w/v) SDS and analyzed by SDS-PAGE on a discontinuous buffer system (17) with 12 or 15% separating gels, respectively. For Western blot analysis, red cell membrane proteins (60 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibodies as described (18). Specifically bound antibodies were detected with a goat anti-IgG conjugated to phosphatase alkaline.

Coupled Transcription-Translation Assays—*In vitro* synthesis of human HUT11 and rabbit UT2 proteins was performed with the transcription-translation coupled reticulocyte lysate system (TNT/T7 Promega, Madison, WI) in the presence of L-[^{35}S]methionine (1.85 GBq/mmol, Amersham, Bucks, United Kingdom). cDNAs from HUT11 and UT2 were subcloned into the *Eco*RV-digested pT7TS plasmid, a pGEM4ZT-derived vector containing the 5′- and 3′-untranslated domains of the *Xenopus laevis* β-globin gene (kindly provided by P. Krieg, Austin, TX) and placed under the control of the T7 promoter. The reaction mixture (50 μl) was incubated at 30 °C for 90 min, and a 5-μl aliquot was directly analyzed by SDS-PAGE followed by autoradiography. The remainder of the reaction product (45 μl) was immunoprecipitated with a preformed complex of the human anti-Jk^3 antiserum and of rabbit anti-human IgG (19). The immunocomplexes were finally washed three times on a sucrose gradient (16) and analyzed by SDS-PAGE as above.

Urea and Water Permeabilities—For urea permeability measurements, resealed one-step ghosts were resuspended to 8×10^8 vesicles/ml in 100 mM NaCl, 5 mM Na_2HPO_4, pH 7.4. For each experiment, 10^8 ghosts were loaded with 1 mM [^{14}C]urea (2 μCi/ml) at 18 °C and washed by ultra-rapid filtration over 200-ms periods of time (20). Urea permeabilities were calculated from the analysis of the exponential time course of [^{14}C]urea washout. For osmotic water permeability measurements, the intact red cells were washed in 150 mM NaCl, 7.5 mM Na_2HPO_4, pH 7.4, supplemented with 1 mM EDTA and 0.5% (v/v) bovine serum albumin and resuspended at 1% hematocrit in the same medium. Volumes of red cells were measured at 25 °C by light scattering (excitation and emission wavelengths, 600 and >515 nm, respectively, as described (21)) after abruptly increasing the extracellular osmolality with sucrose (100 mOsm/kg H_2O). The rate constants, k (s^{-1}), were calculated from the fitted single exponentials describing the time course of red cell shrinkage.

RESULTS AND DISCUSSION

By *in situ* hybridization to human metaphase chromosomes the gene encoding the HUT11 cDNA mapped to the q12-q21 region of the human chromosome 18 (Fig. 1). Interestingly, the Kidd locus has been located in the same region of chromosome 18 (22), consistent with the hypothesis that HUT11 and Kidd antigens might be encoded by the same locus.

In the transcription-translation coupled system, the plasmid pT7TS-HUT11 directed the synthesis of a polypeptide of apparent molecular mass of 36 kDa (Fig. 2, *lane 2*). In the presence of microsomal membranes a major band at 40 kDa and a minor band at 36 kDa were detected (Fig. 2, *lane 1*). The 40-kDa component most likely represented the glycosylation product of the 36-kDa protein, presumably at Asn-211 which is the unique consensus *N*-glycosylation motif in the predicted extracellular loops of HUT11 (10). No protein material was translated using the pT7TS plasmid alone (Fig. 2, *lane 5*) or microsome alone (not shown). As expected, the pT7TS-UT2 cDNA construct directed the synthesis of glycosylated or non-glycosylated proteins with similar electrophoretic mobility as the HUT11 proteins (Fig. 2, *lanes 3* and *4*). The calculated relative molecular mass of the predicted HUT11 and UT2 proteins is close to 43,000 (9, 10), but both migrated on SDS-PAGE with an apparent mass of 36 kDa. A similar observation has been reported for the Rh blood group antigens (23).

The translation product of pT7TS-HUT11 was also examined by immunoprecipitation analysis with an anti-Jk^3 antibody produced by immunized Jk(a−b−) individuals. This antibody immunoprecipitated a 36-kDa ^{35}S-labeled polypeptide (Fig. 2, *lane 7*), but no radioactive material in this region could be immunoprecipitated from transcription-translation of the plasmid vector alone or the luciferase peptide control (Fig. 2, lanes 8 and *9*). These results clearly established that the 36-kDa protein material synthesized from the HUT11 construct expressed a specific Kidd blood group epitope. The anti-Jk^3 antibody immunoprecipitated also a prominent diffuse band of 46–60 kDa from Jk-positive red cells which was not detected with Jk(a−b−) red cells (Fig. 3). These findings suggest that native Jk antigens are carried by a membrane component of 46–60 kDa, consistent with a preliminary estimation of 45 kDa of this antigen (24). After extensive digestion of the immunocomplexes with peptide-*N*-glycosidase F (*N*-glycanase), the size of the 46–60-kDa glycoprotein was reduced to 36 kDa (Fig. 3), the same size described above in the transcription-translation

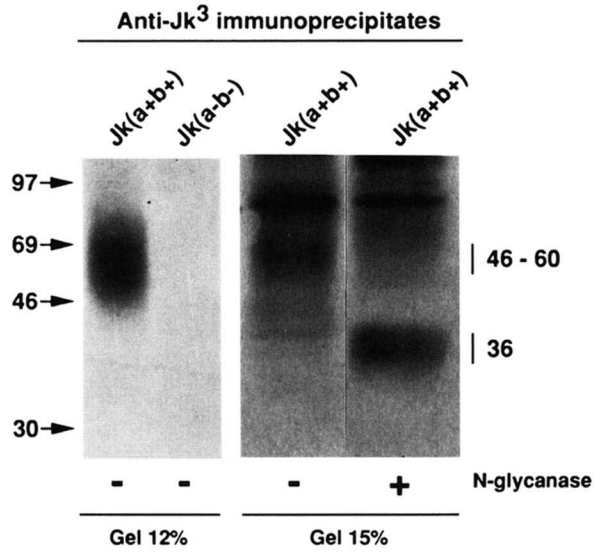

FIG. 3. **Immunoprecipitation of Jk polypeptides with human anti-Jk^3 before and after *N*-glycanase treatment.** Red cell membrane preparations from Jk(a+b+) and Jk(a−b−) individuals were immunoprecipitated with anti-Jk^3 as described under "Materials and Methods." Untreated and *N*-glycanase-treated immunoprecipitates from ^{125}I-labeled erythrocyte membranes were analyzed by SDS-PAGE as indicated. Size (kDa) of markers (*arrows*) and products (*bars*) are given on each side of the gel.

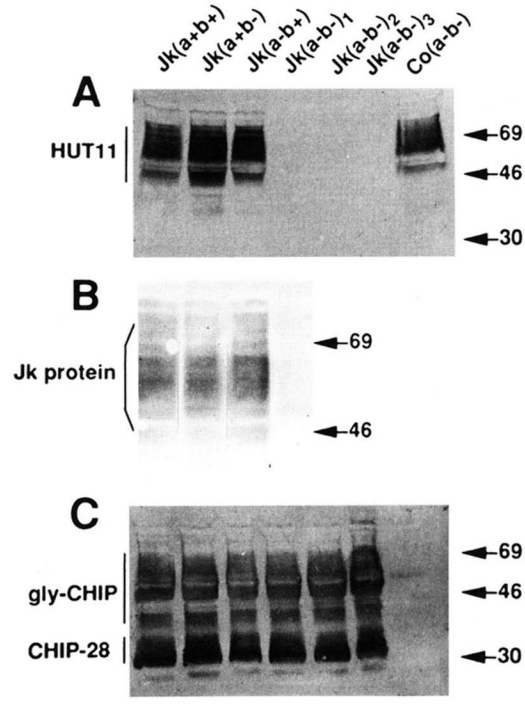

FIG. 4. **Immunoblot analysis of urea carrier and water channel proteins from erythrocyte membranes of individuals with different blood group phenotypes.** Red cell membrane proteins (60 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunostained with anti-HUT11 antiserum (1:400 dilution) (*A*), human anti-Jk^3 antiserum (1:200 dilution) (*B*), and anti-CHIP-28 antibody (*C*) (1:800 dilution).

coupled system (Fig. 2, *lane 2*). Under prolonged exposure of the radioactive material immunoprecipitated from Jk-positive red cell membranes, a minor band at 36 kDa was detected, suggesting that a small proportion of the Jk proteins is unglycosylated in the membrane (not shown).

Additional evidence that the HUT11 polypeptide may be identical to the Jk protein was provided by immunochemical analysis with a rabbit antibody raised against a synthetic peptide derived from the NH_2 terminus of HUT11. This antibody reacted in Western blotting with a diffuse band of 46–60 kDa present in red cell membrane preparations from individuals of different Kidd phenotypes [Jk(a+b+), Jk(a+b−), Jk(a−b+)], but did not react with three samples from unrelated Jk(a−b−) individuals (Fig. 4*A*). Immunoblotting carried out with the anti-Jk^3 antibody resulted in the same pattern (Fig. 4*B* and Fig. 3). Interestingly, the anti-HUT11 similarly reacted with the 46–60-kDa component of Co(a−b−) red cells which are deficient in aquaporin-1, the carrier of Colton blood groups (25, 26). Conversely, a rabbit antibody directed against aquaporin-1 (anti-CHIP28) reacted on immunoblots with membrane components of 28 and 35–60 kDa (CHIP28 and gly-CHIP28, respectively) present in all erythrocytes, except those from Co(a−b−) individuals (Fig. 4*C*). Thus, the null phenotypes Jk(a−b−) and Co(a−b−) carry normal aquaporin-1 (CHIP-28) and HUT11 polypeptides, respectively.

Urea and water permeabilities of Jk(a−b−) red cells were measured and compared with that of red cells that are deficient for Colton and Rh antigens to confirm the expected membrane transport alterations. The urea permeability (P_{urea}) of control ghost preparations was $2.14 \pm 0.20 \times 10^{-3}$ cm/s at 18 °C and was unchanged in Rh_{null} and Co(a−b−) ghosts. This value was reduced to $0.682 \pm 0.052 \times 10^{-3}$ cm/s in two Jk(a−b−) ghost preparations examined (Fig. 5). Measurements of the osmotic water permeability were next carried out on intact red cells from each variant. When placed in a hypertonic medium, red cells from control, Rh_{null}, and Jk(a−b−) individuals shrank very rapidly (rate constant of shrinkage, k, was 2.11 s^{-1}), and equilibrium was reached in 1 to 1.5 s at 25 °C (Fig. 5). In contrast, red cells from Co(a−b−) individuals shrank much more slowly with a rate constant of 0.55 s^{-1}. These results suggest that (i) control and Rh_{null} cells contain functional water channel and urea transporter; (ii) Jk(a−b−) cells carry functional water channel but lack the urea transporter and (iii) Co(a−b−) erythrocytes lack functional water channel (26), but have a functional urea transporter. These findings correlate well with the results of immunoblots carried out with anti-HUT11 and anti-CHIP28 antibodies (see Fig. 4). This is a further proof that the urea and water transports are mediated along different pathways by different membrane protein species.

Our findings provide compelling evidence that the urea transporter encoded by the HUT11 cDNA is the product of the Kidd locus. Both genes co-localize to chromosome 18q12-q21 and immunochemical analysis revealed that anti-HUT11 and anti-Jk^3 antibodies behave similarly when tested *in vitro* on translated products or on erythrocyte membrane preparations from Jk-positive and Jk(a−b−) individuals. Clearly, Jk(a−b−) red cells are characterized by the total absence of the Kidd/urea transport protein and a selective defect of the urea transport capacity. Because the urea transport in human kidney is critical to the urinary concentration mechanism, the question is raised why Jk(a−b−) individuals do not exhibit a severe clinical syndrome, although recent investigations indicate that these patients have impaired urea recycling and a reduced concentrating ability (27). There are at least three possible explanations; (i) the maximal concentrating ability is rarely required in normal condition and the loss of the urea contribution does not strongly reduce the cortico-papillary osmotic gradient; (ii) other urea transporters (*v.g.* an antidiuretic hormone-sensitive one) expressed in the kidney may compensate for the lack of the Kidd/urea transport system. A similar situation exists for the water channels, since Co(a−b−) individuals do not suffer any apparent clinical consequence (26); (iii)

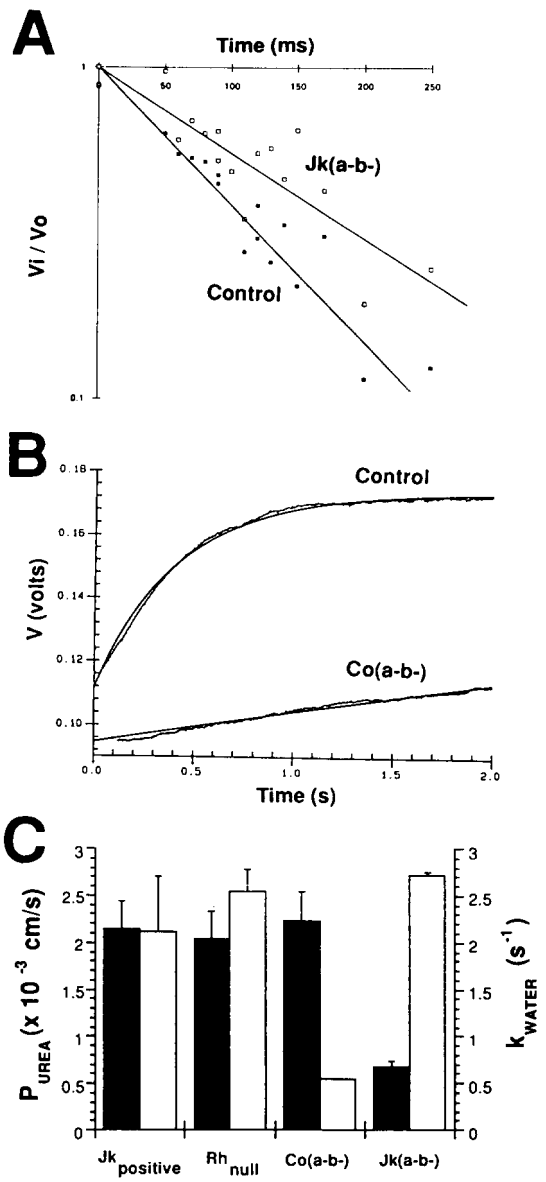

FIG. 5. **Urea and water permeability of ghosts and intact cells from Jk positive, Rh_{null}, Co(a−b−) and Jk(a−b−) human erythrocytes.** *A*, time courses of urea efflux from [^{14}C]urea-loaded resealed ghosts (control and Jk(a−b−)] expressed as the amount of [^{14}C]urea remaining in the vesicles (V_i/V_0). *B*, typical plot of water efflux from red cells placed in hyperosmotic solutions [control and Co(a−b−)]. Experimental time courses of red cell shrinkage are presented. *C*, average plot of all urea and water permeability measurements. Each *bar* represents the mean ± S.E. of 2–3 determinations (urea) or of 6–10 determinations (water), except for the unique Co(a−b−) sample for which only one set of assays was carried out.

Jk(a−b−) individuals have a defect of the urea transporter in erythroid tissues only. A similar situation has been reported for the Duffy antigen/chemokine receptor which has a wide tissue distribution but is absent only from red cells of Fy(a−b−) individuals (28, 29).

Other important biological implications of these studies include an evaluation of the potential role of Kidd antigens in kidney transplantation and a new approach to identify the type 1 diabetes susceptibility gene present on human chromosome 18 (30, 31).

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