Obligatory Amino Acid Exchange via Systems b⁰⁺-like and y⁺L-like
A TERTIARY ACTIVE TRANSPORT MECHANISM FOR RENAL REABSORPTION OF CYSTINE AND DIBASIC AMINO ACIDS*

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Obligatory exchange of amino acids results from the transfer of one amino acid with another amino acid or a small organic molecule. While the amino acid exchanger introduces the transport of some neutral amino acids into the renal proximal tubules (3), the amino acids that induce this transport activity have not been fully clarified. Here we have the following observations:

1. The amino acid exchanger in the renal proximal tubular cell line OK (13) shows that the amino acids that induce this activity are acidic and dibasic amino acids. These amino acids are the substrates of the system b⁰⁺-like (14) and y⁺L-like (15) transport systems.

2. The system b⁰⁺-like transport was first characterized in oocytes expressing rabbit rBAT (16). Very recently, it has been shown that the transport activity expressed by rabbit (16) and rat (17) rBAT cRNA in oocytes is mediated by amino acids. Therefore, the transport activity expressed by rabbit (16) and rat (17) rBAT cRNA in oocytes is mediated by amino acids in the renal proximal tubules of the nephron.

These observations indicate that the amino acid transport system b⁰⁺-like is responsible for the transport of amino acids in the renal proximal tubules.

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Mutations in the rBAT gene cause type I cystinuria, a common inherited aminoaciduria of cystine and dibasic amino acids due to their defective renal and intestinal reabsorption (Calonge, M. J., Gasparini, P., Chillarón, J., Chillón, M., Gallucci, M., Roussaud, F., Zelante, L., Testar, X., Dallapiccola, B., Di Silverio, F., Barceló, P., Estivill, X., Zorzano, A., Nunes, V., and Palacín, M. (1994) Nat. Genet. 6, 420–426; Calonge, M. J., Gasparini, P., Chillarón, J., Zelante, L., Rousaud, F., De Sanctis, L., Beccia, E., Zelante, L., Testar, X., Zorzano, A., Estivill, X., Gasparini, P., Nunes, V., and Palacín, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9667–9671). One important question that remains to be clarified is how the apparently non-concentrative system bo, be clarified is how the apparently non-concentrative system y⁺L-like is coupled enough to ensure a specific accumulation of substrates until the complete replacement of the internal oocyte substrates. Due to structural and functional analogies of the cell surface antigen 4F2hc, respectively, in the epithelial cells of the proximal tubule of the nephron.

Mutations in the human rBAT gene are responsible for classic cystinuria (1–3). This is a common inherited aminoaciduria due to the defective transport of cystine and dibasic amino acids through the epithelial cells of the renal tubule and intestinal tract (4). Patients show urinary hyperecretion of dibasic amino acids and cystine but not of other neutral amino acids; the low solubility of cystine leads to its precipitation and the consequent formation of renal calculi (4). Three types of classic cystinuria have been described on the basis of the amino acid hyperecretion of heterozygotes and the degree of the intestinal transport defect (5–6). It has been demonstrated that rBAT is only responsible for type I, where heterozygotes are silent (7–8).

The rBAT protein is located in the brush border plasma membrane of the proximal straight tubules of the nephron and of the small intestine (9–10). Due to the role of rBAT in cystinuria, it is considered to be responsible for the reabsorption of cystine and dibasic amino acids in the proximal straight tubule. Human rBAT expressed in Xenopus oocytes elicits high affinity sodium-independent transport of cystine, dibasic amino acids, and some neutral amino acids via a b⁰⁺-like transport system (11–12). Very recently, the responsibility of rBAT for this amino acid transport activity has also been demonstrated in the renal proximal tubular cell line OK (13). We refer to this as system b⁰⁺-like, since this activity is very similar to system b⁰⁺-like described in mouse blastocystes (14); these transport activities are not identical, since the latter does not transport L-cystine. An electrogenic exchange diffusion mechanism for dibasic and neutral amino acids has been reported for the transport activity expressed by rabbit (15–16) and rat (17) rBAT cRNA in oocytes. Coady and collaborators (16) showed that neutral amino acids in the trans-side are needed to observe the currents associated with the transport of L-arginine in oocytes expressing rabbit rBAT. Rennie and collaborators (17) suggested that this hetero-exchange could not fully explain the electric activity associated with the induced neutral amino acid transport due to rat rBAT expression in oocytes. In addi-
tion, it has been suggested that an exchange mechanism of transport does not fit the proposed role for system b\(^{+}\)-like/rBAT in the active renal and intestinal amino acid reabsorption (15, 16, 18). Therefore, the concentrative mechanism of amino acid transport that explains the role of system b\(^{+}\)-like in the active renal reabsorption of dibasic amino acids and cystine remains to be clarified. A further objective is the identification of the transport mechanisms linking the structurally related rBAT and 4F2hc proteins (19–20).

Here we offer evidence that system b\(^{+}\)-like, associated with rBAT expression in oocytes, is an obligatory amino acid exchanger that accumulates substrates as a tertiary amino acid transporter. Hetero-exchange between dibasic and neutral amino acids fully explains the electric activity associated with the induced amino acid transport due to human rBAT expression in oocytes, and the exchange of dibasic amino acids (inward) with neutral (outward) amino acids is favored. This amino acid transport activity explains the role of rBAT/system b\(^{+}\)-like in cystinuria. In addition, we offer evidence that the human cell surface antigen 4F2hc also induces, in oocytes, an asymmetric obligatory amino acid exchanger (system y\(^{-}\)L-like) between dibasic (outward) and neutral amino acids (inward).

The participation of these two amino acid transport systems in renal reabsorption is discussed.

MATERIALS AND METHODS

Oocytes, Injections, and cDNA Synthesis—Oocyte origin, management, and injections were as described elsewhere (11). Defolliculated stage VI Xenopus laevis oocytes were injected with saturating concentrations (1–5 ng/oocyte) of human rBAT, human 4F2hc, or mouse CAT1 cDNA. Except where indicated, uninjected oocytes were used as controls; amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those of uninjected oocytes (data not shown). Human rBAT, human 4F2hc (cDNA cloned in EcoR-HindIII pSPORT-1) from the original cDNA cloned in pSP65 by Teixeira and collaborators (21), and mouse CAT1 cDNAs is described elsewhere (11, 22).

Oocyte Uptake Studies—Influx rate measurements of L-[\(^{3}\)H]arginine, L-[\(^{3}\)H]leucine, and L-[\(^{35}\)S]cystine (NEN Radiochemicals) were measured in 100 mM NaCl or 100 mM choline Cl medium at the indicated days after injection and in linear conditions as described elsewhere (11, 19, 23). When presented, cRNA (rBAT, 4F2hc, or CAT1)-induced uptake was calculated by subtracting uptake values in uninjected oocytes from those of the corresponding cRNA-injected oocytes.

For efflux rates measurements, 3 or 4 days after injection of the corresponding cRNA, groups of 5–7 oocytes were incubated, at 25 °C, for 30 min (rBAT and 4F2hc experiments) or 45 min (4F2hc experiments) in medium containing 50 μM L-[\(^{3}\)H]arginine or L-[\(^{3}\)H]leucine (3–10 μCi/10 μl). More than 95% of the oocyte-soluble radioactivity corresponded to the original labeled amino acid (see below). In five independent rBAT experiments, this loading ranged between 145,000 and 295,000 cpm/rBAT-injected oocyte and 139,000 and 315,000 cpm/4F2hc-injected oocyte for L-[\(^{3}\)H]arginine and L-[\(^{3}\)H]leucine uptakes, respectively. In the 4F2hc experiments (n = 4), this loading ranged between 50,000 and 121,000 cpm/4F2hc-injected oocyte and 35,000 and 133,000 cpm/4F2hc-injected oocyte for L-[\(^{3}\)H]arginine and L-[\(^{3}\)H]leucine uptakes, respectively. For those experiments, loading of uninjected oocytes ranged between 8,000 and 21,000 cpm/oocyte and 27,000 and 88,000 cpm/oocyte for L-[\(^{3}\)H]arginine and L-[\(^{3}\)H]leucine uptakes, respectively. After this loading, the radioactive medium was washed 4 times in choline medium at 25 °C. Then, efflux was measured as the appearance of tritium in unlabeled incubation medium (0.6–1 ml of sodium or choline medium as indicated) containing no amino acids (none) or different L-amino acids at the indicated concentrations. When L-cystine was used, efflux was measured in the presence of 10 mM diamide to prevent cystine reduction. In these conditions, diamide did not affect efflux by uninjected oocytes (data not shown). Efflux was measured with aliquots (200 μl) from the medium at zero time and at different times. Efflux rates were calculated by subtracting the radioactivity present at zero time. Previous studies demonstrated that after subtracting the zero value the best fit line passed through the origin, and it was linear for 1, 2, or 5 min for CAT1-injected, rBAT-injected, or 4F2hc-injected oocytes, respectively (data not shown). Efflux rates are expressed either as the radioactivity (cpm × 1,000) appearing in the medium per unit time (2 or 5 min) per group of 5–7 oocytes, when representative experiments are shown, or as the percent of the total radioactivity loaded into the oocyte appearing in the medium per unit time (2 or 5 min), when combined experiments are shown. Statistical comparisons were performed using the Student’s t test.

In the accumulation studies, the radioactivity content of rBAT cRNA-injected oocytes after 3 h incubation with L-[\(^{3}\)H]arginine, L-[\(^{3}\)H]leucine, or L-[\(^{35}\)S]cystine (in the presence of 10 mM diamide) was tested for metabolism. Oocyte homogenates (choline medium) were precipitated with 5% trichloroacetic acid. More than 97% of the radioactivity from L-[\(^{3}\)H]arginine- and L-[\(^{35}\)S]cystine-incubated oocytes remained in the soluble phase; for L-[\(^{3}\)H]leucine-incubated oocytes 15% of the radioactivity was trichloroacetic acid-precipitated, suggesting incorporation into proteins. In the L-[\(^{35}\)S]cystine experiments, oocytes were homogenized in the presence of 20 mM N-ethylmaleimide (NEM)\(^{1}\) to analyze L-[\(^{35}\)S]cystine metabolites-NEM derivatives, as described elsewhere (25). The soluble phase of trichloroacetic acid precipitation was analyzed by thin layer chromatography as described elsewhere (Ref. 26 for L-[\(^{3}\)H]arginine and L-[\(^{3}\)H]leucine experiments and Ref. 25 for L-[\(^{35}\)S]cystine experiments). In all cases, >95% of the radioactivity showed the same chromatographic mobility as the original incubated amino acids, visualized with 0.2% ninhydrin in acetone (L-[\(^{3}\)H]arginine and L-[\(^{3}\)H]leucine experiments) or by autoradiography (L-[\(^{35}\)S]cystine experiments) (data not shown). In the absence of diamide, almost all L-[\(^{35}\)S]cystine radioactivity was recovered, in both the oocytes and the washed medium, as a product with identical chromatographic mobility to the L-[\(^{35}\)S]cystine-NEM derivative.

As a reference value for the space distribution of amino acids in the oocyte in the accumulation studies, the space distribution of water was measured by incubating groups of 7–8 oocytes with [\(^{3}\)H]water (2 μCi; \(\approx 2.4 \times 10^{5}\) cpm/90 μl; Amersham Corp.) for up to 30 min. After incubation, oocytes were washed 3 times in ice-cold choline medium, and the radioactivity of SDS-dissolved single oocytes was counted with scintillation fluid in a β-radioactivity counter, as described elsewhere for uptake studies in oocytes (23). The uptake of [\(^{3}\)H]water increased from 30 s to 2 min and then reached a plateau (4,000–5,000 cpm), which was maintained for the next 30 min. From this we estimated a space distribution of water in stage VI oocytes of 176 ± 14 nl (mean ± S.E. from 10 groups of oocytes in 2 independent experiments).

Oocyte Electric Measurements—Dissection of X. laevis, collection, and handling of the oocytes was described in detail elsewhere (27). Oocytes were injected with cRNA (1 ng/oocyte) or water, and two-electrode voltage and current clamp recordings were performed 3–8 days later in single oocytes in a perfused chamber, as described elsewhere (15). The external control solution (ND96 medium) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl\(_2\), and 5 mM HEPES at pH 7.5. In some experiments, Na\(^{+}\) was replaced by choline. The amino acids were added to the solution at the indicated concentrations, and the tested oocyte was perfused at 20 μl/min, which guaranteed a complete solution exchange in the recording chamber within 10–15 s. The maximal current amplification during amino acid superfusion was recorded as inward currents and outward currents are shown with the prefix -. All data are given as means (± S.E.), where n gives the number of oocyte measurements. Statistical comparisons were performed using the Student’s t test. Experiments were repeated with 2 batches of oocytes; in all repetitions, qualitatively similar data were obtained.

OK Cell Studies—The OK cell line was obtained from Dr. R. D. Goldman (28), derived by selection from the original OK cells (29), between passages 16 and 21, was used in this study. Selected OK cell clones (13) that express human rBAT antisense (AS1) and sense (S1) sequences after permanent transfection of a 669-base pair EcoRI/ClaI fragment from the 5’-end of the full-length human rBAT cDNA (11) were also used. Cell culture conditions were as described (13).

Efflux rates measurements of L-[\(^{3}\)H]arginine into MGA medium (137 mM N-methyl-D-glucamine, 5.4 mM KCl, 2.8 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 10 mM HEPES, pH 7.4) containing or not containing unlabeled L-amino acids were as described elsewhere (13). Results are expressed as cpm, corrected per 150,000 cpm loaded/mg of protein. Statistical comparisons were performed using the Student’s t test. Thin layer chromatography analysis (26) of the efflux medium revealed that ~95% of the efflux radioactivity corresponded to L-arginine (data not shown).

Simulation of the b\(^{+}\)-like Amino Acid Exchanger—To simulate the L-[\(^{3}\)H]-amino-acid, accumulation experiments were performed in oocytes expressing the human rBAT cDNA; we constructed a model based on

\(^{1}\) The abbreviations used are: NEM, N-ethylmaleimide; CAT1, cati-

onic amino acid transporter 1.
the following premises. 1) The induced amino acid transport activity is an obligatory exchanger of dibasic and neutral amino acids with a 1:1 stoichiometry. 2) An endogenous, independent, and labile transport system is necessary to explain the amino acid transport of uninjected oocytes. Fig. 1 shows the obligatory exchange “ping-pong” mechanism considered here for system b^0^+^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^
Amino Acid Exchange via Systems b^0^+^ and y^+^L-like

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**TABLE I**

Kinetic parameters of system b^0^+^like amino acid exchanger activity associated with rBAT expression in oocytes

| Substrate | L-Cystine | L-Arginine | L-Leucine |
|-----------|-----------|------------|-----------|
| V_{max}   |           |            |           |
| (pmol/min/oocyte) | | | |
| 41 ± 7   | 85 ± 7    | 90 ± 12    |
| K_{m}     |           |            |           |
| (µM)      | | | |
| 9 ± 1    | 211 ± 1   | 59 ± 4     |

**FIG. 2.** L-[3H]Arginine and L-[3H]leucine efflux via amino acid transport system b^0^+^like in oocytes. Oocytes were injected with 5 ng of rBAT cRNA (black bars) or uninjected oocytes (open bars). Four days later, amino acid efflux rates were measured in choline medium containing no amino acids (none) or the indicated 1 mM L-amino acids. 200 µM L-cystine (CysC) was used in the presence of 10 mM diamide (A). L-[3H]arginine efflux rates (expressed as the radioactivity (cpm x 1000) appearing into the medium per 2 min per group of 6 oocytes) correspond to the mean ± S.E. of triplicates from a representative experiment. All the L-amino acids, but L-proline and L-glutamate, in the medium significantly increased efflux from rBAT cRNA-injected, but not from uninjected oocytes (p < 0.01). B, L-[3H]Arginine and L-[3H]leucine efflux rates into unlabeled medium containing no amino acids (none) or 1 mM L-arginine (Arg) from rBAT cRNA-injected or uninjected oocytes. Efflux rates (i.e. radioactivity appearing in the medium per 2 min) are lower than in uninjected oocytes (>3% in 2 min) (Fig. 2B). This suggests that either rBAT expression results in the retention of L-arginine inside the oocyte or L-[3H]arginine uptake reaches two different pools in the oocyte, one of these pools being quantitatively important for the uptake measured in uninjected oocytes but not in rBAT-injected oocytes. In any case, L-[3H]arginine efflux was increased 8-fold by 1 mM L-arginine only in oocytes expressing rBAT (Fig. 2B). This demonstrates that L-[3H]arginine efflux via system b^0^+^like is also dependent on the presence of amino acid substrates on the trans-side.

Next, the kinetic parameters of the amino acid transport activity (efflux and influx) induced by rBAT in oocytes were measured. L-Arginine- and L-leucine-elicited efflux of L-[3H]arginine showed saturation (data not shown) with similar apparent Km values for the external amino acids (Table I). Interestingly, the apparent Km values for L-arginine and L-leucine either acting as substrates for influx or stimulating efflux of L-[3H]arginine were similar (µM range, Table I). This is expected for an obligatory exchanger, and it is consistent with an allosteric mechanism of trans-stimulation of efflux. For all the substrates, when efflux or influx was measured, the Hill coefficient was never significantly different from 1 (data not shown), suggesting interaction of one molecule of external substrate per functional molecular unit of transporter. The V_{max} values for influx via system b^0^+^like in oocytes ordered the three substrates considered as follows, L-arginine > L-leucine > L-cystine. Comparison of this with the potency of these amino acids to elicit L-arginine efflux (Fig. 2A and Table I) suggested that in the hetero-exchange events via system b^0^+^like the "slowest" substrate (i.e. L-cystine) limits the transport activity.

The System b^0^+^like Associated with rBAT Expression in OK Cells also Behaves as an Amino Acid Exchanger—We have shown in a previous study that the rBAT gene is necessary for the amino acid transport system b^0^+^like activity in the apical pole of the renal proximal tubular cell line OK; permanent transfection of antisense rBAT sequences results in a specific decrease (60% inhibition in the antisense clone AS1 but not in the sense clone S1) in this transport activity (13). The substrate specificity of the stimulation of L-[3H]arginine efflux in OK cells

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were obtained when the efflux rates of L-[3H]leucine were measured (data not shown). The efflux rates of L-[3H]arginine and L-[3H]leucine from several experiments, and expressed as percent of the previous loading, are shown in Fig. 2B. The efflux rates of L-[3H]leucine into amino acid-free medium were identical in rBAT-injected and uninjected oocytes, whereas they were increased ≤ 7-fold by 1 mM L-arginine only in oocytes expressing rBAT (Fig. 2B). This supports the idea that system b^0^+^like is an obligatory amino acid exchanger; transport via system b^0^+^like occurs only when substrates are present on the trans-side. A similar interpretation of the efflux rates of L-[3H]arginine is difficult to postulate. The efflux rates of L-[3H]arginine, expressed as percent of the previous loading, into medium containing no amino acids in rBAT-injected (>1% in 2 min) are lower than in uninjected oocytes (~3% in 2 min) (Fig. 2B). This suggests that either rBAT expression results in the retention of L-arginine inside the oocyte or L-[3H]arginine uptake reaches two different pools in the oocyte, one of these pools being quantitatively important for the uptake measured in uninjected oocytes but not in rBAT-injected oocytes. In any case, L-[3H]arginine efflux was increased 8-fold by 1 mM L-arginine only in oocytes expressing rBAT (Fig. 2B). This demonstrates that L-[3H]arginine efflux via system b^0^+^like is also dependent on the presence of amino acid substrates on the trans-side.

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the none, L-proline, and L-glutamate groups.

was similar to that of system b\textsuperscript{0,+}-like expressed in oocytes (Figs. 2A and 3). L-Leucine-elicted L-[\textsuperscript{3H}]arginine efflux decreased by 60% in the rBAT-sense-transfected clone (16,800 ± 2,400 and 6,500 ± 700 cpm/mg protein-min in the rBAT-sense S1 and rBAT-antisense AS1 clones, respectively; n = 6). In contrast, L-[\textsuperscript{3H}]arginine efflux into medium containing 1 mM L-glutamate, which is not a system b\textsuperscript{0,+}-like substrate (Figs. 2A and 3), was not affected by antisense expression (L-[\textsuperscript{3H}]arginine efflux was 1,490 ± 840 and 1,480 ± 580 cpm/mg protein-min in the rBAT-sense S1 and rBAT-antisense AS1 clones, respectively, n = 6). This indicates that L-[\textsuperscript{3H}]arginine efflux via system b\textsuperscript{0,+}-like does not occur in the absence of substrates in the medium and demonstrates that L-leucine-elicted L-[\textsuperscript{3H}]arginine efflux occurs via system b\textsuperscript{0,+}-like (associated with rBAT expression). Kinetic analysis of this efflux showed saturability via a single component with an apparent K\textsubscript{m} value of 295 ± 64 μM (data not shown). This value fits reasonably well with the estimated apparent K\textsubscript{m} value for the influx of L-leucine through the component inhibited by L-arginine (175 ± 56 μM; Ref. 13). Then, as already shown in the oocyte studies, system b\textsuperscript{0,+}-like associated with rBAT behaves as an obligatory exchanger in OK cells.

The System b\textsuperscript{0,+}-like Accumulates Its Substrates in the Oocytes as a Tertiary Active Transporter—An obligatory amino acid exchanger is considered a tertiary active transport mechanism; it accumulates substrates as a result of exchange with amino acids on the trans-side. It has been reported that stage VI Xenopus oocytes contain a high amount of free amino acids that are substrates of system b\textsuperscript{0,+}-like (31). If the degree of coupling of exchange of system b\textsuperscript{0,+}-like expressed in oocytes is high enough, uptake via this system should result in the accumulation of these substrates in the oocyte. Uptake studies of 50 μM L-[\textsuperscript{3H}]arginine, L-[\textsuperscript{3H}]leucine, or L-[\textsuperscript{35S}]cystine during long incubation periods (3–6 h) showed a higher plateau of accumulation of these substrates in oocytes expressing rBAT than in uninjected or CAT1-injected oocytes (i.e. oocytes expressing the dibasic amino acid transport y\textsuperscript{-}; Ref. 22) (data not shown and Fig. 4). Similarly, L-[\textsuperscript{3H}]arginine accumulation reached higher levels in 4F2hc-injected oocytes than in uninjected or CAT1-injected oocytes (data not shown); these data are discussed below. Similar uptake values by rBAT-injected oocytes were obtained in the absence and in the presence of sodium (data not shown). With an oocyte space distribution for polar substrates of ~180 nI (see "Material and Methods"), the plateau of uptake values of 50 μM L-[\textsuperscript{3H}]arginine uptake represents a 55-fold accumulation (~40-fold for L-[\textsuperscript{3H}]leucine or L-[\textsuperscript{35S}]cystine uptake) of the substrate in rBAT-injected oocytes (Table II). In contrast, the level of L-[\textsuperscript{3H}]arginine accumulation in CAT1-injected and in uninjected oocytes was 14–20-fold (Table II). This demonstrates that under these conditions accumulation of substrates via system b\textsuperscript{0,+}-like is clearly higher than via system y\textsuperscript{-}.

If the accumulation of substrates via system b\textsuperscript{0,+}-like is due to exchange with the intracellular oocyte substrates, their total oocyte content (~1,000 pmol/oocyte; Ref. 31) would limit this accumulation. The increase in L-[\textsuperscript{3H}]arginine concentration from 10 to 1,000 μM resulted in a nonlinear increase in L-arginine accumulation, which reached a maximum of ~1,000 pmol/oocyte at ~500 μM L-arginine (Fig. 4). This is at odds with the uptake via system y\textsuperscript{-}, associated with CAT1 expression, which increased from ~140 pmol/oocyte at equilibrium with 50 μM L-[\textsuperscript{3H}]arginine to ~750 pmol/oocyte, without reaching equilibrium after 4 h, with 500 μM L-[\textsuperscript{3H}]arginine (data not shown). This level of L-arginine uptake is nearly 4 times the reported dibasic amino acid content of stage VI oocytes (31). To test whether a tertiary active transport mechanism could explain the accumulation of substrates in rBAT-injected oocytes, we simulated the L-[\textsuperscript{3H}]arginine accumulation curves shown in Fig. 4 with a model that considers system b\textsuperscript{0,+}-like as an obligatory exchanger with 1:1 stoichiometry (see "Material and Methods"). Interestingly, this modeling reproduces the experimental results (see lines in Fig. 4). At the highest L-[\textsuperscript{3H}]arginine concentration used (i.e. 1 mM), the model predicts that at equilibrium nearly 98% of the internal substrates of system b\textsuperscript{0,+}-like have been replaced by L-arginine with the initial spe-
Amino Acid Exchange via Systems $b^\text{0,+] and y'^{L-like}$

**Table II**

| Substrate | Un-injected | rBAT | 4F2hc | CAT1 |
|-----------|-------------|------|-------|------|
| L-Arginine| 14 ± 1      | 55 ± 5 | 34, 39 | 19 ± 2 |
| L-Leucine | 19 ± 3      | 48 ± 11 | 27, 37 |       |
| L-Cystine | 1.1 ± 0.6   | 40 ± 42 |       |       |

Accumulated gradient of amino acids at equilibrium

Oocytes were injected (1-5 ng/oocyte) with cRNA (rBAT, 4F2hc, or CAT1) or noninjected, 3-4 days later the uptake of 50 μM L-[(35S)cystine, L-[(3H)]leucine, or L-[(3H)]arginine was measured in groups of 6-8 oocytes at equilibrium (i.e., 3-6 h of uptake incubations). The accumulated gradient of substrates into the oocyte expressed as times the initial concentration of substrate in the medium, was calculated assuming a space distribution of [H2O]water of 176 ml (see "Materials and Methods").

Data for rBAT-, CAT1-, and un.injected oocytes are the mean ± S.E. The corresponding to 6-15 determinations (3-7 independent experiments).

**Table III**

Simulated internal concentrations and specific activities during L-[(3H)]arginine accumulation in oocytes expressing rBAT

| Internal concentration (μM) | Time = 0 | Time = 4 h |
|----------------------------|----------|-----------|
| L-Arginine                 | 697      | 5653.0 (97.7%) |
| L-Leucine                  | 231      | 3.1 (0.05%)  |
| L-Neutal                   | 4288     | 64.3 (1.1%)   |
| L-Dibasic                  | 574      | 63.0 (1.1%)   |
| Total                      | 5790     | 5783.4      |

**The Amino Acid Transport System y' L-like Behaves as an Obligatory Exchanger with Asymmetry—Due to the structural and functional homology between rBAT and 4F2hc, we tested for the accumulation of substrates in 4F2hc-injected oocytes.** As indicated above, uptake of 50 μM L-[(3H)]arginine or L-[(3H)]leucine reached levels of accumulation in 4F2hc-injected oocytes higher that those obtained via system y' or in uninjected oocytes (Table II). These data demonstrated an active mechanism of transport for system y' L-like associated with 4F2hc expression in oocytes. Interestingly, L-arginine and L-leucine, but not L-tryptophan, in the medium stimulated efflux of L-[(3H)]arginine via system y' L-like, associated with 4F2hc expression in oocytes (Fig. 7A). As already shown (Fig. 2), these amino acids did not stimulate efflux in un.injected oocytes (Fig. 7). L-Leucine-elicited efflux of L-[(3H)]arginine in 4F2hc-injected oocytes was barely detectable at 0.1 mM in the absence of sodium (choline medium), but it increased dramatically in the presence of sodium; the sodium effect was much less apparent at 10 mM L-leucine (Fig. 7A). This substrate specificity corresponds to that of influx via system y' L-like in oocytes. Thus, the 4F2hc-induced influx of 100 μM L-[(3H)]leucine was 1 ± 0.3 pmol/min per oocyte in the absence of sodium, and 34 ± 3 pmol/min per oocyte in the presence of 100 mM sodium (n = 6 oocytes). In agreement with this, the y' L amino acid transport activity described in human erythrocytes and placenta carries dibasic amino acids with high affinity, neutral amino acids, like L-leucine, with high affinity only in the presence of sodium, but not L-tyrosine (36-38). All this demonstrated that efflux via the y' L-like transport activity associated with 4F2hc expression in oocytes is highly dependent on the presence of substrates on the trans-side. This, together with the effective accumulation of substrates, such as L-arginine and L-leucine, in oocytes expressing 4F2hc strongly suggests that system y' L-like is a tertiary active amino acid transport system with an obligatory exchanger mechanism.

Next, we studied L-[(3H)]leucine efflux via system y' L-like associated with 4F2hc expression in oocytes. To our surprise, L-[(3H)]leucine efflux was not stimulated by external L-arginine (Fig. 7B) or L-leucine (data not shown). Thus, efflux rates, expressed as percent of the radioactivity loaded per 5 min, by 4F2hc-injected (3.3% ± 0.2 in the absence of external amino acids and 3.4% ± 0.3 in the presence of 1 mM L-arginine, n = 3

The main conclusion of this theoretical study is that accumulation of L-[(3H)]arginine in rBAT-injected oocytes can be fully explained by an obligatory exchanger of 1:1 stoichiometry, in which the driving force of the accumulation is the high internal concentration of amino acids. An interesting consequence of this is that we can almost completely exchange the internal content of substrates of system b0,1-like substrates, respectively (data not shown).

As indicated above, uptake of 50 μM L-[(3H)]arginine or L-[(3H)]leucine for 3 h results in 95 and 79% replacement of the internal oocyte system b0,1-like substrates, respectively (data not shown).

This was tested experimentally. Indeed, continuous superfusion of rBAT-injected oocytes with 50 μM L-arginine or L-leucine for 3 h resulted in a dramatic decrease in the inward positive current elicited by L-arginine and the outward positive current elicited by L-leucine, respectively (Fig. 5). In these conditions, L-arginine- and L-leucine-induced currents tended to be zero. This indicates that all the electric activity of system b0,1-like is due to the hetero-exchange between neutral and dibasic amino acids and that the stoichiometry of the amino acid homo-exchange is n.n. In contrast, superfusion with L-arginine increased the L-leucine-induced currents, and superfusion with L-leucine increased L-arginine-induced currents (Fig. 5). In these conditions, at -50 mV membrane potential, the maximal L-arginine-induced currents (i.e. by exchange with the internal L-leucine) are approximately twice as strong as the maximal L-leucine-induced currents (i.e. by exchange with the internal L-arginine). This demonstrates that the exchange via system b0,1-like of L-arginine inward, L-leucine outward is favored versus the reverse direction.

To determine the stoichiometry of the exchange of amino acids via system b0,1-like, rBAT-injected oocytes were incubated for 4 h with 1 mM labeled L-[(3H)]arginine or L-[(3H)]leucine or the corresponding unlabeled substrates, and influx and L-amino acid-elicited efflux transport rates were measured immediately. Fig. 6 shows that, for every type of homo- and hetero-exchange between L-arginine and L-leucine, the transport rates for influx and for the L-amino acid-elicited efflux were identical. This demonstrates an n.n. stoichiometry for the obligatory exchange of amino acids via system b0,1-like. This stoichiometry is most probably 1:1 since for all the kinetic studies of L-arginine and L-leucine influx and efflux transport rates, the Hill coefficient was never different from 1 (data not shown). Again, the hetero-exchange L-arginine inward, L-leucine outward is favored versus the reverse direction of exchange (Fig. 6).
independent experiments) and uninjected oocytes (3.6% ± 0.7 in the absence of external amino acids and 3.2% ± 0.9 in the presence of 1 mM L-arginine) were similar. This is at odds with the 7-fold increase in L-[3H]leucine efflux due to trans-L-arginine in rBAT-injected oocytes (Fig. 2B). This demonstrates functional asymmetry of the amino acid exchange via system y^+L-like expressed in oocytes and suggests that hetero-exchange of arginine inward/neutral amino acid outward, if it occurs, is clearly weaker than the reverse hetero-exchange. To provide further evidence for amino acid hetero-exchange (i.e. neutral inward/dibasic outward) via system y^+L-like, the electronegativity of this system was studied in 4F2hc-injected oocytes. These oocytes, but not uninjected oocytes, showed a small but significant and reproducible positive outward current when 10 mM L-leucine was present in the external medium in the absence of sodium (1.2 ± 0.1 and -0.1 ± 0.1 nA for 4F2hc-injected and uninjected oocytes, respectively, n = 7–8 oocytes, p ≤ 0.01). In the presence of sodium, this current was not detectable (-1.7 ± 0.2 and -1.8 ± 0.3 nA for 4F2hc-injected and uninjected oocytes, respectively, n = 7–8 oocytes). This is most probably due to the activity of an endogenous sodium-dependent transporter for leucine and/or co-transport of sodium with L-leucine via system y^+L-like. In agreement with this, it has been shown that the placenta system y^+L is largely insensitive to alterations of the membrane potential, suggesting co-transport of sodium and neutral amino acids.

### Discussion

We have shown that amino acid transport systems b^0^+-like, associated with rBAT expression in oocytes and OK cells, and y^+L-like, associated with 4F2hc expression in oocytes, are highly coupled obligatory exchangers (i.e. tertiary active transporters). The exchange via systems b^0^+-like and y^+L-like is asymmetric, favoring the uptake and the release of dibasic amino acids, respectively. This offers a functional explanation for the role of system b^0^+-like in type I cystinuria and allows us to propose a role of system y^+L in the active efflux of dibasic amino acids.

Several studies have shown induction of the exchange of amino acids in oocytes expressing rabbit and rat rBAT expression (15–17). Coady and collaborators (16) described an obligatory hetero-exchange mechanism between dibasic and neutral amino acids via system b^0^+-like to explain the currents associated with the function of this transport system in oocytes. In contrast, Rennie and collaborators (17) suggested that this hetero-exchange could not fully explain the electric activity associated with the induced transport of neutral amino acids due to rat rBAT expression in oocytes. Here we demonstrate that the amino acid transport activity induced by human rBAT in oocytes can be fully explained by system b^0^+-like activity, as
Amino Acid Exchange via Systems b\textsuperscript{0}L-like and y \textsuperscript{+}L-like

Fig. 7. L-[\textsuperscript{3}H]Arginine efflux via amino acid transport system y \textsuperscript{+}L-like in oocytes. Oocytes were injected with 1 ng of 4F2hc cRNA (black bars). Uninjected oocytes (open bars) were used as controls. Four days after, rates of L-[\textsuperscript{3}H]arginine or L-[\textsuperscript{3}H]leucine efflux into sodium or choline medium were measured. In addition, medium contained no amino acids (none) or l-amino acids at the indicated concentrations (mM). Efflux rates are expressed as the radioactivity (cpm × 1,000) appearing in the medium/5 min per group of 6 or 7 oocytes. A, L-[\textsuperscript{3}H]arginine efflux rates. Data correspond to the mean ± S.E. of triplicates from a representative experiment. The presence of L-arginine (Arg) and L-leucine (Leu), but not L-tryptophan (Trp), in the medium increased significantly efflux by 4F2hc-cRNA-injected, but not by uninjected, oocytes (p < 0.05). B, L-[\textsuperscript{3}H]leucine efflux rates. Efflux rates by 4F2hc-injected and uninjected oocytes in the presence of L-arginine (Arg) were similar to those in the absence of amino acids or in the presence of tryptophan (Trp). Data correspond to the mean ± S.E. from triplicates from a representative experiment. Another 3 independent experiments gave similar results.

An obligatory exchanger, most probably with 1 (inward):1 (outward) stoichiometry for the homo- and hetero-exchange of its amino acid substrates. In addition, the expression of the rBAT gene in the renal proximal tubular cell line OK is also associated with system b\textsuperscript{0}L-like activity, with characteristics of obligatory amino acid exchange.

We have also shown that the amino acid exchange activity of system b\textsuperscript{0}L-like is tightly coupled and allows intracellular concentration of amino acid substrates until the complete replacement of the internal system b\textsuperscript{0}L-like substrates of the oocyte. The maximum level of accumulation of substrates via system b\textsuperscript{0}L-like (~ 1,000 pmol/oocyte) fits well with the reported content of free amino acid substrates of this system in stage VI oocytes (31). Interestingly, the level of accumulation of substrates at low \mu M concentration reached in rBAT-injected oocytes exceeds that obtained in uninjected or in CAT1-injected oocytes (i.e. expressing system y \textsuperscript{+}L-like amino acid transport activity). In contrast to system b\textsuperscript{0}L-like, system y \textsuperscript{+}L is an equilibrative transport activity that shows a high trans-stimulation effect (Ref. 24; 6-fold in CAT1-injected oocytes, data not shown), but with significant transport activity in the absence of substrates on the trans-side (24, 39), and which leads to a higher accumulation of substrates than that given by the membrane potential in oocytes (present study) and in fibroblasts (40). All this strongly suggests that system b\textsuperscript{0}L-like should be considered as a tertiary active transporter. In contrast, primary and secondary active transport mechanisms could not explain accumulation of substrates via system b\textsuperscript{0}L-like for the following reasons. (i) The cut-open oocyte model is able to show system b\textsuperscript{0}L-like activity in rBAT-injected oocytes without the addition of triphosphate nucleotides to the external perfusion system (16). (ii) Sodium is not necessary for the accumulation of substrates via system b\textsuperscript{0}L-like (present study), and neither sodium, potassium, nor chloride ions are needed for system b\textsuperscript{0}L-like activity in oocytes (11, 16, 23). Rennie and collaborators (17) suggested hetero-exchange of neutral amino acids (inward) and potassium (outward) in rat rBAT-injected oocytes. In contrast, both for human and for rabbit rBAT-injected oocytes, potassium does not affect the currents induced by L-arginine or L-leucine (15). In conclusion, system b\textsuperscript{0}L-like, associated with rBAT expression, is a tightly coupled exchanger with 1:1 stoichiometry. Whether system b\textsuperscript{0}L-like has a concerted or a ping-pong mechanism of exchange is beyond the scope of the present study and needs further research.

Here we provide evidence that system y \textsuperscript{+}L-like, associated with 4F2hc expression in oocytes, is an obligatory amino acid exchanger that mediates efflux of dibasic amino acids (e.g. L-arginine) and, in the presence of sodium, influx of neutral amino acids at \mu M concentration. It has been suggested (41) that the amino acid transport activity associated with human 4F2hc expression in oocytes is identical to the y \textsuperscript{+}L activity described in human erythrocytes and placenta (36–38, 42). In contrast to this general system, y \textsuperscript{+}L shows transport activity in the absence of substrates in the trans-side, as an equilibrative transporter with trans-stimulation (36–38). It is also possible that system y \textsuperscript{+}L may indeed be an obligatory exchanger because the functional isolation of system y \textsuperscript{+}L from the co-existing system y \textsuperscript{-}L in erythrocytes has been accomplished by NEM treatment, since the latter system is sensitive to the reagent, whereas the former system is resistant (42). A possible modification of the hypothetical coupled exchange mechanism of transport of system y \textsuperscript{+}L by NEM treatment has not been ruled out. In fact, mitochondrial exchangers, such as the ATD/ADP carrier, act as equilibrative transport systems after sulfhydryl reagent treatment (43).

To our knowledge this is the first study demonstrating active transport via systems y \textsuperscript{+}L-like and b\textsuperscript{0}L-like, associated with 4F2hc and rBAT expression in oocytes, respectively. These two amino acid transport systems are very alike. (i) Both are high affinity systems with a broad specificity for dibasic and neutral amino acids. (ii) Both proteins are homologous, with a similar hydrophobic profile and most probably linked by disulfide bridges to putative “light” subunits of 30–50 kDa (44–45). This fostered the hypothesis that both transporters are heterodimeric, both subunits being essential, but not sufficient, for the transport activity of systems b\textsuperscript{0}L-like and y \textsuperscript{+}L-like (46). The tertiary active transport mechanism shown here for these two transport systems indicates that they belong to a common family of obligatory amino acid exchangers.

A Role for the Amino Acid Exchanger Systems b\textsuperscript{0}L-like and y \textsuperscript{+}L-like in Renal Reabsorption—Recent studies have demonstrated that the human rBAT gene is responsible for type I cystinuria (1, 7–8). Patients show hyperexcretion of dibasic amino acids and cystine, but not of neutral amino acids, due to a defect in the active reabsorption of those amino acids in kidney (4). The tertiary active transport mechanism of the high affinity system b\textsuperscript{0}L-like, described here, explains the responsibility of rBAT in cystinuria. We propose a model for the role of system b\textsuperscript{0}L-like (rBAT) in the active renal reabsorption of cystine and dibasic amino acids by obligatory exchange with intracellular neutral amino acids (Fig. 8). The direction of

\textsuperscript{2}A. E. Busch, unpublished results.
exchange dibasic-inward/neutral-outward has been shown to be favored in the present study and agrees with the fact that hyperexcretion of neutral amino acids does not occur in cystinuric patients. The negative membrane potential, the intracellular reduction of L-cystine to L-cysteine, and the high intracellular concentration of neutral amino acids would be ensured by concentrative (Na⁺/cotransport) neutral amino acid transport activities in the apical pole (system Bo, neutral brush border) and the basolateral pole (systems ASC and others). T transporters shown in the scheme), t, sodium-independent neutral amino acid transporters (e.g., system L1). ATPase, Na⁺/K⁺-ATPase. Co-localization of the cell surface antigen 4F2hc and rBAT in the epithelial cells of the proximal straight tubule is hypothetical.

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Amino Acid Exchange via Systems b_{0}^{+} and y^{+}L-like

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