Relació estructura-funció en la família de transportadors d'aminoàcids heteromultimèrics. Identificació d'una nova família de transportadors lisosomals

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RELACIÓ ESTRUCTURA-FUNCIÓ EN LA FAMÍLIA DE TRANSPORTADORS D'AMINOÀCIDS HETEROMULTIMÈRICS IDENTIFICACIÓ D'UNA NOVA FAMÍLIA DE TRANSPORTADORS LISOSOMALS

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Obligatory amino acid exchange via systems $b^{0,+}$-like and y^+L-like. A tertiary active transport mechanism for renal reabsorption of cystine and dibasic amino acids.

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* Aquests autors han contribuit per igual.

El doctorand és autor de la figura 6 i coautor de les figures 2,4 i 7, i de les taules1 i 2. L'experiment de la figura 5 va ser dissenyat pel doctorand.

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Obligatory Amino Acid Exchange via Systems $b^{o,+}$-like and y^+L-like

A TERTIARY ACTIVE TRANSPORT MECHANISM FOR RENAL REABSORPTION OF CYSTINE AND DIBASIC AMINO ACIDS*

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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., Rousaud, 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., Volipini, V., Bisceglia, 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 $b^{o,+}$-like, associated with rBAT expression, participates in the active renal reabsorption of these amino acids. Several studies have demonstrated exchange of amino acids induced by rBAT in *Xenopus* oocytes. Here we offer evidence that system $b^{o,+}$-like is an obligatory amino acid exchanger in oocytes and in the "renal proximal tubular" cell line OK. System $b^{o,+}$-like showed a 1:1 stoichiometry of exchange, and the hetero-exchange dibasic (inward) with neutral (outward) amino acids were favored in oocytes. Obligatory exchange of amino acids via system $b^{o,+}$-like fully explained the amino acid-induced current in rBAT-injected oocytes. Exchange via system $b^{o,+}$-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 to rBAT, we tested for amino acid exchange via system y^+L-like. 4F2hc-injected oocytes accumulated substrates to a level higher than CAT1-injected oocytes (*i.e.* oocytes expressing system y^+) and showed exchange of amino acids with the substrate specificity of system y^+L and L-leucine-induced outward currents in the absence of extracellular sodium. In contrast to L-arginine, system y^+L-like did not mediate measurable L-leucine efflux from the oocyte. We propose a role of systems $b^{o,+}$-like and y^+L-like in the renal reabsorption of cystine and dibasic amino acids that is based on their active tertiary transport mechanism and on the apical and basolateral localization of rBAT and 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 hyperexcretion 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 hyperexcretion 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 $b^{o,+}$-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 $b^{o,+}$-like, since this activity is very similar to system $b^{o,+}$ described in mouse blastocysts (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-

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tion, it has been suggested that an exchange mechanism of transport does not fit the proposed role for system $b^{o,+}$-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 $b^{o,+}$-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 $b^{o,+}$-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 $b^{o,+}$-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 cRNA 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 cRNA. Except where indicated, noninjected 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). Synthesis of human rBAT, human 4F2hc (cDNA cloned in *Eco*RI-*Hin*dIII pSPORT-1, from the original cDNA cloned in pSP65 by Teixeira and collaborators (21)), and mouse CAT1 cRNAs 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 CAT1 experiments) or 60 min (4F2hc experiments) in medium containing 50 μM L-[^{3}H]arginine or L-[^{3}H]leucine (3–10 μCi/90 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/rBAT-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 always measured in the presence of 10 mM diamide to prevent L-cystine reduction. In these conditions, diamide did not affect efflux by uninjected oocytes (data not shown). Efflux was measured taking 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 metabolization. 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 medium, as a product with identical chromatographic mobility to the L-[^{35}S]cysteine-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; ~2.4 × 10^6 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 ml/min, which guaranteed a complete solution exchange in the recording chamber within 10–15 s. The maximal current amplitude induced during amino acid superfusion was measured. Inward 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 clone 3B/2 (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 *Eco*RI/*Cla*I fragment from the 5'-end of the full-length human rBAT cDNA (11) were also used. Cell culture conditions were as described (13, 30).

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·min. Statistical comparisons were done 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 $b^{o,+}$-like Amino Acid Exchanger—To simulate the L-^{3}H-amino-acid, accumulation experiments were performed in oocytes expressing the human rBAT cRNA; we constructed a model based on

[1] The abbreviations used are: NEM, *N*-ethylmaleimide; CAT1, cationic 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 equilibrative 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 $b^{o,+}$-like. An alternative concerted mechanism of obligatory exchange, tested in parallel, gave similar results for the accumulation behavior to the ping-pong mechanism. The computer program written to simulate the amino acid transport activity in oocytes expressing rBAT is available upon request.

The simulation was prepared according to the following steps.

(i) Characteristics of the simulated system. The experimental system of L-[^{3}H]arginine accumulation studies (Fig. 5) was reproduced as two separate compartments of 90 μl (the outside, uptake medium) and 180 nl per oocyte (the inside, the space distribution for [^{3}H]water, see above). Initial inner concentrations of the amino acid substrates of system $b^{o,+}$-like for stage VI *Xenopus* oocytes were set according to Taylor and Smith (31). The concentrations of L-arginine (and L-[^{3}H]arginine), L-leucine, and L-cystine were treated individually, whereas the rest of the amino acids were considered in two groups, neutral and dibasic. Transport rates were evaluated from the relative concentrations of transporter binary and empty complexes at either side, according to the formalism of Cha (32), which allows us to combine equilibrium and steady-state steps in a single mechanism. Binding steps were considered as equilibria (defined as dissociation constants, K_o, outside and K_i, inside), whereas translocation steps were considered as steady-state (defined as two translocation rate constants, k_{in}, k_{out}) (Fig. 1). During the simulation, amino acid concentrations at either side were modified by numerical integration of the transport rates. The equilibrium assumptions do not significantly alter the calculated rates with respect to a full steady-state mechanism. For the experiment shown in Fig. 6, superfusion experimental conditions were simulated by fixing the external concentrations to the initial values, and only internal concentrations were changed. This is reasonable as the external solution is exchanged in 10–15 s (see above).

The transport rates of dibasic amino acids are markedly influenced by the membrane potential (Φ). The effect of this parameter was introduced by modification of dissociation and translocation constants according to the following equation:

$$P_i = P_i^0 exp\{\partial Z_i F\Phi/\pi RT\} \quad \text{(Eq. 1)}$$

where P_i is the given parameter, P_i^0, its value at zero potential, Z_i, the amino acid charge, F, 96,500 coulomb/mol, R, 8.31 J/mol·K, and T, the absolute temperature (fixed to 298 K), n was set to 1 for equilibrium constants, and 2 for rate constants. ∂ represents the effective fraction of the membrane potential that influences the step indicated by P. This fraction was arbitrarily set to 0.2 for binding and to 0.6 for translocation steps. This parameter (∂) has little influence on the accumulation curves (data not shown). Membrane potential was fixed to −50 mV for resting oocytes and to −35 mV for 50 μM L-arginine uptake (see legend to Fig. 5). Initial potentials for other concentrations were varied according to the expected initial rates. The membrane potential was returned to the resting value after some time (see legend to Fig. 5). This effect was also included in the simulation by using a time constant of 100 min, which is a good representation of the experimental evolution.

(ii) Amino acid kinetic parameters. The simulation system prepared as indicated above permits us to follow the evolution of amino acid concentrations and, therefore, to simulate experimental influx and efflux rates in any experimental condition. However, before using the system, values are required for the kinetic parameters (see Fig. 1) for every amino acid and the concentration of transporters. No direct experimental determination of transporter concentration is available; therefore, V_{max} values as reported in Table I represent inseparable combinations of the transporter concentration and the translocation constants. For this reason, the translocation constants (k_{in} and k_{out}) for L-arginine were set arbitrarily to 1, and used as reference. The translocation constants for the remaining amino acids were then used as relative values. In this way, changes in the transporter concentration are represented by a single parameter (adjusted as indicated below), which affects every amino acid in the right proportion.

Relative values of translocation constants were estimated as follows. The efflux rates determined according to the experiment of Fig. 2 were simulated using several combinations of parameters. An analytic dependence of the calculated rates and the parameters was then deduced, and the best estimates of the parameters were obtained. Apparent parameters for the pools of neutral or dibasic amino acid were obtained by averaging the individual values of each amino acid at its relative concentration. The whole simulation and fitting cycle was repeated until self-consistence. The final values of the relative translocation constants ($k_{in} = k_{out}$) at zero membrane potential were 2.1 for L-leucine, 1.05 for the pool of neutral amino acids, and 0.78 for the pool of dibasic amino acids. Dissociation constants (K_o, K_i) were set to 90 μM for all the amino acids, according to the external apparent K_m values obtained (Table I). Due to the high internal concentrations of substrates, the $b^{o,+}$-like transporter remained saturated during the simulation. Therefore, the precise values of the internal dissociation constants have little influence on the results obtained.

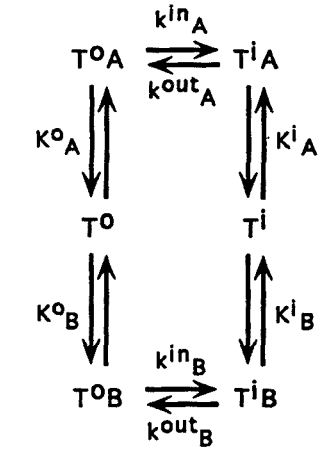

FIG. 1. **Reaction mechanism for the amino acid exchanger model.** A ping-pong mechanism (only one amino acid, either A or B, is transported at once) of obligatory exchange is shown. Binding steps were considered as equilibria (defined as dissociation constants K_o and K_i), whereas translocation steps were considered as steady state (defined as two translocation rate constants k^{in}, k^{out}). The system has been assumed to be symmetric (*i.e.* $k^{in} = k^{out}$, $K_o = K_i$). The equilibrium assumptions do not significantly alter the calculated rates with respect to a full steady-state mechanism. Translocation of the empty transporter (T^o, facing outside; T^i, facing inside) is not considered, since system $b^{o,+}$-like is assumed to be an obligatory exchanger. The same mechanism of transport was used for the endogenous transporter but allowing free translocation of the empty transporter, as this is considered an equilibrative system.

(iii) Simulation of the accumulation experiments. To reproduce accumulation studies shown in Fig. 5, first the concentration and kinetic parameters of the endogenous transporter were manually adjusted to reproduce the accumulation curves obtained in uninjected oocytes (see legend to Fig. 5). Second, in the presence of this endogenous activity, the V_{max} of L-arginine influx, which includes the concentration of $b^{o,+}$-like transporter and the actual value of the L-arginine translocation constant, was set to fit the initial influx rates of rBAT-injected oocytes (see legend to Fig. 5). Finally, with the concentrations chosen and the parameters indicated above the system was simulated for the desired time.

RESULTS

Substrate Specificity and Kinetic Parameters of the $b^{o,+}$-like Amino Acid Exchanger Expressed in Oocytes—Several studies have suggested that the amino acid transport system $b^{o,+}$-like, associated with rBAT expression in oocytes, is an amino acid exchanger (15–17). If this is correct, the substrate specificity and the apparent kinetic parameters should be identical when influx or the amino acid-dependent stimulation of efflux are measured. The efflux of L-[^{3}H]arginine via system $b^{o,+}$-like in rBAT-injected oocytes was stimulated by amino acids in the external medium with the same substrate specificity shown in uptake studies (influx) through this transport activity (Fig. 2*A*). Thus, 1 mM dibasic and neutral L-amino acids (or 200 μM L-cystine) in the external medium, which are substrates for system $b^{o,+}$-like (23, 33–35), increased efflux of L-arginine in rBAT-injected but not in uninjected oocytes. L-Proline and L-glutamate, which are not substrates for system $b^{o,+}$-like (23, 33–34), did not increase efflux of L-[^{3}H]arginine in rBAT-injected oocytes (Fig. 2*A*). Similar data of substrate specificity

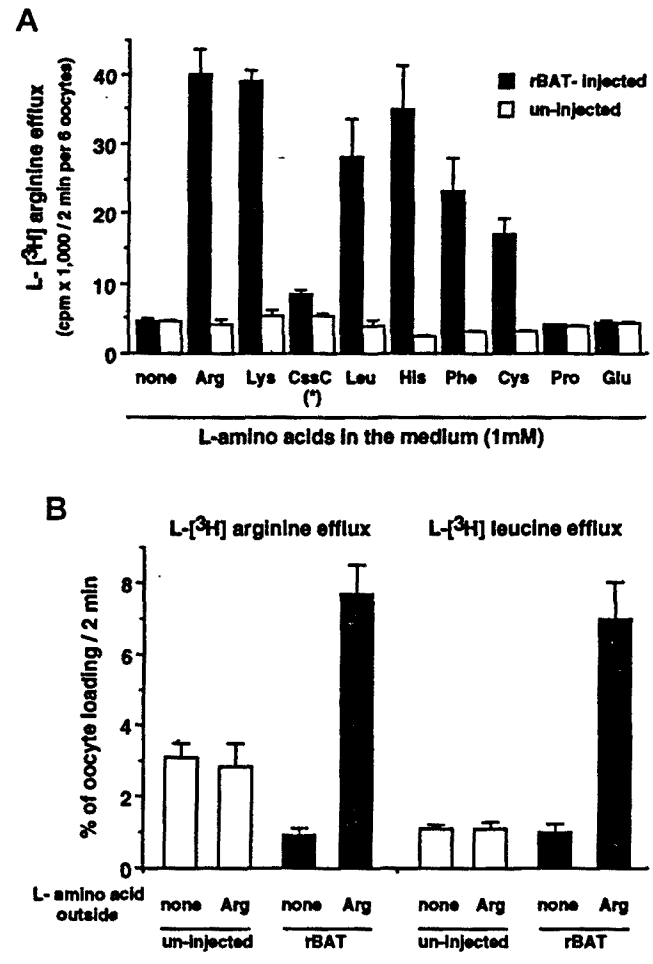

FIG. 2. L- [^{3}H]Arginine and L- [^{3}H]leucine efflux via amino acid transport system $b^{o,+}$-like in oocytes. Oocytes were injected with 5 ng of rBAT cRNA (*black bars*) or uninjected oocytes (*open bars*). Four days later, amino acid efflux rates were measured in choline medium containing no amino acids (none) or the indicated 1 mM L-amino acids. 200 μM L-cystine (*CssC*) was used in the presence of 10 mM diamide. *A*, L- [^{3}H]arginine efflux rates (expressed as the radioactivity (cpm × 1,000) 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 ($p \leq 0.01$). *B*, L-[^{3}H]Arginine and L-[^{3}H]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/2 min) are expressed as percent of the total radioactivity loaded into the oocyte. Data (mean ± S.E.) are from 9–12 determinations (5 independent experiments, L-arginine efflux) and from 9 determinations (3 independent experiments, L-leucine efflux). L-Arginine significantly induced L-[^{3}H]arginine and L-[^{3}H]leucine efflux from rBAT cRNA-injected ($p \leq 0.001$) but not from uninjected oocytes. L-[^{3}H]Arginine efflux into medium containing no amino acids (*none*) was lower from rBAT cRNA-injected than from uninjected oocytes ($p \leq 0.001$), whereas L-[^{3}H]leucine efflux into medium containing no amino acids (none) was similar in the two groups.

were obtained when the efflux rates of L-[^{3}H]leucine were measured (data not shown). The efflux rates of L-[^{3}H]arginine and L-[^{3}H]leucine from several experiments, and expressed as percent of the previous loading, are shown in Fig. 2*B*. The efflux rates of L-[^{3}H]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. 2*B*). This supports the idea that system $b^{o,+}$-like is an obligatory amino acid exchanger; transport via system $b^{o,+}$-like occurs only when substrates are present on the trans-side. A similar interpretation of the efflux rates of L-[^{3}H]arginine is difficult to postulate. The efflux rates of L-[^{3}H]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. 2*B*). This suggests that either rBAT expression results in the retention of L-arginine inside the oocyte or L-[^{3}H]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-[^{3}H]arginine efflux was increased 8-fold by 1 mM L-arginine only in oocytes expressing rBAT (Fig. 2*B*). This demonstrates that L- [^{3}H]arginine efflux via system $b^{o,+}$-like is also dependent on the presence of amino acid substrates on the trans-side.

TABLE I

Kinetic parameters of system $b^{o,+}$-like amino acid exchanger activity associated with rBAT expression in oocytes

rBAT-induced influx rates for L-cystine, L-arginine, and L-leucine were measured 3–4 days after injection of rBAT cRNA (5 ng/oocyte) at 7–8 different substrate concentrations (10–250 μM) and calculated as described under "Materials and Methods." The amino acid-elicited L-[^{3}H]arginine efflux rates were assayed at 6-different concentrations (10–1,000 μM) of the external substrate and calculated by subtracting the rates of efflux into medium containing no amino acids. K_m for efflux represents the concentration of external amino acid needed for the semi-maximal amino acid-elicited efflux. Influx data (mean ± S.E.) correspond to representative kinetic experiments run in triplicate. Efflux data (mean ± S.E.) correspond to 3–6 determinations from 3 independent experiments. ND, not determined.

Substrate	L-Cystine	L-Arginine	L-Leucine
Influx			
K_m (μM)	41 ± 7	85 ± 7	90 ± 12
V_{max} (pmol/min/oocyte)	9 ± 1	211 ± 11	59 ± 4
Efflux (L-arginine)			
K_m (μM)	ND	65 ± 5	67 ± 26
V_{max} (% oocyte loading/2 min)	ND	6.1 ± 0.2	2.9 ± 0.6

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-[^{3}H]arginine showed saturability (data not shown) with similar apparent K_m values for the external amino acids (Table I). Interestingly, the apparent K_m values for L-arginine and L-leucine either acting as substrates for influx or stimulating efflux of L-[^{3}H]arginine were similar (μM range, Table I). This is expected for an obligatory exchanger, and it is inconsistent 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 $b^{o,+}$-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. 2*A* and Table I) suggested that in the hetero-exchange events via system $b^{o,+}$-like the "slowest" substrate (*i.e.* L-cystine) limits the transport activity.

The System $b^{o,+}$-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 $b^{o,+}$-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-[^{3}H]arginine efflux in OK cells

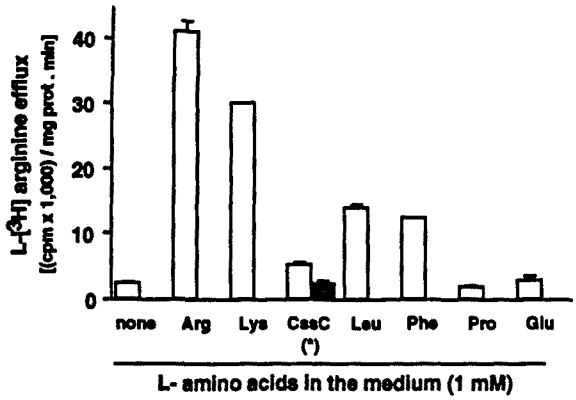

FIG. 3. L- [^{3}H]Arginine efflux in OK cells. L-[^{3}H]Arginine efflux rates were measured in media containing no amino acids (*none*) or the indicated L-amino acids at 1 mM, except for L-cystine (*CssC*), which was present at 200 μM plus 5 mM diamide (as controls, cells were incubated in amino acid-free medium plus 5 mM diamide, *hatched bar*). Efflux rates (mean ± S.E.) are cpm × 1,000 measured in the medium (corrected for 150,000 cpm loaded into the cells)/mg of protein per min from 3 to 12 determinations (5 independent experiments). Data for L-lysine and L-phenylalanine groups are the mean from two determinations in a representative experiment. Efflux rates in the L-cystine, L-leucine, and L-arginine groups were significantly different ($p \leq 0.05$) from those of the none, L-proline, and L-glutamate groups.

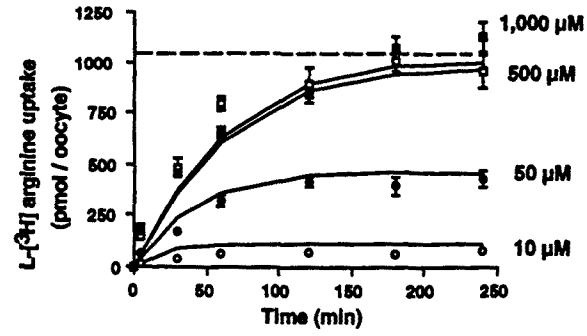

FIG. 4. Concentration dependence of L-arginine accumulation in rBAT-injected oocytes. Oocytes were injected with 5 ng of rBAT cRNA or uninjected (not shown). Three days later the uptake of L-[^{3}H]arginine (3–5 μCi/90 μl) was measured at 10 (*open circles*), 50 (*closed circles*), 500 (*open squares*), and 1,000 (*closed squares*) μM concentration of substrate for the time indicated and in sodium uptake medium. Simulations (*curved lines*) of the accumulation progress for the rBAT-injected oocytes were estimated with the obligatory exchange mechanism described under "Materials and Methods." The V_{max} for $b^{o,+}$-like transport activity was adjusted to fit the influx velocity of L-[^{3}H]arginine by rBAT-injected oocytes measured in this experiment. The endogenous transport activity was adjusted to reproduce the influx velocity of L-arginine (3.8 ± 0.4, 4.9 ± 0.7, 7.0 ± 0.8, and 16.5 ± 1.3 pmol/5 min per oocyte at 10, 50, 500, and 1,000 μM L-[^{3}H]arginine), the evolution of the accumulation curves, and the plateau of accumulation (~30, ~125, ~160, and ~230 pmol in 4 h per oocyte at 10, 50, 500, and 1,000 μM L-[^{3}H]arginine) for the uninjected oocytes measured in this experiment; at 500 and 1,000 μM concentrations, the uptake in uninjected oocytes increased over the time (4 h) assayed without reaching a plateau. The total content of internal substrates for system $b^{o,+}$-like (31) is shown by a *horizontal dashed line*. The uptake data (pmol/oocyte) are the mean ± S.E. from 7 oocytes in a representative experiment. When not visible the errors are smaller than symbols. Similar data were obtained in another two independent experiments.

was similar to that of system $b^{o,+}$-like expressed in oocytes (Figs. 2A and 3). L-Leucine-elicited L-[^{3}H]arginine efflux decreased by 60% in the rBAT antisense-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-[^{3}H]arginine efflux into medium containing 1 mM L-glutamate, which is not a system $b^{o,+}$-like substrate (Figs. 2A and 3), was not affected by antisense expression (L-[^{3}H]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-[^{3}H]arginine efflux via system $b^{o,+}$-like does not occur in the absence of substrates in the medium and demonstrates that L-leucine-elicited L- [^{3}H]arginine efflux occurs via system $b^{o,+}$-like (associated with rBAT expression). Kinetic analysis of this efflux showed saturability via a single component with an apparent K_m value of 295 ± 64 μM (data not shown). This value fits reasonably well with the estimated apparent K_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 $b^{o,+}$-like associated with rBAT behaves as an obligatory exchanger in OK cells.

The System $b^{o,+}$-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 $b^{o,+}$-like (31). If the degree of coupling of exchange of system $b^{o,+}$-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-[^{3}H]arginine, L-[^{3}H]leucine, or L-[^{35}S]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^+; Ref. 22) (data not shown and Fig. 4). Similarly, L- [^{3}H]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 nl (see "Material and Methods"), the plateau of uptake values of 50 μM L-[^{3}H]arginine uptake represents a 55-fold accumulation (~40-fold for L-[^{3}H]leucine or L-[^{35}S]cystine uptake) of the substrate in rBAT-injected oocytes (Table II). In contrast, the level of L-[^{3}H]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 $b^{o,+}$-like is clearly higher than via system y^+.

If the accumulation of substrates via system $b^{o,+}$-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-[^{3}H]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^+, associated with CAT1 expression, which increased from ~140 pmol/oocyte at equilibrium with 50 μM L-[^{3}H]arginine to ~750 pmol/oocyte, without reaching equilibrium after 4 h, with 500 μM L-[^{3}H]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- [^{3}H]arginine accumulation curves shown in Fig. 4 with a model that considers system $b^{o,+}$-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-[^{3}H]arginine concentration used (*i.e.* 1 mM), the model predicts that at equilibrium nearly 98% of the internal substrates of system $b^{o,+}$-like have been replaced by L-arginine with the initial spe-

TABLE II

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-[^{35}S]cystine, L-[^{3}H]leucine, or L-[^{3}H]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 [^{3}H]water of 176 nl (see "Materials and Methods"). Data for rBAT-, CAT1-, and uninjected oocytes are the mean ± S.E. corresponding to 6–15 determinations (3–7 independent experiments). 4F2hc and L cystine (rBAT) data are the mean values (3–6 determinations) in two independent experiments.

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		

cific activity of the substrate and with hardly any change in the total substrate content of the oocyte (Table III). Similarly, the model also predicts that superfusion (20 ml/min) of rBAT-injected oocytes with 50 μM L-arginine or L-leucine for 3 h results in 95 and 79% replacement of the internal oocyte system $b^{o,+}$-like substrates, respectively (data not shown). The main conclusion of this theoretical study is that accumulation of L-[^{3}H]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 $b^{o,+}$-like and then estimate the influx and efflux rates through the transporter under conditions of homogeneous exchange of substrates.

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 $b^{o,+}$-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 $b^{o,+}$-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 $b^{o,+}$-like, rBAT-injected oocytes were incubated for 4 h with 1 mM labeled (L-[^{3}H]arginine or L-[^{3}H]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 $b^{o,+}$-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).

TABLE III

Simulated internal concentrations and specific activities during L-[^{3}H]arginine accumulation in oocytes expressing rBAT

The internal concentration (μM and % of total substrates of system $b^{o,+}$-like) of the analyzed groups of amino acids at time = 0 (values from Ref. 31) and after 4 h of 1,000 μM L-[^{3}H]arginine uptake in rBAT-injected oocytes are shown (upper). The evolution of the external and internal specific activity of L-[^{3}H]arginine is also indicated (lower). The initial specific activity of L-[^{3}H]arginine, defined as (labeled L-arginine)/(total L-arginine), was set to 0.0001. The actual value of the specific activity does not alter the conclusion, provided that the concentration of labeled L-[^{3}H]arginine does not significantly change the total L-arginine concentration.

	Internal concentration (μM)	
	Time = 0	Time = 4 h
L-Arginine	697	5653.0 (97.7%)
L-Leucine	231	3.1 (0.05%)
L-Neutral	4288	64.3 (1.1%)
L-Dibasic	574	63.0 (1.1%)
Total	5790	5783.4
	L-[^{3}H]Arginine specific activity (×10^5)	
	Time = 0	Time = 4 h
External	10	9.90
Internal	0	9.88

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-[^{3}H]arginine or L-[^{3}H]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-[^{3}H]arginine via system y^+L-like, associated with 4F2hc expression in oocytes (Fig. 7*A*). As already shown (Fig. 2), these amino acids did not stimulate efflux in uninjected oocytes (Fig. 7). L-Leucine-elicited efflux of L-[^{3}H]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. 7*A*). This substrate specificity corresponds to that of influx via system y^+L-like in oocytes. Thus, the 4F2hc-induced influx of 100 μM L-[^{3}H]leucine was 1 ± 0.3 pmol/5 min per oocyte in the absence of sodium, and 34 ± 3 pmol/5 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-tryptophan (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-[^{3}H]leucine efflux via system y^+L-like associated with 4F2hc expression in oocytes. To our surprise, L-[^{3}H]leucine efflux was not stimulated by external L-arginine (Fig. 7*B*) 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

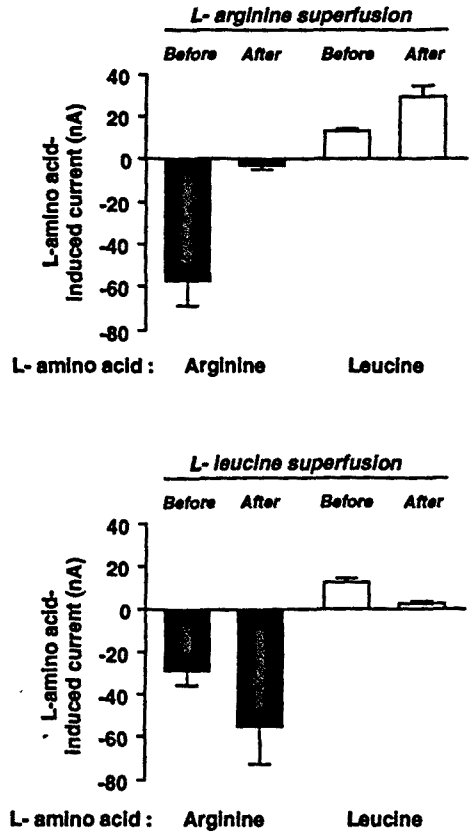

FIG. 5. **Effect of L-arginine and L-leucine superfusion on L-amino acid-induced currents in rBAT-injected oocytes.** Oocytes were injected with 1 ng of rBAT cRNA. 50 μM L-arginine-induced and 50 μM L-leucine-induced currents were measured, at a clamped potential of −50 mV, before and after 3 h superfusion with 50 μM L-arginine (*upper graph*, 8 days after injections) or with 50 μM L-leucine (*lower graph*, 3 days after injections). In current clamped conditions, the membrane resting potential (−54 ± 3 and −60 ± 2 mV in the L-arginine and L-leucine superfusion experiments, respectively) was immediately depolarized to −33 ± 3 mV by L-arginine and repolarized to −66 ± 6 mV by L-leucine. After 3 h of 50 μM L-amino acid superfusion, the membrane potential was almost restored to −46 ± 4 mV (L-arginine superfusion experiment) and to −60 ± 3 mV (L-leucine superfusion experiment). The whole protocol was performed in sodium ND96 medium. Data (nA) are the mean ± S.E. from 5 oocytes (L-arginine superfusion) and 4 oocytes (L-leucine superfusion) from two different batches.

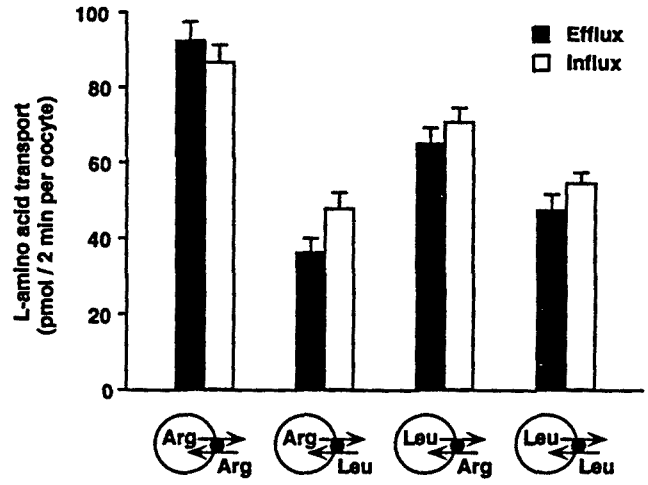

FIG. 6. **Efflux and influx transport rates via system $b^{o,+}$-like in rBAT-injected oocytes.** Oocytes were injected with 5 ng of rBAT cRNA or uninjected, and 4 days later they were assayed for efflux (*closed bars*) and influx (*open bars*) transport of the indicated amino acids in the oocyte schemes shown at the *foot* of the figure. For efflux studies, groups of 7 oocytes were incubated in choline uptake medium for 4 h in the presence of 1 mM L-[^{3}H]arginine or L-[^{3}H]leucine (3 μCi/90 μl). Then efflux rates were measured in the absence or in the presence of the indicated 250 μM L-amino acids. For influx studies, groups of 7 oocytes were incubated in choline uptake medium (90 μl) for 4 h in the presence of 1 mM cold L-arginine or L-leucine. Then media were removed and the oocytes were washed as indicated for efflux studies. Influx of 250 μM L-[^{3}H]arginine or L-[^{3}H]leucine were immediately assayed for 2 min. Efflux data correspond to the amino acid-elicited efflux rates (*i.e.* efflux into media containing amino acids minus efflux into medium containing no amino acids). To express efflux rates as pmol/2 min per oocyte, the initial specific activity of the tracer during the accumulation phase of the study was used (see Table III). Influx rates correspond to the rBAT-induced transport activity (*i.e.* transport in rBAT-injected minus that in uninjected oocytes). Data (mean ± S.E.) are from 4 (efflux) and 12–14 (influx) determinations from a representative experiment. The corresponding efflux and influx rates ere not significantly different in the four groups. Efflux and influx rates in the hetero-exchange Leu (outward)/Arg (inward) were significantly higher ($p \leq 0.01$) than those corresponding to the reverse hetero-exchange.

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-[^{3}H]leucine efflux due to *trans*-L-arginine in rBAT-injected oocytes (Fig. 2*B*). 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 electrogenicity 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, $p \leq 0.01$). 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 L-neutral amino acids (38).

DISCUSSION

We have shown that amino acid transport systems $b^{o,+}$-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 $b^{o,+}$-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 $b^{o,+}$-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 $b^{o,+}$-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 $b^{o,+}$-like activity, as

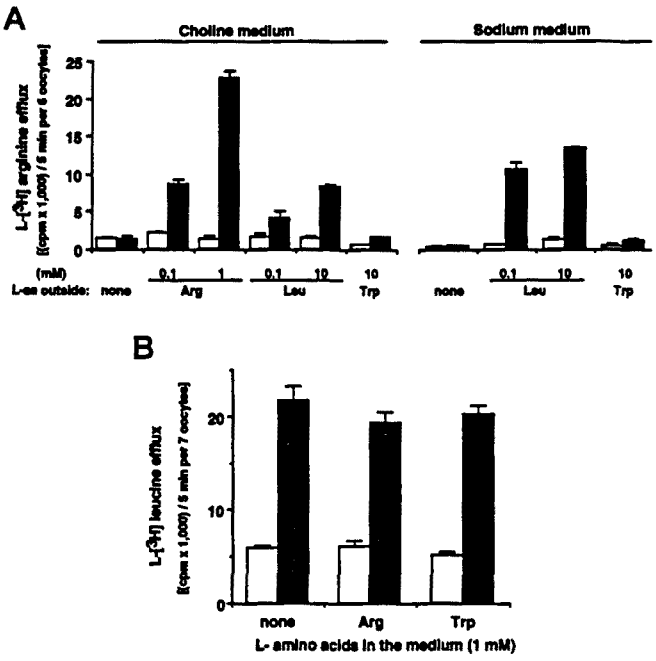

FIG. 7. L- [^{3}H]Arginine efflux via amino acid transport system y^+L-like in oocytes. Oocytes were injected with 1 ng of 4F2hc cRNA (*black bars*). Uninjected oocytes (*open bars*) were used as controls. Four days after, rates of L-[^{3}H]arginine or L-[^{3}H]leucine efflux into sodium or choline medium 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-[^{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 ($p \leq 0.05$). *B*, L-[^{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 $b^{o,+}$-like activity, with characteristics of obligatory amino acid exchange.

We have also shown that the amino acid exchange activity of system $b^{o,+}$-like is tightly coupled and allows intracellular concentration of amino acid substrates until the complete replacement of the internal system $b^{o,+}$-like substrates of the oocyte. The maximum level of accumulation of substrates via system $b^{o,+}$-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 μM concentration reached in rBAT-injected oocytes exceeds that obtained in uninjected or in CAT1-injected oocytes (*i.e.* expressing system y^+ amino acid transport activity). In contrast to system $b^{o,+}$-like, system y^+ 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 $b^{o,+}$-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 $b^{o,+}$-like for the following reasons. (i) The cut-open oocyte model is able to show system $b^{o,+}$-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 $b^{o,+}$-like (present study), and neither sodium, potassium, nor chloride ions are needed for system $b^{o,+}$-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).[2] In conclusion, system $b^{o,+}$-like, associated with rBAT expression, is a tightly coupled exchanger with 1:1 stoichiometry. Whether system $b^{o,+}$-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^+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 μM concentration. It has been suggested (41) that the amino acid transport activity associated with human 4F2hc expression in oocytes is identical to the y^+L activity described in human erythrocytes and placenta (36–38, 42). In contrast to this general system, y^+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^+L may indeed be an obligatory exchanger because the functional isolation of system y^+L from the co-existing system y^+ 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^+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^+L-like and $b^{o,+}$-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 hydrophobicity 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 $b^{o,+}$-like and y^+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 $b^{o,+}$-like and y^+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 $b^{o,+}$-like, described here, explains the responsibility of rBAT in cystinuria. We propose a model for the role of system $b^{o,+}$-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

[2] A. E. Busch, unpublished results.

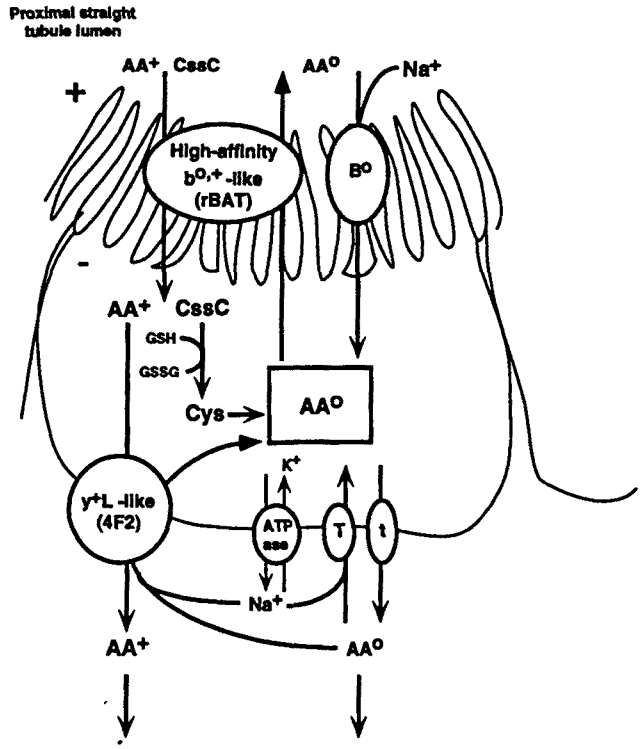

FIG. 8. **Model for the renal reabsorption of dibasic amino acids via systems $b^{o,+}$-like and y^+L-like.** Obligatory amino acid exchange with neutral amino acids (*AA°*) via systems $b^{o,+}$-like (apical) and y^+L-like (basolateral) would mediate the active reabsorption of dibasic amino acids (*AA*$^+$) and cystine (*CssC*) in a epithelial cell from the proximal straight tubule. Influx of dibasic amino acids and cystine from the lumen would be favored by the negative membrane potential and by the reduction of cystine to cysteine associated with glutathione oxidation (GSH → GssG), respectively. A high intracellular concentration of neutral amino acid would be ensured by concentrative (Na^+ cotransport) neutral amino acid transport activities in the apical pole (system *B°*, 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 L). *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.

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, which are substrates for system $b^{o,+}$-like, may be the determinants of the reabsorption of L-dibasic amino acids and L-cystine via system $b^{o,+}$-like. The tightly coupled mechanism of obligatory exchange of system $b^{o,+}$-like would ensure efficient active reabsorption of cystine and dibasic amino acids. In addition this mechanism would prevent the net loss of amino acids via system $b^{o,+}$-like toward the lumen at the end of the proximal straight tubule, where rBAT is expressed (9–10) and a low concentration of amino acids is present. Further studies are needed to examine the factors affecting the activity and the direction of amino acid exchange via system $b^{o,+}$-like.

The obligatory exchange of amino acids via system y^+L-like, associated with 4F2hc expression in oocytes, may have important physiological consequences. It has been reported that efflux across the basolateral membrane is the rate-limiting step in the intestinal absorption of dibasic amino acids (48–49). Furthermore, leucine at low μM concentration increases (6–10-fold) the trans-epithelial flux of lysine (49–50). Countertransport between lysine (outward) and leucine (inward) or allosterism were considered to be responsible for this process. System y^+L can sustain lysine-leucine exchange with an apparent K_m for leucine of ~10 μM in the presence of sodium (36). If such a system is found in the basolateral membranes of intestinal or renal epithelial cells, system y^+L will support the countertransport hypothesis (37). The surface antigen 4F2hc has a basolateral localization in renal epithelial cells from the proximal tubule (51). System y^+L-like, associated with 4F2hc expression, could be responsible for the active release of dibasic amino acids through the basolateral membrane of epithelial cells (Fig. 8). The evidence offered here, that the direction of exchange that is favored is L-arginine (outward) with low μM concentration of leucine (inward) in the presence of sodium, strongly supports this hypothesis. Further research is needed to elucidate the mechanism (*e.g.* a weak interaction of neutral amino acids from inside due to the low intracellular concentration of sodium) responsible for this asymmetric exchange.

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Treball 3

The amino acid transport system y^+L/4F2hc is a heteromultimeric complex.

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El doctorand és autor de totes les figures i taules excepte la figura 4. Vaig participar en la redacció de l'article (figures, legend to figures, experimental procedures). L'estrategia experimental d'aquest treball va ser proposada pel doctorand.

The amino acid transport system y^+L/4F2hc is a heteromultimeric complex

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ABSTRACT **4F2hc is an almost ubiquitous transmembrane protein in mammalian cells; upon expression in *Xenopus laevis* oocytes, it induces amino acid transport with characteristics of system y^+L. Indirect evidence fostered speculation that function requires the association of 4F2hc with another protein endogenous to oocytes and native tissues. We show that expression of system y^+L-like amino acid transport activity by 4F2hc in oocytes is limited by an endogenous factor and that direct covalent modification of external cysteine residue(s) of an oocyte membrane protein blocks system y^+L/4F2hc transport activity, based on the following. *1)* Induction of system y^+L-like activity saturates at very low doses of human 4F2hc cRNA (0.1 ng/oocyte). This saturation occurs with very low expression of 4F2hc at the oocyte surface, and further increased expression of the protein at the cell surface does not result in higher induction of system y^+L-like activity. *2)* Human 4F2hc contains only two cysteine residues (C109 and C330). We mutated these residues, singly and in combination, to serine (C109S; CS1, C330S; CS2 and C109S-C330S, Cys-less). Mutation CS2 had no effect on the expressed system y^+L-like transport activity, whereas C109S-containing mutants (CS1 and Cys-less) retained only partial y^+L-like transport activity (30 to 50% of wild type). *3)* Hg^{2+}, the organic mercury compounds pCMB, and the membrane-impermeant p-CMBS almost completely inactivated system y^+L-like induced by human 4F2hc wild type and all the mutants studied. This was reversed by β-mercaptoethanol, indicating that external cysteine residue(s) are the target of this inactivation. *4)* Sensitivity to Hg^{2+} inactivation is increased by pretreatment of oocytes with β-mercaptoethanol or in the C109S-containing mutants (CS1 and Cys-less). The increased Hg^{2+} reactivity of C109S-containing mutants supports the possibility that C109 may be linked by a disulfide bond to the Hg^{2+}-targeted cysteine residue of the associated protein. These results indicate that 4F2hc is intimately associated with a membrane oocyte protein for the expression of system y^+L amino acid transport activity. To our knowledge, this is the first direct evidence for a heteromultimeric protein structure of an organic solute carrier in mammals.—Estévez, R., Camps, M., Rojas, A. M., Testar, X., Devés, R., Hediger, M. A., Zorzano, A., Palacín, M. The amino acid transport system y^+L/4F2hc is a heteromultimeric complex. *FASEB J.* 12, 1319–1329 (1998)**

Key Words: oocyte · mutagenesis · erythrocyte · y^+L transport activity · homologous protein

TWO HOMOLOGOUS PROTEINS, rBAT and 4F2hc, were identified as members of a protein family related to plasma membrane amino acid transport because they induce high-affinity, broad-specificity amino acid exchanger systems $b^{0,+}$-like (1–3) and y^+L-like (4–5), respectively, in oocytes. The role of rBAT in the high-affinity renal reabsorption and intestinal absorption of cystine and basic amino acids is well established: *1)* rBAT is expressed in the apical plasma membrane of epithelial cells of the proximal straight tubule of the nephron and the small intestine (6, 7), *2)* the expression of rBAT is necessary for the $b^{0,+}$-like activity present in the apical plasma membrane of the renal cell line OK (8), and *3)* mutations in the *rBAT* gene cause cystinuria type I (9–13), an inherited aminoaciduria of cystine and basic amino acids (14). In contrast, the amino acid transport activity associated with 4F2hc expression is controversial. Human and rat 4F2hc induce y^+L-like activity (sodium-independent transport for basic amino acids, and mainly sodium-dependent transport for neutral amino acids) (4, 5, 15). Similarly, mRNA from human choriocarcinoma cells and rat lung induces y^+L-like activity in oocytes, which is hybrid-depleted by 4F2hc antisense oligonucleotides (ref 16; R. Estévez, A. Zorzano, and M. Palacín, unpublished results). In contrast, mRNA from C6-BU-1 rat glioma cells induces system L-like (sodium-independent transport for neutral amino

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acids) amino acid transport activity in oocytes, which is hybrid-depleted by antisense sequences of rat 4F2hc cDNA (17, 18); transient transfection of rat 4F2hc in CHO cells results in a moderate increase in L-isoleucine transport with characteristics of system L (19).

The mechanism by which rBAT and 4F2hc induce amino acid transport is not clear. Neither is very hydrophobic, and they both have a structural prognosis as type II membrane glycoproteins with a single transmembrane domain (2, 3, 20, 21). Tate's group (22) offered experimental evidence that rBAT has four transmembrane domains with cytoplasmic amino and carboxy termini. Nevertheless, this is in contrast to other identified amino acid transporters and transporters in general, which contain 10 to 14 transmembrane domains. This fosters the hypothesis that rBAT and 4F2hc are modulators or subunits of the transporters. The cell surface antigen 4F2 is a heterodimer (~125 kDa) composed of a heavy chain of 85 kDa (4F2hc, i.e., the homologous protein to rBAT) and an unidentified light chain of 40 kDa, which are believed to be linked by disulfide bridges (23, 24). Similarly, Tate's group (25) reported the presence of rBAT complexes in brush border preparations from kidney and intestine or in oocytes. In our hands, renal rBAT is immunodetected in nonreducing conditions as complexes of ~240 kDa and ~125 kDa (26). It therefore seems that rBAT has a heterodimeric structure (125 kDa) consisting of a 'heavy chain' (~90 kDa), probably linked by disulfide bridges to a putative 'light chain' of 40–50 kDa.

The aim of this study was to investigate whether 4F2hc by itself constitutes the system y^+L-like transporter. Here we show dissociation between the expression of 4F2hc at the oocyte surface and induction of system y^+L-like activity, which indicates that this expression is limited by an endogenous factor. In addition, we show that y^+L transporters induced in oocytes by cysteine-free human 4F2hc are sensitive to sulfhydryl-specific reagents that modify cysteine residue(s) exposed to the aqueous solvent. Sensitivity to inactivation is increased in reducing conditions and in 4F2hc mutants in which the cysteine residue at position 109 has been mutated to serine. These results indicate that 4F2hc is intimately associated with a membrane oocyte protein for the expression of system y^+L amino acid transport activity. The increased Hg^{2+} reactivity of C109S-containing mutants supports the possibility that C109 may be linked by a disulfide bond to the Hg^{2+}-targeted cysteine residue of the associated protein.

MATERIALS AND METHODS

Oocytes, injections, and uptake measurements

Oocyte origin, management, and injections were as described elsewhere (10). Defolliculated stage IV *Xenopus laevis* oocytes were injected with different amounts of human 4F2hc or mouse CAT1 or CAT2 cRNA, as indicated. For saturating induction of amino acid transport, oocytes were injected with 1 to 20 ng of human 4F2hc cRNA. Except where indicated, noninjected oocytes were used as controls; amino acid uptake rates obtained with oocytes injected with water (50 nl) were similar to those of noninjected oocytes (data not shown). Synthesis of human 4F2hc [cDNA cloned in *Eco*RI-*Hin*dIII pSPORT-1, from the original cDNA cloned in pSP65 by Teixeria et al. (21)], and mouse CAT1 (27) and CAT2 (28) cRNAs is described elsewhere (15).

Influx rates of L-[^{3}H]arginine and L-[^{3}H]leucine were measured in 100 mM NaCl (sodium medium) or 100 mM choline Cl (choline medium) media on the days indicated after injection and in linear conditions, as described elsewhere (15). When present, the induced uptake was calculated by subtracting uptake values in noninjected oocytes from those of the corresponding cRNA-injected oocytes.

The thiol-specific reagents in this study [pCMB (p-chloromercuribenzoic acid), pCMBS (p-chloromercuriphenylsulfonic acid; monosodium salt), and $HgCl_2$][2] to treat oocytes were from Sigma (St. Louis, Mo.) and were used as described in the corresponding figure legends.

Uptake measurements in human erythrocytes

L-[^{14}C]Lysine influx was determined as described previously (29). Briefly, cells were suspended at 10% hematocrit in a medium containing NaCl (140 mM), KCl (4 mM), and sodium phosphate (5 mM) at pH 6.8, and the uptake of labeled lysine was measured as a function of time. All determinations were performed in duplicate and rates (mean±SEM) were determined from linear regression analysis of six time points, up to 4 min. System y^+L activity was estimated as the transport component inhibited by 2 mM L-leucine; the residual activity was attributed to system y^+, as demonstrated in previous studies (29, 30). When necessary, cells (5% hematocrit) were treated at 25°C with pCMBS and/or β-mercaptoethanol for 10 min. The same buffer was used throughout. The treatment was stopped by centrifugation at 4°C, and the cells were washed three times between treatments or prior to uptake measurements. Uptake data are expressed in nmol/l cell water per min.

Site-directed mutagenesis

For construction of the C109S and C330S human 4F2hc mutants, we used the QuickChange Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's protocol. The mutagenic oligonucleotides were 5'-TAGCTCGCGA(G)AACG-CGGCGC-3' (antisense strand; the mutated nucleotide at position 326 is indicated in parentheses) and 5'-ACTCCAG-CTG(G)ACCAGCGATT-3' (antisense strand; the mutated nucleotide at position 989 is indicated in parentheses) for the C109S and C330S, respectively. Mutants were identified by sequencing; a cassette between *Sph*I and *Sac*I sites for the C109S mutant and one between *Bsm*I and *Eco*47III sites for the C330S mutant were completely sequenced. The first cassette was then substituted into human 4F2hc cDNA inserted in pSP65 (21), where *Sph*I and *Sac*I sites were removed by digestion with these enzymes and being polished with Klenow. The second cassette was substituted into human 4F2hc inserted in pSPORT (15) with a similar strategy, using *Bsm*I and EcoIII sites. Finally, the

[2] Abbreviations: pCMB, p-chloromercuribenzoic acid; pCMBS, p-chloromercuriphenylsulfonic acid; PBS, phosphate-buffered saline; PBSm, modified PBS; FBS, fetal bovine serum.

two inserted cassettes were checked by complete sequencing. For the construction of the Cys-less mutant, a fragment of the pSP65-human 4F2hc-C109S, comprised between the restriction sites *Eco*RI and *Sac*I, was purified and ligated into p-SPORT-human 4F2hc-C330S, which was cut with the same enzymes. The cassettes were checked again by sequencing after ligations.

Confocal immunofluorescence microscopy

Groups of five oocytes were prepared for immunofluorescence 4 days after injection of a maximal (0.1 ng per oocyte) or supramaximal (20 ng per oocyte) dose of human 4F2 cRNA or were not injected. The oocytes were placed on a 1 cm^2 piece of Whatman 3M paper, embedded in O.C.T. compound (Agar Scientific Ltd., Essex, England), frozen on dry ice, and stored at −80°C. Sections (15 μm) were mounted on glass slides coated with 0.5% gelatin and dried at 37°C for 10 min. The sections were fixed in 3% paraformaldehyde phosphate-buffered saline (PBS) for 10 min, incubated in 100 mM glycine-PBS for 10 min, permeabilized in 1% Triton-X-100 in PBS for 10 min, washed three times in PBS, blocked in 10% fetal bovine serum (FBS) in PBS for 30 min, and exposed to primary antibody (mouse monoclonal antibody CD98 from Immunotech, Marseille, France), diluted 1/50 in 10% FBS-PBS, at room temperature for 1 h. Slides were washed three times in PBS, incubated with 7.5 μg/ml Texas red-conjugated goat anti-mouse (Molecular Probes, Leiden, The Netherlands) at room temperature for 1 h, washed three times in PBS, and mounted in immunofluore (ICN; Madrid, Spain). With a similar protocol, the oocyte β1-integrin was detected using 8C8 antibody, kindly provided by Dr. A. H. J. Muller (Max Planck Institute für Entwicklingsbiologie, Tübingen, Germany). Confocal microscopy was performed at the Serveis Científico Tècnics of the Universitat de Barcelona.

Binding assays of 4F2hc on the oocyte surface

Four days after injection, binding of the primary antibody (mouse monoclonal antibody anti-CD98 from Immunotech, Marseille, France) to oocytes expressing human 4F2hc and to noninjected oocytes was assayed. Eight oocytes in each experimental group were transferred to a 1.5 ml Eppendorf tube containing a modified PBS buffer (PBSm) [137 mM NaCl, 9 mM Na_2HPO_4, 1.4 mM NaH_2PO_4 (pH 7.4) and 2% (w/v) ovalbumin]. The oocytes were then washed three times in buffer, incubated for 3 h in 0.5 ml of 1:25 primary antibody diluted in PBSm at 4°C, washed three times in PBSm, incubated in 0.4 ml of 1:200 dilution of biotinylated goat anti-mouse antibody (Sigma) for 1 h at 4°C, washed three times, and finally incubated in 0.3 ml with 0.3 mCi [^{125}I] streptavidin (Amersham, Arlington Heights, Ill.) for 1 h at room temperature and washed. The radioactivity was assessed directly by a gamma counter. The background binding (i.e., radioactivity associated with noninjected oocytes) was 5600 ± 500 cpm in three groups of eight oocytes.

RESULTS

Expression of the 4F2hc-associated amino acid transport in *Xenopus* oocytes is saturated at very low amounts of injected cRNA

Figure 1 shows the dose-response curve of expression of sodium-independent L-[^{3}H]arginine uptake by human 4F2hc cRNA compared with that induced by the mouse cationic amino acid transporter CAT1 (mCAT1). At 3 days after injection, maximal transport activity was reached at 0.05–0.1 ng cRNA of human 4F2hc per oocyte (Fig. 1); 6 days after injection, saturation of transport expression was reached at an even lower amount of cRNA (0.01–0.05 ng cRNA per oocyte; n=two independent experiments with different cRNA preparations and oocyte batches; data not shown). The 4F2hc-induced system y^+L-like transport activity in oocytes is characterized by sodium-independent cationic amino acid transport and sodium-dependent transport of neutral amino acids (4, 5). Similarly, the 4F2hc-induced sodium-dependent L-leucine uptake in oocytes showed the same saturation curve as the induced sodium-independent L-arginine uptake (data not shown). Finally, the amino acid transport induced both at high (25 ng per oocyte) and low (0.1 ng per oocyte) amounts of 4F2hc cRNA injected showed the characteristic pattern of inhibition of system y^+L-like activity (i.e., sodium-independent L-arginine transport inhibited by L-leucine in the presence of sodium, and sodium-dependent L-leucine transport inhibited by L-arginine) (data not shown; n=7 independent experiments). In summary, human 4F2hc induced y^+L-like transport activity that saturates at very low amounts of injected cRNA. This is at odds with the dose-response curve of the uptake induced by the expression of proteins with the 'typical' structure of a transporter protein, i.e., proteins with 12 to 14 transmembrane domains. Thus, the transport induced by mCAT1, mCAT2, and GLUT1 saturates at 1 ng (see Fig. 1), 1–5 ng (data not shown), and 10–15 ng (31) per oocyte, respectively.

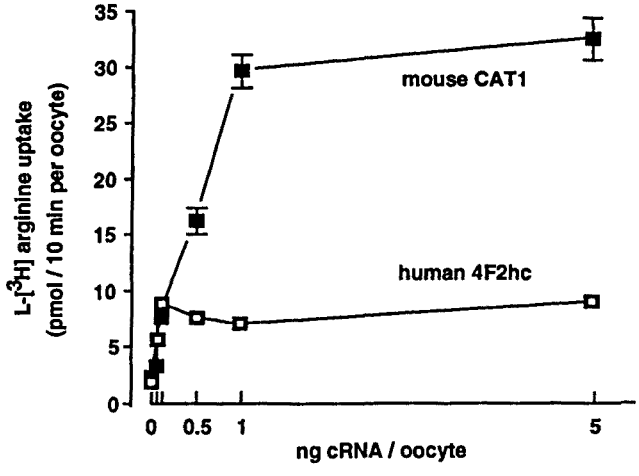

Figure 1. Dose-response curves for induction of L-arginine uptake by human 4F2hc and mouse CAT1 cRNAs in oocytes. Oocytes were injected with different amounts of 4F2hc or CAT1 cRNAs (0, 0.05, 0.1, 0.5, 1, and 5 ng per oocyte). Three days after the injection, the uptake of 50 μM L-[^{3}H]arginine in 100 mM choline Cl medium was determined in linear conditions (for 10 min incubation in human 4F2hc-injected oocytes and for 5 min incubation in mouse CAT-1-injected oocytes). Data are expressed as pmol/10 min per oocyte and are the mean ±SEM for seven oocytes in a representative experiment. When not visible, errors are smaller than symbols. Similar results were obtained in three independent experiments.

Dissociation between 4F2hc at the cell surface and induced y^+L-like activity in oocytes

We next examined whether the 4F2hc protein expressed at the oocyte surface correlates with the induced y^+L-like transport activity. We first performed experiments of immunocytochemistry in oocytes by using an antibody directed to CD98 (human 4F2hc) with noninjected oocytes and with oocytes injected with 0.1 ng (maximal dose) or 20 ng (supramaximal dose) of human 4F2hc cRNA (**Fig. 2*A***). The intensity of fluorescence at the oocyte periphery was higher for oocytes injected with 20 ng than with 0.1 ng (Fig. 2*A*), but the induced sodium-independent L-arginine uptake in both sets of oocytes was similar (data not shown). By quantifying the density of fluorescence (i.e., intensity of fluorescence/measured area), we found a difference of 38-fold between oocytes injected with 0.1 or 20 ng of human 4F2hc (658 ± 120 arbitrary units for 20 ng; n=three independent experiments, 24 ± 7 arbitrary units for 0.1 ng; n=four independent experiments, and 7 ± 1 arbitrary units for noninjected oocytes; n=3 independent experiments). The 4F2hc-associated fluorescence at the oocyte surface was similar to that of the β_1-integrin protein, which localized to the oocyte plasma membrane (data not shown). Part of the endoplasmic reticulum in the *Xenopus* oocyte is very close to the plasma membrane, so it is difficult to conclude that the 4F2hc-associated fluorescence signal at the oocyte periphery corresponds to 4F2hc expressed at the oocyte plasma membrane. For this reason, we performed surface binding assays with anti-4F2hc antibody in intact oocytes, a method similar to that described by Wang and Goldstein (32). Injection of a supramaximal dose of 4F2hc cRNA (20 ng per oocyte) resulted in higher expression of the 4F2hc protein at the oocyte surface than did injection of a maximal dose of 4F2hc cRNA (0.1 ng per oocyte) (Fig. 2*B*). In contrast, the sodium-independent 4F2hc-induced uptake of L-[^{3}H]arginine was similar in the oocytes injected with either amount of 4F2hc cRNA (see legend to Fig. 2*B*). We therefore conclude that there is dissociation between 4F2hc at the surface and induced

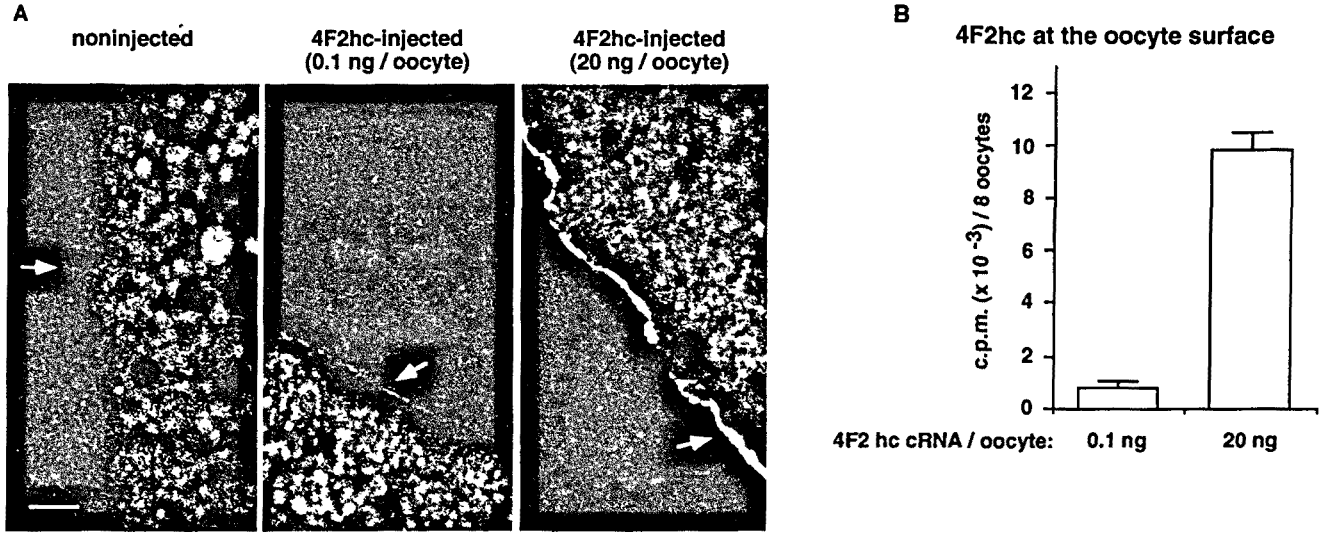

Figure 2. Dissociation between the expression of 4F2hc in the cell surface and induced y^+L-like activity in oocytes. Oocytes were injected with amounts of 4F2hc cRNA that correspond to a maximal (0.1 ng/oocyte) or supramaximal (20 ng/oocyte) dose with respect to the induction of amino acid transport activity. Four days after injections, oocytes were processed for protein expression or amino acid transport induction. *A)* Expression of 4F2hc protein in oocytes. Sections of injected and noninjected oocytes were processed for indirect immunofluorescence using a mouse monoclonal antibody anti-CD98 (i.e., 4F2hc) from Immunotech (Marseille, France) as primary antibody. The micrographs are overexposed in order to show that 4F2hc signal (arrows) is clearly visible in the surface of the oocyte injected with 20 ng of 4F2hc cRNA, whereas it is hardly visible in the surface of the oocyte injected with 0.1 ng of 4F2hc cRNA and no signal is detected in the surface of noninjected oocytes. The signal obtained without primary antibody in the injected groups was equal to that obtained with primary antibody in noninjected oocytes (not shown). Intracellular labeling was similar in the three groups of oocytes. The induction of sodium-independent L-[^{3}H]arginine and of sodium-dependent L-[^{3}H]leucine uptake were similar in oocytes, from the same batch, injected with both doses of cRNA (data not shown). These results are representative of three or four independent experiments where five oocytes for each group were analyzed. Scale bar, 10 μm. *B)* Expression of 4F2hc protein in the oocyte surface. Injected and noninjected oocytes were processed in groups of eight for detection of 4F2hc in the surface of intact oocytes. This was quantified by the radioactivity associated with the specific binding of [^{125}I]streptavidin to the complex of biotinylated goat anti-mouse antibody and mouse monoclonal anti-4F2hc antibody. Data are the mean ±SEM for five groups of eight oocytes in a representative experiment. In oocytes from the same batch, the induction of 50 μM L-[^{3}H]arginine uptake in choline Cl medium was similar in oocytes injected with 0.1 or 20 ng of 4F2hc cRNA (8.1±1.0 and 6.0±0.7 pmol/oocyte per 10 min, respectively; uptake in noninjected oocytes was 2.0±0.1 pmol/oocyte in 10 min). Data are the mean ±SEM for eight oocytes in a representative experiment.

y^+L-like activity in oocytes. This supports the hypothesis that an oocyte factor limits the 4F2hc-induced y^+L-like transport activity.

Human 4F2hc-induced amino acid uptake was inactivated by covalent modification of external cysteine residue(s)

Organic mercury compounds (pCMB and pCMBS) and Hg^{2+} blocked human 4F2hc-induced amino acid transport activity in oocytes. As shown in **Fig. 3**, both the water-soluble, membrane-impermeant pCMBS and the membrane-permeant, pCMB thiol-specific reagents inactivated this amino acid transport activity to the same extent. Similarly, Hg^{2+} blocked this induced transport activity (see Figs. 5, 8, and 9). Exposure of oocytes expressing human 4F2hc to 200 μM $HgCl_2$ for 20 min almost completely inactivated the induced L-[^{3}H]arginine uptake (the remaining activity was 10 ± 1% of induced control uptake; mean ± SEM from 21 oocytes in three independent experiments). Inactivation of 4F2hc-induced amino acid transport activity by these thiol-specific reagents exhibited characteristics expected for covalent modification of cysteine residues; it was not reversed upon washout, but was almost completely reversed by β-mercaptoethanol (see Figs. 3 and 7). These results indicate that covalent modification of external cysteine residues inactivates 4F2hc-induced amino acid transport activity (i.e., system y^+L) in oocytes.

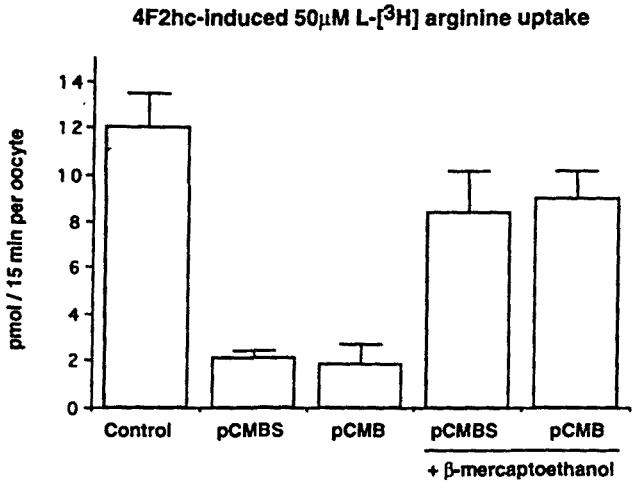

Figure 3. Hg agents inactivate 4F2hc-induced amino acid uptake by modification of extracellular cysteine residue(s). Three days after injection with saturating amounts of human 4F2hc cRNA, groups of seven or eight 4F2hc-injected and noninjected oocytes were incubated with 1 mM of the indicated organic mercury agents (pCMBS or pCMB) for 5 min. These agents were dissolved in 100 mM choline Cl medium (see ref 15 and Materials and Methods) containing 0.01% DMSO and 10 μM EDTA (to chelate free Hg^{2+}) for pCMB and 10 μM EDTA for pCMBS. Control oocytes were incubated in the same conditions and medium, but without the organic mercury agents and other chemicals. Previous experiments showed that 0.01% DMSO and 10 μM EDTA did not modify 4F2hc-induced amino acid uptake (data not shown). Then, oocytes treated with organic mercury agents and control oocytes were rinsed three times in 3 ml of 100 mM choline Cl medium and incubated for 5 min with 100 mM choline Cl medium with or without 5 mM β-mercaptoethanol, as indicated. Prior to 50 μM L-[^{3}H]arginine uptake measurement in 100 mM choline Cl medium, oocytes were again rinsed three times. Uptake values in the noninjected oocytes were (pmol/15 min per oocyte; mean ± SEM): 11 ± 1, control; 1.2± 0.3, pCMBS; 1.2 ± 0.2, pCMB; 11 ± 2, pCMBS + β-mercaptoethanol; 8.5 ± 1.2, pCMB + β-mercaptoethanol. 4F2hc-induced uptake (see Materials and Methods) is expressed in pmol/15 min per oocyte (mean ± SEM).

System y^+L was first described in human erythrocytes (29). So we next examined whether covalent modification of external cysteine residues also inactivated erythrocyte system y^+L. Treatment with 50 μM pCMBS for 10 min halved the uptake of 1 μM L-[^{14}C] lysine (**Fig. 4**). System y^+L and y^+ are responsible for L-lysine uptake in human erythrocytes; flux of 1 μM L-lysine through system y^+L is fully inhibited by 2 mM L-leucine in the presence of 140 mM sodium, whereas that through system y^+ is unaffected (29, 30). Here we show that ~50% of the total flux of 1 μM L-lysine occurs via system y^+L in human erythrocytes (Fig. 4), which is consistent with previous reports (29, 30). System y^+L was fully inactivated by 50 μM pCMBS for 10 min whereas system y^+ was unaffected (Fig. 4). This inactivation of L-lysine uptake was reversed by β-mercaptoethanol (Fig. 4). Therefore, the results in this section indicate that covalent modification of external cysteine residues inactivated system y^+L both in human erythrocytes and in oocytes expressing human 4F2hc.

Pretreatment with β-mercaptoethanol increased sensitivity of 4F2hc-induced amino acid transport to inactivation by Hg^{2+}

Protein labeling and immunoprecipitation studies suggested that 4F2hc is linked by disulfide bridges to a light subunit (23, 24). Pretreatment with the reducing agent β-mercaptoethanol (**Fig. 5**) did not alter the amino acid transport induced by 4F2hc in oocytes. In contrast, pretreatment with β-mercaptoethanol increased sensitivity to inactivation by Hg^{2+} (Fig. 5). This suggests that reduction of disulfide bridges increases the exposure of cysteine residue(s) of 4F2hc/system y^+L to Hg^{2+} either because the Hg^{2+}-targeted residues are involved in these bonds or due to indirect steric effects. In addition, these results indicate that the presence of disulfide bridges might not be necessary for the amino acid transport function of 4F2hc.

C109S-containing mutants retain partial y^+L transport activity

Human 4F2hc contains only two cysteine residues (C109 and C330), which face the extracellular space

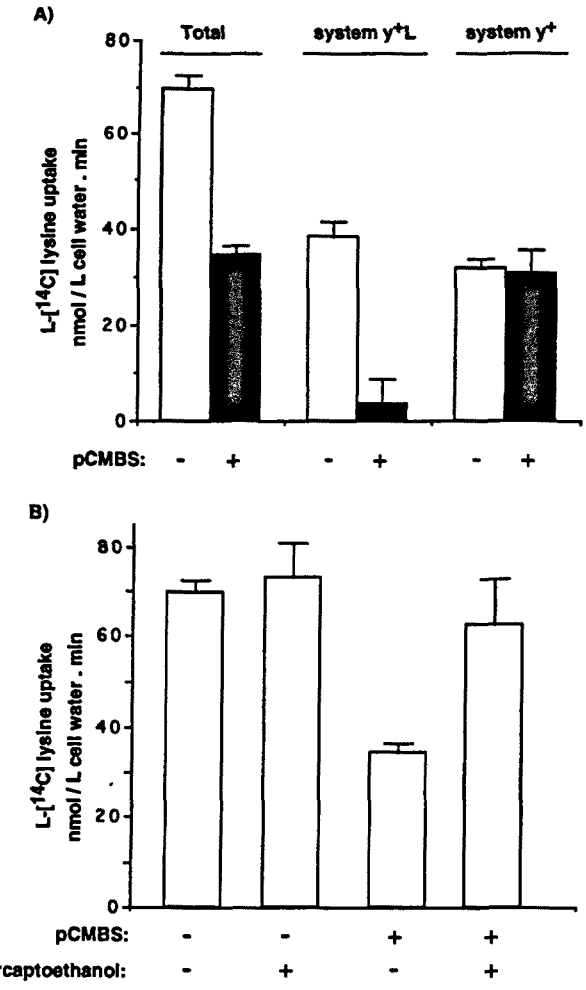

Figure 4. pCMBS inactivates human erythrocyte system y^+L by modification of extracellular cysteine residue(s). *A)* Uptake of 1 μM L-[^{14}C] lysine was measured (see Materials and Methods) in cells treated (closed bars) or not (open bars) with 50 μM pCMBS for 10 min. Amino acid transport flux inhibited by 2 mM L-leucine was attributed to system y^+L and the remnant flux to system y^+. *B)* The uptake of 1 μM L-[^{14}C] lysine uptake was measured in cells treated or not (as indicated) with p-CMBS (50 μM for 10 min) first and then with β-mercaptoethanol (5 mM for 10 min). Transport rates (nmol/l cell water·min) are the mean ± SEM from a representative experiment. A second, independent experiment gave similar results.

with a topology model as a type II membrane glycoprotein (20, 21). This may explain the inactivation of amino acid transport by pCMBS and the other thiol-specific reagents. To look for the cysteine residue that is the target of this inactivation, we mutated these two residues to serine either singly (C109S, CS1; C330S, CS2) or in combination (C109S-C330S, Cys-less). CS2/4F2hc induced system y^+L-like amino acid transport activity (i.e., sodium-independent transport of L-arginine, sodium-dependent transport of L-leucine, and very little sodium-independent transport of L-leucine) in oocytes to an extent similar to wild type 4F2hc (**Fig. 6*A***). In contrast, C109S-containing mutants (i.e., CS1 and Cys-less) retained only partial (30 to 50% of wild type) y^+L-like transport activity when expressed in oocytes (Fig. 6*A* and **Table 1**). CS1 and Cys-less showed reduced V_{max} but unaltered $K_{0.5}$ of the induced transport in oocytes (Table 1).

It is known that system 4F2hc/y^+L has an exchange mechanism of transport (15). To test whether CS1 mutant affected this transport mechanism, the efflux of L-[^{3}H]arginine was measured in oocytes expressing wild type or CS1 4F2hc. To load both sets of oocytes with a similar amount of labeled L-arginine, oocytes injected with wild type and CS1 4F2hc mutant were loaded with 50 μM L-[^{3}H]arginine for 30 and 90 min, respectively (see inset in Fig. 6*B*). Then, efflux of L-[^{3}H]arginine toward media containing no amino acid substrates or 1 mM L-arginine was measured. As previously described (15), efflux from wild type 4F2hc-injected oocytes was totally dependent on the presence of substrate (i.e., L-arginine) in the medium (Fig. 6*B*). This was also true for CS1 4F2hc mu-

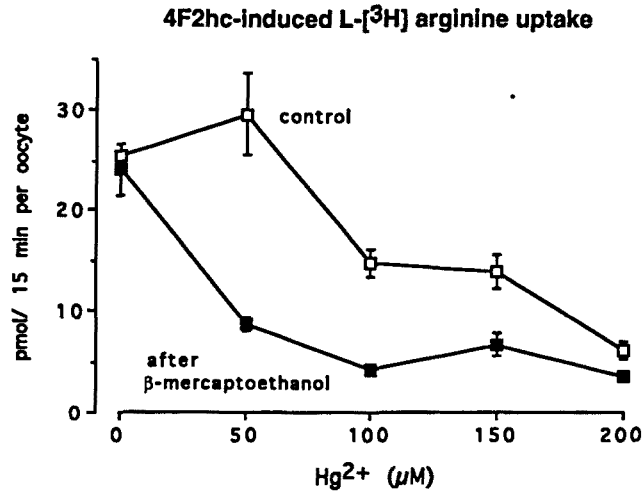

Figure 5. Effect of reducing conditions on 4F2hc-induced amino acid uptake: sensitivity to Hg^{2+} inactivation. Four days after injection with saturating amounts of human 4F2hc cRNA, groups of 7–8 4F2hc-injected and noninjected oocytes were incubated for 20 min with 100 mM choline Cl medium containing (solid squares) or not containing (control; open squares) 10 mM β-mercaptoethanol, as indicated. Oocytes were then rinsed three times with 100 mM choline Cl medium, as described in legend to Fig. 4, and incubated for 5 min to allow the release of possible intracellularly accumulated β-mercaptoethanol. Prior to 50 μM L-[^{3}H]arginine uptake measurement, oocytes were incubated for 20 min with the indicated concentrations of $HgCl_2$ and rinsed as described in legend to Fig. 4. Uptake values in the noninjected oocytes were (pmol/15 min per oocyte; mean±SEM): 2.2 ± 0.2, 0.4 ± 0.1, 0.2 ± 0.03, 0.3 ± 0.03, and 0.2 ± 0.02 for 0, 50, 100, 150, and 200 μM $HgCl_2$ in nontreated oocytes, and 2.3 ± 0.2, 0.5 ± 0.1, 0.2 ± 0.03, 0.3 ± 0.03, and 0.5 ± 0.1 for 0, 50, 100, 150, and 200 μM $HgCl_2$ in oocytes treated with β-mercaptoethanol. 4F2hc-induced uptake (see Materials and Methods) is expressed in pmol/15 min per oocyte (mean±SEM from three independent experiments).

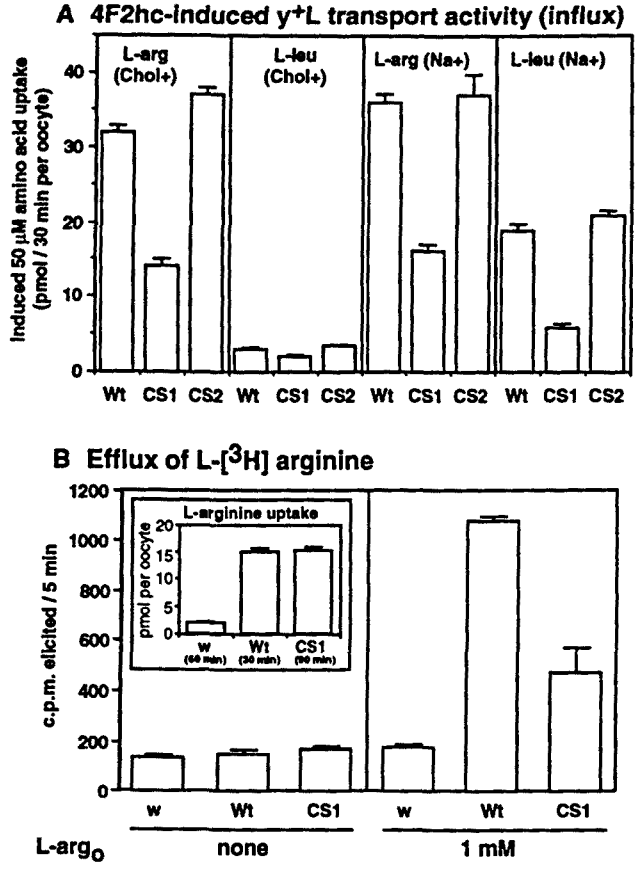

Figure 6. Cysteine-to-serine mutants of human 4F2hc are still functional. Three to four days after injection of saturating amounts of wild type (Wt), CS1 (solid bars) or CS2 mutants of human 4F2hc cRNA, oocytes were analyzed for amino acid uptake. *A)* Influx of 50 µM L-[^{3}H]arginine (L-arg) or L-[^{3}H]leucine (L-leu) was measured in the absence (Chol$^+$) or presence (Na$^+$) of 100 mM sodium, as indicated in Materials and Methods. Uptake values in the noninjected oocytes were (pmol/30 min per oocyte; mean±SEM from 7–8 oocytes per group): 2.5 ± 0.4, L-arg (Chol$^+$); 0.8± 0.1, L-leu (Chol$^+$); 14.5 ± 0.4, L-arg (Na$^+$); 14.8 ± 0.5, L-leu (Na$^+$). 4F2hc-induced uptake (see Materials and Methods) is expressed in pmol/30 min per oocyte (mean±SEM from 7–8 oocytes per group). *B)* For L-[^{3}H]arginine efflux measurements, groups of five oocytes, cRNA-injected (Wt or CS1) or noninjected (w), were incubated with 50 µM L-[^{3}H]arginine for 30 min (Wt) or 90 min (CS1) in order to reach a similar accumulation in both groups of oocytes; noninjected oocytes (w) were incubated for an intermediate time of 60 min (see inset). Oocytes were rinsed and the radioactivity efflux was measured in linear conditions, as described elsewhere (ref 15), to medium (L-arg$_o$) containing no amino acids (none) or 1 mM L-arginine. Efflux bars (cpm elicited/5 min) represent mean ± SEM from four groups of oocytes.

tant injected-oocytes, but the efflux rate was ~50% of that of wild type 4F2hc-injected oocytes. This demonstrated that the CS1 mutant does not affect the exchange mechanism of system 4F2hc/y$^+$L, but reduces the induced system y$^+$L activity.

Therefore, all the results in this section are compatible with reduced intrinsic transport activity of system y$^+$L induced by C109S-containing 4F2hc mutants and/or reduced active transport protein in the plasma membrane.

Cys-less 4F2hc-induced y$^+$L transport activity is inactivated by covalent modification of external cysteine residue(s)

The Cys-less 4F2hc mutant showed induced y$^+$L-like transport activity, which was completely inactivated by 0.1 mM Hg^{2+} and the water-soluble, membrane-impermeant thiol-specific reagent pCMBS (**Fig. 7**). Again, this inactivation had the characteristics expected for covalent modification of cysteine residues: it was not reversed upon washout and was completely reversed by β-mercaptoethanol (Fig. 7). This suggests that complete inactivation of the y$^+$L transporter function is caused by the modification of a non-4F2hc external cysteine-bearing protein.

Increased sensitivity to Hg^{2+} inactivation of C109S-containing mutants

The above results suggested an increased sensitivity to inactivation by thiol-specific reagents of the y$^+$L transport activity induced by Cys-less 4F2hc in comparison to that of wild type 4F2hc. Thus, 0.1 mM Hg^{2+} inactivated ~50% wild type 4F2hc-induced system y$^+$L-like and abolished almost completely Cys-less 4F2hc-induced system y$^+$L-like activity (Fig. 7). Indeed, sensitivity to Hg^{2+} inactivation increased in C109S-containing mutants (i.e.,.CS1 and Cys-less) (**Fig. 8**). Hg^{2+} at 10 µM completely inactivated the arginine uptake induced by CS1 and Cys-less mutants in 20 min, whereas under these conditions and in this experiment, wild type and CS2 4F2hc were inactivated only partially (approx 40%). Inactivation of wild type 4F2hc-induced system y$^+$L-like activity varies with different batches of oocytes, and in this experiment it was larger than in the experiments shown in Fig. 5. In an additional experiment, the increased sensitivity to Hg^{2+} inactivation of Cys-less 4F2hc-induced system y$^+$L-like activity was confirmed: 10 µM Hg^{2+} in 30 s completely inactivated Cys-less 4F2hc-induced arginine uptake but did not affect wild type 4F2hc-induced arginine uptake (data not shown). This, together with the increased sensitivity to thiol-specific reagents after β-mercaptoethanol treatment (Fig. 5), could be explained in two ways: *1)* residue C109 forms a disulfide bond with a cysteine residue of a 4F2hc-associated protein, which is the target of the thiol-specific reagent inactivation, or *2)* mutation of residue C109 to serine increases the accessibility of Hg^{2+} to an otherwise hidden external cysteine residue(s) of a native 4F2hc-associated protein. In any case, these data demonstrate the functional interaction of expressed 4F2hc and an oocyte plasma membrane protein to form active y$^+$L amino acid transporters.

TABLE 1. *Kinetic parameters of the amino acid uptake induced by wild type and cysteine-to-serine mutants of human 4F2hc*[a]

	L-arginine (choline medium)		L-leucine (sodium medium)	
	$K_{0.5}$ (μM)	V_{max} (pmol/15 min per oocyte)	$K_{0.5}$ (μM)	V_{max} (pmol/15 min per oocyte)
Wt	28 ± 1	20 ± 2	24 ± 3	35 ± 4
CS1	30 ± 3	10 ± 1	n.d.	n.d.
Cys-less	n.d.	n.d.	23 ± 3	15 ± 2

[a] Three days after injection of saturating amounts of wild type (Wt), CS1 mutant, or cysteine-free (Cys-less) human 4F2hc cRNA, the uptake of L-[^{3}H]arginine in the absence of sodium (100 mM choline Cl medium) or the uptake of L-[^{3}H]leucine in the presence of 100 mM sodium (sodium medium) at seven different concentrations of amino acid substrate (from 5 to 250 μM) were measured in groups of 7–8 oocytes. Simultaneously, amino acid uptake in the same conditions were measured in control noninjected oocytes (data not shown). The kinetic parameters (mean ±SEM) of the induced uptake (uptake in cRNA-injected minus that of noninjected oocytes) were estimated with Sigma Plot program. The L-[^{3}H]arginine and L-[^{3}H]leucine uptakes correspond to different batches of oocytes. n.d., not determined.

DISCUSSION

We have shown that induction of system y^+L amino acid transport activity by 4F2hc in oocytes is limited by endogenous factors: saturation of induction occurs with very low expression of 4F2hc at the oocyte surface, and further increased expression of the protein at the cell surface does not result in higher induction of the transport activity. In addition, we demonstrate that Hg^{2+} and two organic mercury compounds inactivate 4F2hc-induced y^+L activity. The reagents act in the extracellular medium since the membrane-impermeant thiol-specific reagent pCMBS inactivates transport to the same extent as the membrane-permeant pCMB. Similarly, human erythrocyte system y^+L is also inactivated by pCMBS. These reagents inactivate cysteine-free 4F2hc/system y^+L. In all cases, inactivation is reversed by β-mercaptoethanol. This shows that external cysteine residue modification of a plasma membrane protein of the oocyte leads to inactivation of 4F2hc-induced y^+L activity. Finally, reduction with β-mercaptoethanol of oocytes expressing 4F2hc increased sensitivity to Hg^{2+} inactivation of system y^+L. Similarly, C109S-containing mutants (CS1 and cysteine-free 4F2hc; Cys-less) showed increased sensitivity to Hg^{2+} inactivation. These results support that the y^+L amino acid transport system is formed through the intimate association of 4F2hc and an oocyte plasma membrane protein. These results also suggest that cysteine residue 109 of human 4F2hc may be linked by a disulfide bond to the Hg^{2+}-targeted cysteine residue of the 4F2hc-associated protein. The fact that reduction by β-mercaptoethanol does not result in decreased system 4F2hc/y^+L activity suggests that the 4F2hc-associated protein does not diffuse away from 4F2hc due to protein interactions other than disulfide bonds or that, after diffusion from 4F2hc, the associated protein remains fully active for transport. The experimental approach used in this study cannot discriminate between these two possibilities, nor does it show the number of associated proteins that form the functional heteromultimeric structure of 4F2hc/system y^+L or their identity. In contrast, it allows us to propose that the oocyte factor limiting y^+L activity and the 4F2hc-associated protein bearing external cysteine residue(s), which is susceptible to modification-inactivation, might be the light chain of 4F2hc revealed by previous labeling and immunoprecipitation studies (23, 24).

The mechanisms by which 4F2hc and rBAT induce amino acid transport systems y^+L and $b^{o,+}$, respectively, in oocytes are not clear. Structural and indirect functional evidence fosters the hypothesis that these transporters have a basic heterodimeric structure (for review, see opening paragraphs and refs 26, 33). Both proteins are considered to be complexed into heterodimers with corresponding light subunits (40–50 kDa), which seem to be linked by disulfide bridges (23, 26). These subunits have not been microsequenced or cloned. Indirect evidence suggests that these heterodimers are the functional units of the rBAT/system $b^{o,+}$-like and 4F2hc/system y^+L-like transporters. *1)* Transfection of rBAT in COS cells resulted in no amino acid transport expression (22, 26), and the 125 kDa rBAT complex is not detected (26). *2)* The loss of function of Met467Thr rBAT, the most frequent cystinuria type I mutant known, is due to a defect in trafficking to the plasma membrane that affects the V_{max} of the induced transport in oocytes (10). Long oocyte expression periods and injection of oversaturating amounts of mutant rBAT cRNA resulted in total recovery of the induced amino acid transport. Under these conditions, the amount of Met467 Thr rBAT on the oocyte surface is only <10% of the corresponding wild type protein (10). *3)* A carboxy terminus-deleted (D511-685) human rBAT induces an amino acid transport activity in oocytes with some of the characteristics of 4F2hc/system y^+L-like (34). This suggests that the carboxy ter-

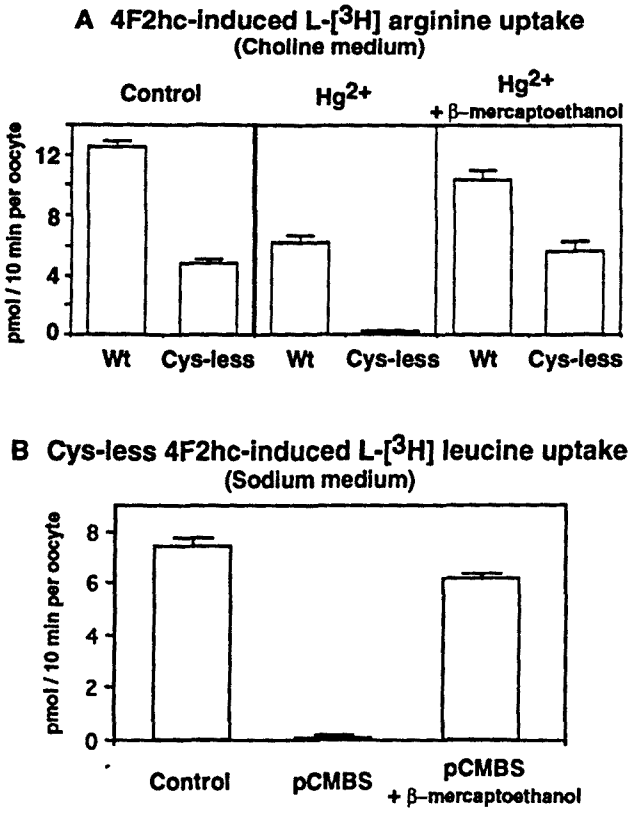

Figure 7. Cysteine-free human 4F2hc-induced amino acid uptake is sensitive to cysteine reagents. *A)* Three days after injection with saturating amounts of wild type (Wt) or cysteine-free (Cys-less; solid bars) human 4F2hc cRNA, groups of 7–8 cRNA-injected and non-injected oocytes were treated (Hg^{2+} and Hg^{2+}+β-mercaptoethanol) or not (control) with 0.1 mM of $HgCl_2$ and inactivation reversed for 20 min with 10 mM β-mercaptoethanol or not, as indicated. After all treatments, 50 μM L-[^{3}H]arginine uptake was measured in choline Cl medium. Uptake values in the noninjected oocytes were (pmol/10 min per oocyte; mean±SEM): 1.3 ± 0.1, control group; 0.04 ± 0.02, Hg^{2+}-treated group; 0.4 ± 0.03, Hg^{2+}- and β-mercaptoethanol-treated group. 4F2hc-induced uptake (see Materials and Methods) is expressed in pmol/10 min per oocyte (mean±SEM). *B)* Four days after injection of saturating amounts of cysteine-free human 4F2hc (Cys-less; solid bars) cRNA, groups of 7–8 cRNA-injected and noninjected oocytes were treated or not (control) with 0.1 mM pCMBS, as indicated. Then inactivation was reversed for 20 min with 10 mM β-mercaptoethanol or not, as indicated. After all treatments, 50 μM L-[^{3}H]leucine uptake was measured in the presence of 100 mM sodium (sodium Cl medium). Uptake values in the noninjected oocytes were (pmol/10 min per oocyte; mean±SEM): 1.7 ± 0.1, control group; 1.7 ± 0.05, pCMBS-treated group; 1.6 ± 0.05, pCMBS- and β-mercaptoethanol-treated group. 4F2hc-induced uptake (see Materials and Methods) is expressed in pmol/10 min per oocyte (mean±SEM). *A, B)* All reagents were dissolved in 100 mM choline Cl medium or 100 mM sodium Cl medium, depending on the transport to be measured: sodium-independent L-arginine or sodium-dependent L-leucine uptake, respectively. These media were used as control solutions; oocytes were rinsed after each treatment, as indicated in the legend to Fig. 4.

minus of rBAT and 4F2hc might be relevant for the interaction with the putative transporter or subunit in which the substrate specificity of systems $b^{o,+}$-like and y^+L-like might reside. Finally, *4)* Ahmed's and Taylor's groups (35, 36) reported that rBAT induces several amino acid transport systems in oocytes, including system $b^{o,+}$-like and a sodium-dependent histidine transport system with comparable activity, whereas the others are not very conspicuous. Similarly, Bröer's group (18, 37) reported increased amino acid transport activity in oocytes injected with rat 4F2hc compatible with the simultaneous induction of systems y^+L and $b^{o,+}$. Then, either because of the overexpression of rBAT or 4F2hc or reflecting a true mechanism of activation, rBAT and 4F2hc induce several amino acid transport activities in the oocyte. All this is compatible with rBAT and 4F2hc being necessary, but not sufficient, for the expression of systems $b^{o,+}$-like and y^+L-like, respectively: endogenous factors might be needed for the expression of these transport activities when rBAT or 4F2hc cRNA are injected into oocytes.

The results presented here indicate that 4F2hc is intimately associated with a membrane oocyte protein for the expression of system y^+L amino acid transport activity. Induction of transport activities in oocytes by the expression of foreign proteins that associate with endogenous subunits has been described for Na^+/K^+ ATPase (38) and potassium multimeric channels (39–41). Indeed, as for 4F2hc/system y^+L,

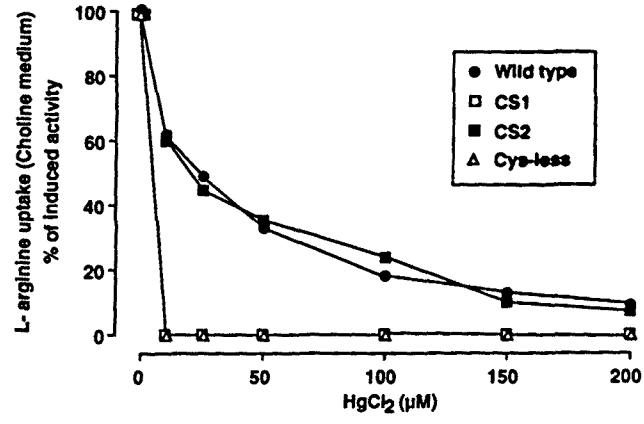

Figure 8. Increased sensitivity to Hg agents of the amino acid uptake induced by cysteine-109 to serine human 4F2hc mutants. Oocytes were injected with saturating amounts of wild type, CS1, CS2, or cysteine-free (Cys-less) human 4F2hc cRNA. Two days after injections, groups of 7–8 cRNA-injected or non-injected oocytes were incubated with varying concentrations of $HgCl_2$ dissolved in choline Cl medium for 20 min. After washouts (see legend to Fig. 4), 50 μM L-[^{3}H]arginine uptake was measured in choline Cl medium. Induced L-[^{3}H]arginine uptake values (see Materials and Methods) are expressed in percentage (mean±SEM) of the corresponding control values. Uptake values in the noninjected oocytes were (pmol/15 min per oocyte; mean±SEM): 2.1 ± 0.2, control group, and 0.6 ± 0.02, 0.5 ± 0.01, 0.5 ± 0.03, 0.5 ± 0.01, 0.7 ± 0.1, and 0.6 ± 0.05 for 10, 25, 50, 100, 150, and 200 μM $HgCl_2$ groups. cRNA-induced uptake values in control conditions were (pmol/15 min per oocyte; mean±SEM): 13.5 ± 0.6 for wild type 4F2hc; 6.5 ± 0.5 for CS1 mutant; 13.5 ± 0.8 for CS2 mutant; and 6.3 ± 0.7 for cysteine-free 4F2hc (Cys-less).

the first direct evidence for a heteromultimeric structure of MinK channels came from studies of cysteine residue modification of the channel activity induced by cysteine-free MinK in oocytes (42). The increased sensitivity to Hg^{2+} inactivation of the C109S-containing mutants shown here suggests that this residue participates in a disulfide bridge with a cystine residue of an oocyte 4F2hc-associated protein, which is the target of the Hg^{2+}inactivation of system y^+L. A similar approach has been used to identify an intracatenary disulfide bridge in the second external loop of the serotonine transporter (43). Human 4F2hc-C109 residue is conserved in all known 4F2hc sequences (18, 20, 21, 44) and is the only cysteine residue of 4F2hc proteins conserved in all known rBAT sequences (cysteine residue 114 in human rBAT) (1–3, 45, 46). Additional work is needed to demonstrate functional association of rBAT with an endogenous plasma membrane protein through residue C114 in human rBAT. It has been shown that rBAT/system $b^{o,+}$ is inactivated mainly through modification of intracellular cysteine residues (47). This suggests that the experimental approach used in the present study may not be suitable for rBAT/system $b^{o,+}$.

We are far from establishing the physiological role of 4F2hc in amino acid transport. Several labs reported induction of amino acid transport in oocytes injected with 4F2hc cRNA. Human (4, 5, 15, 16; this study) and rat (R. Estévez, A. Zorzano, and M. Palacín, unpublished results) 4F2hc induced y^+L-like transport activity. In contrast, Bröer's group (17, 18) found induction of a system L-like transport activity by injecting rat glioma culture cell mRNA in oocytes, which is hybrid-depleted by rat 4F2hc antisense sequences. Expression cloning from this transport signal resulted in the isolation of rat 4F2hc cDNA (18). Injection of this cRNA into oocytes resulted in the induction of amino acid transport compatible with the simultaneous expression of systems y^+L-like and $b^{o,+}$-like (18, 37). These puzzling results can now be explained after the demonstration of the heteromultimeric structure of 4F2hc/system y^+L presented here: different 4F2hc-associated proteins or subunits, expressed in different tissues or in *Xenopus* oocytes, might explain induction of amino acid transport systems y^+L-like, L-like, and $b^{o,+}$-like after injection of oocytes with human or rat 4F2hc (4, 5, 15), and with mRNA from human choriocarcinoma cells (16), rat glioma culture cells (18), and rat lung (R. Estévez, A. Zorzano, and M. Palacín, unpublished results). Purification studies of the 4F2hc complex and screening of a rat lung cDNA library after coexpression of system y^+L activity by injecting saturating amounts of 4F2hc cRNA and rat lung mRNA (R. Estévez, A. Zorzano, and M. Palacín, unpublished results) are currently in progress in an attempt to identify 4F2 light chain subunit(s). FJ

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Identification and characterization of a membrane protein (y^+L amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity y^+L. A candidate gene for lysinuric protein intolerance.

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Identification and Characterization of a Membrane Protein (y^+L Amino Acid Transporter-1) That Associates with 4F2hc to Encode the Amino Acid Transport Activity y^+L

A CANDIDATE GENE FOR LYSINURIC PROTEIN INTOLERANCE*

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We have identified a new human cDNA (y^+L amino acid transporter-1 (y^+LAT-1)) that induces system y^+L transport activity with 4F2hc (the surface antigen 4F2 heavy chain) in oocytes. Human y^+LAT-1 is a new member of a family of polytopic transmembrane proteins that are homologous to the yeast high affinity methionine permease MUP1. Other members of this family, the *Xenopus laevis* IU12 and the human KIAA0245 cDNAs, also co-express amino acid transport activity with 4F2hc in oocytes, with characteristics that are compatible with those of systems L and y^+L, respectively. y^+LAT-1 protein forms a ≈135-kDa, disulfide bond-dependent heterodimer with 4F2hc in oocytes, which upon reduction results in two protein bands of ≈85 kDa (*i.e.* 4F2hc) and ≈40 kDa (y^+LAT-1). Mutation of the human 4F2hc residue cysteine 109 (Cys-109) to serine abolishes the formation of this heterodimer and drastically reduces the co-expressed transport activity. These data suggest that y^+LAT-1 and other members of this family are different 4F2 light chain subunits, which associated with 4F2hc, constitute different amino acid transporters. Human y^+LAT-1 mRNA is expressed in kidney ≫ peripheral blood leukocytes ≫ lung > placenta = spleen > small intestine. The human *y^+LAT-1* gene localizes at chromosome 14q11.2 (17cR ≈ 374 kb from D14S1350), within the lysinuric protein intolerance (LPI) locus (Lauteala, T., Sistonen, P., Savontaus, M. L., Mykkanen, J., Simell, J., Lukkarinen, M., Simmell, O., and Aula, P. (1997) *Am. J. Hum. Genet.* 60, 1479–1486). LPI is an inherited autosomal disease characterized by a defective dibasic amino acid transport in kidney, intestine, and other tissues. The pattern of expression of human y^+LAT-1, its co-expressed transport activity with 4F2hc, and its chromosomal location within the LPI locus, suggest *y^+LAT-1* as a candidate gene for LPI.

rBAT and 4F2hc are homologous proteins that induce amino acid transport in *Xenopus* oocytes (1, 2). These two proteins are slightly hydrophobic, which prompted the hypothesis that rBAT and 4F2hc are subunits or modulators of the corresponding amino acid transporter. This has been supported by several indirect observations: (i) rBAT and 4F2hc are involved in the induction of several activities in *Xenopus* oocytes (3–6); (ii) these two proteins can be immunodetected or immunoprecipitated as complexes of ≈125 kDa in the absence of reducing agents and as two proteins of ≈85 kDa (4F2hc or rBAT) and ≈40 kDa in the presence of reducing agents (7–9); and (iii) in oocytes, there is a dissociation between the expression of 4F2hc and rBAT at the plasma membrane and the induction of system y^+L and $b^{0,+}$ activity, respectively, indicating that this expression is limited by an endogenous factor (10, 11). We have recently provided new evidence that the amino acid transport system y^+L has a heterodimeric structure (11). Thus, we have shown that the y^+L activity induced in oocytes by a cysteine-less mutant of human 4F2hc is also inactivated by membrane-impermeant thiol-specific reagents, implying that another protein is required for this function, which would have external cysteine(s) that are targets of these reagents. Moreover, the sensitivity to inactivation is increased by reducing conditions and in 4F2hc mutants in which cysteine 109 has been mutated. These results indicate that Cys-109 may be linked by a disulfide bond to the cysteine target of these agents of the associated protein.

ASUR4 (Y12716), an adrenal steroid up-regulated cDNA from *Xenopus laevis* A6 cells (12) induces an L-type amino acid transport activity when co-expressed with 4F2hc in oocytes.[1] When comparing the amino acid sequence of ASUR4 to protein data bases, many highly homologous eukaryotic and prokaryotic amino acid transporter-related proteins are listed within the amino acids, polyamines, and choline (APC) family of transporters. Among these, and with highest degree of identity (between 38 and 82% when corresponding protein regions are compared) to ASUR4, are found its counterpart in human, E16 (Q01650) (13), and in rat, TA1 (Q63016) (14); a human cDNA, termed KIAA0245 (D87432) (15); five different *Caenorhabditis elegans* open reading frames deduced from genomic DNA sequence (Z68216, U50308, Z74042, U56963, and U70850) (16); and a *Schistosoma mansoni* cDNA, SPRM1 (L25068). In the same list appeared a yeast high affinity methionine permease, MUP1 (17) (U40316), and many other prokaryotic amino acid permeases. ASUR4 showed low, although significant, identity

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF092032.

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[1] F. Verrey, personal communication.

(between 26 and 31%) with the mammalian transporters for cationic amino acids CAT1[2] and CAT2 (18, 19). Human E16 was first identified from peripheral blood leukocytes and related to lymphocyte activation (13). Rat TA1 was cloned later on the basis of its differential expression between hepatoma cells and normal liver (14). E16, TA1, and ASUR4 cDNA were first described as proteins 241 amino acids long. The presence in the data base of a thyroid hormone regulated *X. laevis* cDNA, termed IU12 (AF019906) (20), which was 507 amino acid long and practically identical to ASUR4 (only one amino acid was different in the corresponding protein region), suggested that the former three cDNAs were indeed longer. Very recently, F. Verrey has submitted a new ASUR4 cDNA GenBank entry (accession number Y12716), which also has 507 amino acids. Although IU12 and the new entry of ASUR4 still differ in four disperse amino acids, we can consider that both sequences correspond to the same gene in *Xenopus*. We can now assume that E16 and TA1 are actually longer proteins.

In this study, we have identified a new human member of this group of amino acid permease-related proteins This protein, which we have named y⁺L amino acid transporter-1 (y⁺LAT-1) does not induce transport of amino acids in oocytes when injected alone, but y⁺L activity is co-expressed when it is injected with 4F2hc. We demonstrate here that it forms an heterodimer with 4F2hc linked by disulfide bridges with residue cysteine 109 of human 4F2hc. Its pattern of expression and its chromosomal localization indicate that this gene could be responsible for lysinuric protein intolerance (21), an inherited disorder of cationic amino acid transport.

EXPERIMENTAL PROCEDURES

Oocytes, Injections, and Uptake Measurements—Oocyte origin, management, and injections were as described elsewhere (1, 2) Defolliculated stage VI *X laevis* oocytes were injected with different amounts of human 4F2hc, human y⁺LAT-1, human y⁺LAT-2 (KIAA0245), or *X laevis* IU12 cRNA Amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those of uninjected oocytes (data not shown) Synthesis of human 4F2hc cRNA (22) was as described (11) IU12 was a gift from Shi and co-workers (20), and the cRNA was obtained by cutting the cDNA, cloned in pBluescript SK⁻ between the sites *Xho*I and *Eco*RI with *Apa*I and using T3 polymerase The open reading frame of y⁺LAT-1 was obtained from the Integrated and Molecular Analysis of Genomes and their Expression (IMAGE) cDNA clone 727811 cloned in the vector pT7T3 between the restriction sites *Eco*RI and *Not*I. To obtain the y⁺LAT-2 cRNA, because it has a long 3′-untranslated region and is not expressed properly in *Xenopus* oocytes, we inserted the open reading frame of KIAA0245 (obtained from Takahiro Nagase from the Kasuza DNA Research Institute, (15) and cloned in pBluescript II SK⁺) in another vector with a shorter 3′ tail Subcloning was done by cutting pBluescript-KIAA0245 with *Apa*I and filling with Klenow, the clone was then ethanol-precipitated, cut again with *Pst*I, and finally ligated into pSPORT1-human rBAT that had been cut with *Pst*I and *Bst*1107I Influx rates of L-[^{3}H]arginine, L-[^{3}H]leucine were measured in 100 mM NaCl or 100 mM choline Cl medium at the indicated days after injection and under linear conditions When presented, the induced uptake was calculated by subtracting uptake values in uninjected oocytes from those of the corresponding cRNA-injected oocytes

PCR Amplification and Sequencing—For PCR amplification, first strand cDNA was synthesized from 5 μg of total RNA purified from opossum kidney (23) cells using the SuperScript II kit (Life Technologies, Inc) Two degenerate forward and reverse primers were designed based on two highly conserved regions among KIAA0245, IU12, E16, TA1, and SPRM1 proteins From region 1 sequence (A/S)REGHLP (corresponding to residues 347–353 of KIAA0245), a forward 5′-C(C/T)-(C/A)G(C/A/G)GA(G/A)GG(C/G)CA(C/T)CT(G/C/T/A)CC-3′ primer (1F) was synthesized, as well as a reverse (2R) 5′-A(T/G)G(A/C)(T/A)(A/G)-AA(C/G)A(C/A)(C/G)A(C/T)(T/A/G)GG-3′ primer deduced from region 2 sequence P(I/V)(V/F)F(I/C)(I/L) (corresponding to residues 429–434 of KIAA0245) Amplification was carried out in a Perkin-Elmer 9600 thermocycler, and conditions were as follows hot start of 3 min at 94 °C, 15 cycles of denaturing (94 °C for 25 s), annealing (starting 65–50 °C lowering 1 °C each cycle for 30 s), and extension (74 °C for 70 s), 25 cycles of denaturing (94 °C for 25 s), annealing (50 °C for 30 s), and extension (74 °C for 70 s), and a final extension of 4 min at 72 °C PCR-amplified DNA fragments with the expected length were subcloned into pGEM-T easy vector (Promega) and sequenced in one direction The DNA sequence obtained and all frames of the deduced amino acid sequences were then compared with DNA and protein sequence data bases. All sequences carried out in this work were performed in one or both directions (in the case of clone 727811) with D-Rhodamine Dye Terminator Cycle Sequencing Ready Reaction (Perkin-Elmer) Analysis of the sequence reactions was done with an Abi Prism 377 DNA sequencer

Computer Analysis—Amino acid or nucleotide sequence homology searching was performed using basic local alignment tool (BLAST) via on-line connection to the National Center of Biotechnology Information The programs BLASTn, BLASTp, and BLASTx were run using default parameters Data base searching was done against nonredundant or dbEST, when searching for nucleotide sequence homology, and *versus* nonredundant when comparing to peptide sequences. The clusters of Expressed Sequence Tag were identified and analyzed with the IMAGE data base and Telethon Institute of Genetics and Medicine EST assembly machine tool. Multiple nucleotide or amino acid sequence comparisons were done with CRUSTALW via on-line connection to the Genome World Wide Web server (University of Tokyo) and to the Baylor College of Medicine Search Launcher (University of Texas) Amino acid sequence deduction and other sequence analysis were done with Genetics Computer Group utilities

The prediction of transmembrane segments of the proteins y⁺LAT-1, y⁺LAT-2, IU12, and SPRM1 was established on the basis of the combination of three criteria (i) the prediction of transmembrane segments by the programs of Aloy *et al.* (23) and TopPred II (24) using the algorithms of G. von Heijne (25), Goldman, Engelman, and Steitz (26), and K_D (27) to determine the position of hydrophobicity peaks, (ii) the prediction of α-helix in the predicted secondary structure using a program that combines the algorithms of Chou-Fasman and Rose (28), and (iii) the surface probability and flexibility index plots, according to the algorithms of Boger (29) and Karplus and Schulz (30), respectively

Northern Blot Analysis—A human adult poly(A⁺) membrane containing 12 different tissues, purchased from CLONTECH (Palo Alto, CA) was used Insert of clone 727811 was separated from the pT7T3D-727811 vector with *Apa*I-*Not*I digestion This 2250-bp-long DNA fragment was purified, labeled with [α-^{32}P]dCTP (Amersham) using a random oligonucleotide-priming labeling kit (Amersham), and used as a probe Hybridization, carried out in Express HybTM Hybridization solution (CLONTECH), and wash conditions were as recommended by the manufacturer To rule out differences in sample loading, the CLONTECH membrane was hybridized with human β-actin probe A nonradioactive fluorescein and anti-fluorescein peroxidase-conjugated antibody detection kit was used (Amersham) Hybridization, washes, and detection conditions were as suggested by the supplier

Chromosome Mapping—Chromosome mapping was done using the Stanford Human Genome Center G3 Radiation Hybrid panel (medium resolution) DNA samples of this panel, along with total genomic DNA and pT7T3-727811 (used as a positive controls), were PCR-screened for the presence of the genomic sequences flanked by the primers F7 (5′-GGAAGTTGAAAAGGAAAGC-3′) and R7 (5′-AAGGAGACAGGAAATTGG-3′), which are located at the 3′-untranslated region of the cDNA PCR amplifications were carried out in a Perkin-Elmer 9600 thermocycler, using 200 μM dNTP, 3 pmol of each primer and DNA *Taq* polymerase (Boehringer Mannheim) in PCR buffer Amplification conditions were as follows· 35 cycles of denaturing (94 °C for 30 s), annealing (56 °C for 40 s), and extension (74 °C for 30 s) PCR results were classified as 0 (for no amplification), 1 (for positive amplification), or r (for uncertain) and submitted to the Radiation Hybrid Mapping E-mail server at the Stanford Human Genome Center (SHGC) Resulting chromosomal location, referred to a SHGC marker, was obtained automatically via E-mail from this server

Site-directed Mutagenesis—Construction of the mutants C109S and C330S of human 4F2hc was as described in Ref. 11

Immunoprecipitation of Methionine-labeled Proteins from Xenopus Oocytes—Oocytes were injected with 10 ng of human 4F2hc or C109S human 4F2hc (CS1) or C330S human 4F2hc (CS2) alone or in combi-

[2] The abbreviations used are CAT, cationic amino acid transporter, LAT, L amino acid transporter, LPI, lysinuric protein intolerance, IMAGE, Integrated and Molecular Analysis of Genomes and their Expression; SHGC, Stanford Human Genome Center, PCR, polymerase chain reaction, bp, base pair(s)

nation with 10 ng of y⁺LAT-1 cRNA. After 24 h, [^{35}S]methionine (0.5 μCi in 50 nl of water; ICN) was injected, and the oocytes (usually 20 oocytes) were incubated for 48 h at 18 °C in 1 ml of modified Barth's solution. Oocytes were then harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethanesulfonyl fluoride. Extracts were centrifuged twice at 1000 × *g* in order to remove the yolk granules. Aliquots of 10^6 cpm were rotated overnight at 4 °C with 20 μl of human 4F2hc antibody (Immunotech, Marseille, France) previously bound to protein G-Sepharose (Sigma). The beads were washed five times in a buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 500 mM LiCl, and 0.5% Nonidet P-40 and five times in the same buffer without LiCl. The resulting immunoprecipitates were heated in sample buffer with or without 100 mM dithiothreitol for 5 min at 95 °C before gel loading. The labeled proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography after enhancement with 1 M sodium salicylate.

RESULTS

Our goal was to identify any new member of the amino acid transporter-related family expressed in kidney and potentially involved in reabsorption of amino acids. For this purpose reverse transcription PCR amplification of total RNA from opossum kidney cells (32) was performed. Degenerated primers were designed on the basis of two highly conserved protein regions (see under "Experimental Procedures") revealed after a multiple amino acid sequence comparison among KIAA0245, E16, TA1, IU12, and SPRM1 proteins. Electrophoretic analysis of the PCR showed one band of 228 bp, which was subcloned into pGEMT-easy vector and amplified in *Escherichia coli*. Several clones were then analyzed by sequencing. Nucleotide sequence of clone b4c2 showed a significant degree of identity to the amino acid transporter-related proteins when compared, using BLASTx program, to nonredundant peptide data bases. Deduced amino acid sequence comparison showed an identity of 75, 52, 49, 47, and 20% for b4c2 with KIAA0245, IU12, TA1, E16, and SPRM1, respectively. This high degree of identity with KIAA0245 suggested that we had cloned a fragment of the KIAA0245 ortholog cDNA in opossum. To rule out the presence of other human genes having high homology to KIAA0245, the coding region sequence of this gene was run as a query with BLASTn program against dbEST data bases. Flanking 3′- and 5′-untranslated regions were avoided to minimize the presence of KIAA0245 EST in the results. We identified an IMAGE EST cluster (46303) that corresponds to a new unidentified human gene with a high degree of identity (75%) to KIAA0245. EST AA393488 (located 5′ of this cluster) and EST AA400789 (located 3′ of this cluster and presenting a poly(A⁺) tail) are flanking regions of IMAGE cDNA clone 727811 and comprised the whole cDNA. We named this clone y⁺LAT-1 (y⁺L amino acid transporter), and KIAA0245 tentatively as a y⁺LAT-2 because they yielded system y⁺L amino acid transport activity when co-injected with 4F2hc in oocytes (see below).

Two direction sequencing of clone 727811 (Fig. 1) showed a cDNA 2245 bp long. Sequence comparison of the corresponding region of y⁺LAT-1 with the opossum b4c2 clone revealed 82 and 81% identity for DNA and protein, respectively. We then assumed that b4c2 clone is a fragment of the corresponding y⁺LAT-1 in opossum. The size of the human y⁺LAT-1 cDNA corresponds to the transcript seen in Northern blots (see Fig. 5). The first ATG codon lies within a good consensus initiation sequence (5′-CCACC) (33). The open reading frame continues to the first stop codon (TAA) at base 1820 and codes for a protein of 511 amino acid residues with a predicted molecular mass of 55,988 Da. The nucleotide sequence of y⁺LAT-1 has been deposited in the GenBank data base (accession number: AF092032).

Hydrophobicity studies (see under "Experimental Procedures") show 12 transmembrane domains with both C- and N-terminal segments intracytoplasmatic, a typical protein structure similar to some previously reported organic solute transporters (34–36). There is only one putative N-glycosylation site underlined in Fig. 1 (Asn-Ala-Ser) between the putative transmembrane segments VIII and IX. In our prediction model, this segment is cytoplasmic and cannot be glycosylated. There are also two putative casein kinase II phosphorylation sites (threonine 8 and serine 11, located in the putative cytoplasmic N-terminal segment) and one putative protein kinase C phosphorylation site (threonine 96, located intracellularly between the putative transmembrane segments II and III). A multiple sequence alignment of the predicted amino acid sequence of y⁺LAT-1, y⁺LAT-2 (KIAA0245), IU12, E16, and SPRM1 is shown in Fig. 2. The percentages of identity between y⁺LAT-1 and y⁺LAT-2, E16, IU12, SPRM1, and the yeast permease MUP1 are 75, 51, 53, 39, and 31%, respectively. The predicted structural model of these proteins is also very similar. Only the consensus for the position of the transmembrane segment III can vary for the proteins presented in Fig. 2. For y⁺LAT-1 and y⁺LAT-2, this segment could be located in the position indicated in Fig. 2. However, in the case of IU12, the fragment is displaced 10 amino acids to the C-terminal end, and in the protein SPRM1, the fragment is moved 5 amino acids to the N-terminal end. Because 4F2hc is associated with y⁺LAT-1 in a disulfide bond-dependent manner (see Fig. 7), we looked for cysteine residues conserved in these proteins. There are only two cysteines conserved in all these proteins: cysteine 151 of y⁺LAT-1, located extracellularly in our structure model prediction, corresponding to residues 159, 164, and 137 of y⁺LAT-2, IU12, and SPRM1, respectively; and cysteine 174, located in the transmembrane domain IV and corresponding to residues 182, 187, and 160 of y⁺LAT-2, IU12, and SPRM1, respectively. These two cysteines are not conserved in the yeast permease MUP1.

The human *y⁺LAT-1* gene was chromosome mapped by using a radiation hybrid panel (see under "Experimental Procedures") with primers corresponding to the 3′-untranslated region of the y⁺LAT-1 cDNA. From this screening, we obtained 13 positive, 70 negative and 2 uncertain results. Chromosome mapping results, obtained from the SGHC server, linked *y⁺LAT-1*, with a logarithm odds score of 10.4, to a distance of 17 cR (374 kb) from the marker SHGC-13532 (D14S1350) located at chromosome 14q11.2. When uncertain samples were submitted as positive, the localization was linked to the T-cell receptor α chain marker, which lies ≈150 kb telomeric of SHGC-13532.

cRNA from y⁺LAT-1, y⁺LAT-2, and IU12 was prepared and injected into oocytes alone or in combination with an equimolar quantity of human 4F2hc cRNA and tested for transport of arginine and leucine (50 μM) in the presence or in the absence (choline) of sodium (100 mM) (Fig. 3). These three proteins do not induce any amino acid transport activity when injected alone, but interestingly, they induce different activities when co-injected with 4F2hc. In the case of y⁺LAT-1 and y⁺LAT-2 (KIAA0245), the pattern of induced activity resembles that described as system y⁺L (37) (*i.e.* sodium-independent uptake of dibasic amino acids and sodium-dependent uptake of some neutral amino acids). IU12, by contrast, induced an activity above that of 4F2hc alone, which is compatible with the activity described as system L (*i.e.* sodium-independent uptake of neutral amino acids). For y⁺LAT-1, the induced activity is very similar to the component of y⁺L activity induced by 4F2hc alone, but the level of induction is higher. From 10 independent experiments, the average fold induction relative to the induction of 4F2hc alone was 3.8 ± 0.9 (range, 2–14-fold). To explain this increase, we performed kinetic analysis, and from an in-

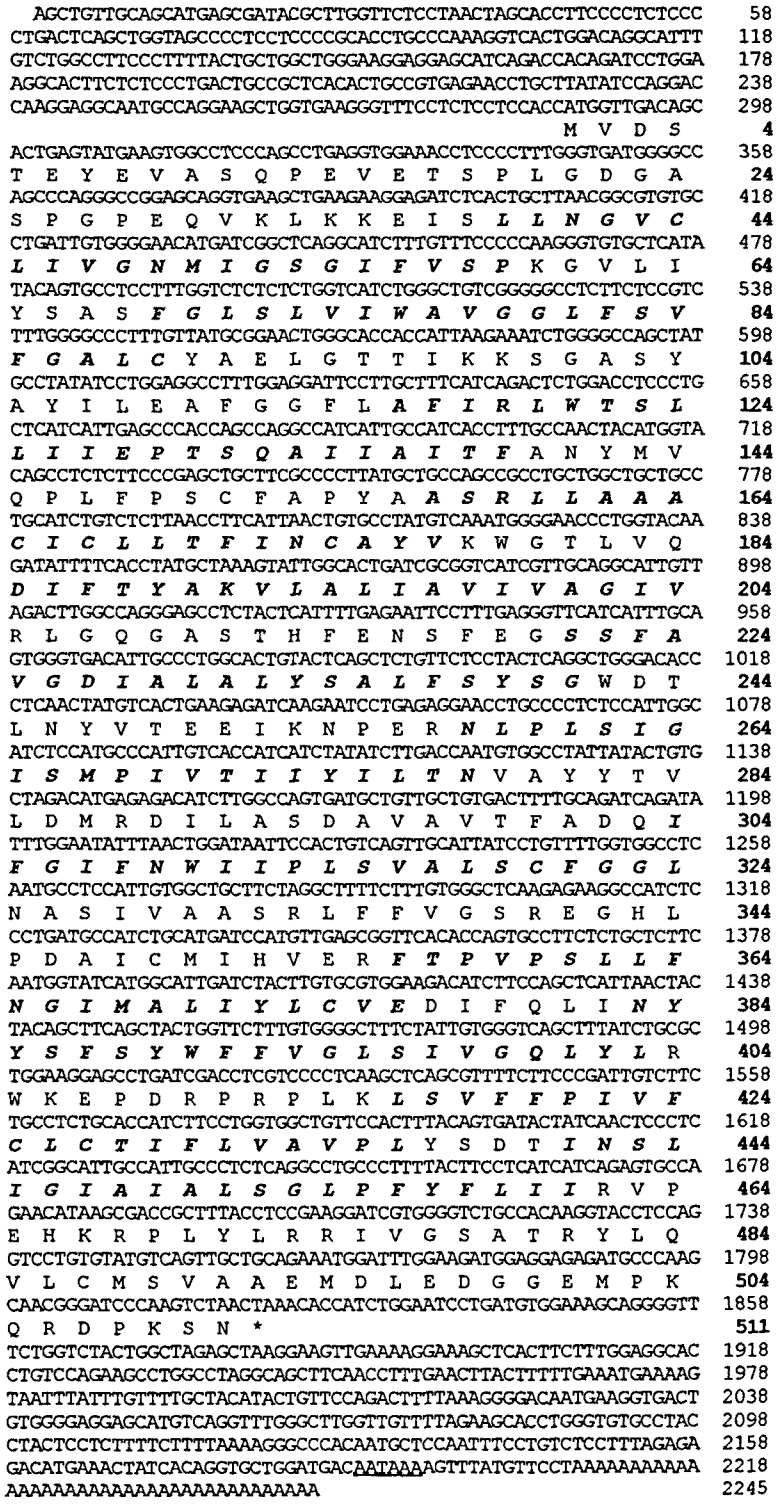

FIG. 1. **Nucleotide and deduced amino acid sequence of the y⁺LAT-1 cDNA.** The size of the clone is 2245 bp and it contains a 5'-untranslated region of 268 bp, followed by an open reading frame of 511 amino acids and a 3'-untranslated region of 423 bp that contains a poly(A) tail of 38 bp. The stop codon (TAA) is indicated by one *star*. The possible polyadenylation signal is *underlined*. The putative transmembrane domains, deduced by hydrophobicity analysis (see under "Experimental Procedures"), are shown in *boldface*.

dividual experiment, the kinetic parameters showed an increase in V_{max} without apparent change in the $K_{0.5}$ parameter (4F2hc-induced uptake: $K_{0.5}$, 55 ± 15 μM; V_{max}, 18 ± 4 pmol of arginine (choline medium)/15 min per oocyte; 4F2hc plus y⁺LAT-1-induced uptake: $K_{0.5}$, 45 ± 18 μM; V_{max}, 36 ± 5 pmol of arginine (choline medium)/15 min per oocyte). A further characterization of this transport activity co-expressed by y⁺LAT-1 and y⁺LAT-2 is in progress.[3]

To further characterize the uptake activity co-expressed by y⁺LAT-1 and 4F2hc, we measured the inhibition of arginine uptake by different amino acids at a 100-fold excess concentration (5 mM). As shown in Table I, dibasic amino acids inhibit 50 μM arginine uptake in a sodium-independent manner, but in contrast, neutral amino acids inhibit more in the presence of sodium. In order to define better the effect of sodium on the inhibition by neutral amino acids, the uptake of L-arginine (50 μM) was measured in the presence or absence of sodium and in the presence of different concentrations of L-leucine (Fig. 4). These results showed clearly that sodium increased the affinity of L-leucine. This effect is indistinguishable in 4F2hc alone or

[3] M. Pineda, R. Estévez, and M. Palacín, manuscript in preparation.

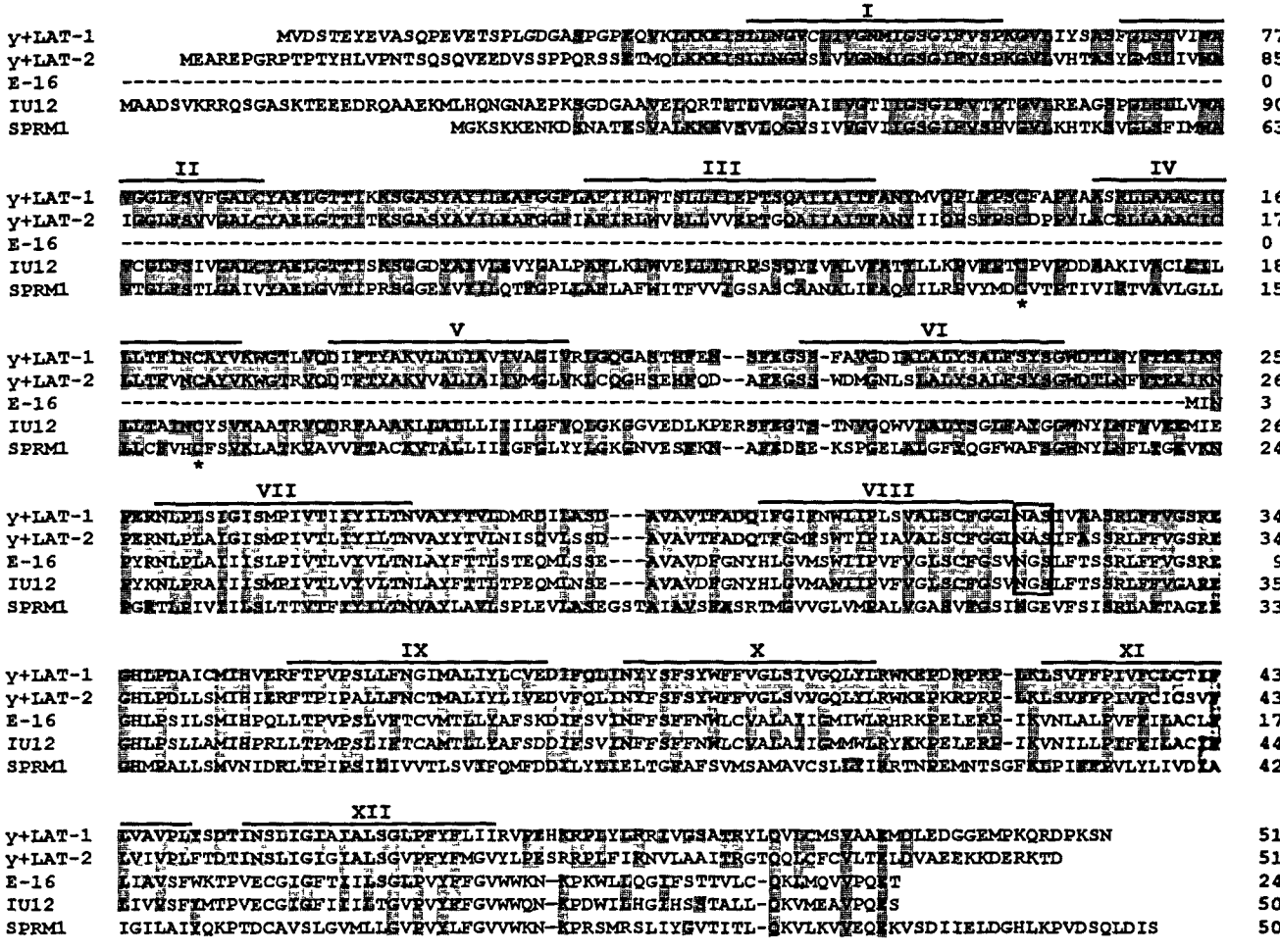

FIG. 2. **Amino acid sequence comparison of five members of the family of amino acid transporter-related proteins.** Multialignment was done using the program CLUSTALW Sequence Alignment from Baylor College of Medicine The *thin horizontal lines* indicate the putative 12 transmembrane domains determined by computer analysis (see under "Experimental Procedures"). The amino acid residues identical to y LAT-1 sequence are indicated by *gray boxes*. The *solid frame box* indicates a potential N-glycosylation site, but according to our membrane topology prediction, this site is intracellular and cannot be glycosylated. Two cysteine residues conserved in all the proteins presented here are indicated by a *star*.

4F2hc plus y⁺LAT-1-injected oocytes. All of this is consistent with the expression of y⁺L transport activity (38).

The tissue expression of the mRNA corresponding to y⁺LAT-1 was examined by Northern blot analysis at high stringency conditions (Fig. 5). The mRNA species of ≈2.4 kb hybridizes with the y⁺LAT-1 cDNA. Transcript expression is as follows: kidney ≫ peripheral blood leukocytes ≫ lung > placenta = spleen > small intestine.

Recently (11), we have postulated that residue cysteine 109 of human 4F2hc could be involved in the formation of a disulfide bond with a putative membrane protein already present in the *Xenopus* oocyte to express system y⁺L transport activity. To test whether this is also the case with human y⁺LAT-1 protein, we performed co-injection experiments with C109S (CS1) or C330S (CS2) human 4F2hc mutants (Fig. 6). The CS1 mutant injected alone led to a decrease of 56% in the induced activity compared with the wild type. This agrees with previous results (11) that showed a V_{max} decrease of 50% without changes in the $K_{0.5}$ parameter for this mutant. Moreover, CS1 co-injected with y⁺LAT-1 showed a 74% decrease in transport expression compared with wild type 4F2hc co-injected with y⁺LAT-1. In contrast, the CS2 4F2hc mutant showed no decrease in the induced activity when injected alone (similar to previous results; Ref. 11) or co-injected with y⁺LAT-1.

In the batch of oocytes used in the experiment shown in Fig. 6, we checked whether y⁺LAT-1 and 4F2hc proteins could form a heterodimeric structure via a disulfide bond. This was done by [^{35}S]methionine labeling and immunoprecipitation using a monoclonal antibody directed to human 4F2hc (Fig. 7). Under nonreducing conditions, two 4F2hc-specific protein bands were detected in 4F2hc-injected oocytes with ≈85- and ≈169-kDa electrophoretic mobilities. A band of ≈110 kDa was also visible, but it did not correspond to 4F2hc because it was also detected after immunoprecipitation of extracts from oocytes co-expressing 4F2hc and y⁺LAT-1 with protein G-Sepharose without 4F2hc antibody. The 85-kDa band corresponds to 4F2hc, as detected in activated lymphocytes (9). This band is also detected in oocytes not injected with 4F2hc cRNA, suggesting that *Xenopus* oocytes express a homologous 4F2hc protein. The 169-kDa band is not visible in reducing conditions or in oocytes expressing CS1 4F2hc, suggesting that this band might represent 4F2hc homodimers linked by a disulfide bridge involving cysteine residue 109. In oocytes co-injected with wild type or CS2 4F2hc plus y⁺LAT-1, a new 4F2hc-specific band of ≈135 kDa appears. Under reducing conditions, this band is drastically reduced and a new y⁺LAT-1-specific ≈40-kDa band appears (Fig. 7). In contrast, neither the 135- nor the 40-kDa band is visible, even after very long film exposures, in oocytes co-injected with CS1 4F2hc and y⁺LAT-1. This indicates that the 135-kDa band corresponds to a heterodimer of 4F2hc and

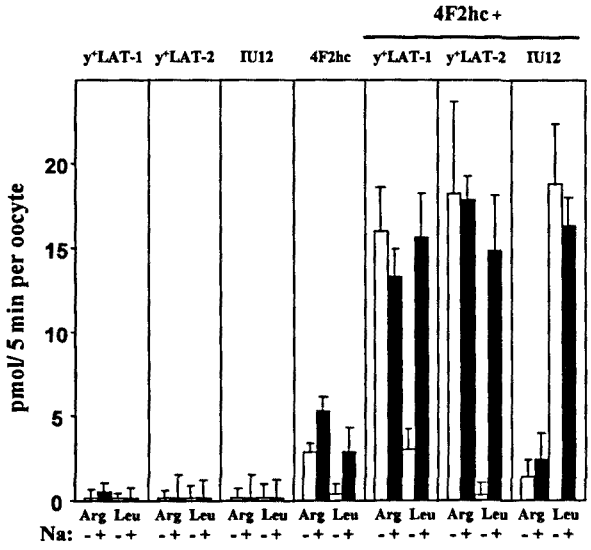

FIG 3 **Different co-expressed transport activities with three members of the family of amino acid transporter-related proteins and 4F2hc.** Oocytes were injected with 10 ng of y⁺LAT-1 (IMAGE clone 727811), y⁺LAT-2 (KIAA0245), or IU12 alone or coinjected with 10 ng of human 4F2hc Three days after the injection, the uptake of 50 μM L-[³H]arginine (*Arg*) and 50 μM L-[³H]leucine (*Leu*) in the presence (+, *closed bars*) or absence (−, *open bars*) of 100 mM NaCl was determined for 5 min Amino acid uptake (pmol/oocyte × 5 min) was calculated by subtracting the uptake in uninjected from that of the cRNA injected groups Amino acid uptake in uninjected oocytes was as follows (i) L-[³H]arginine uptake 1 1 ± 0 2 (choline medium) and 3 5 ± 0 5 (sodium medium), (ii) L [³H]leucine uptake 3 1 ± 0 6 (choline medium) and 3 8 ± 0 4 (sodium medium)

TABLE I
Inhibition of y⁺LAT 1/4F2hc induced transport activity by different amino acids

Uptake was measured at 50 μM arginine concentration in either the absence (choline medium) or the presence (sodium medium) of 100 mM sodium and inhibited by different amino acids at a concentration of 5 mM Each data point is the mean of values obtained in seven oocytes and expressed as the residual percentage of uptake Basal values of uptake (mean ± S E), expressed in pmol/10 min per oocyte, were 32 7 ± 2 3 (choline medium) and 43 3 ± 3 3 (sodium medium) for y⁺LAT-1 plus 4F2hc-injected oocytes and 3 4 ± 0 2 (choline medium) and 7 8 ± 0 5 (sodium medium) for uninjected oocytes

Inhibitor (5 mM)	Residual arginine (50 μM) uptake	
	Choline medium	Sodium medium
	%	
Arginine	2 ± 0 3	ND[a]
Lysine	2 ± 0 3	1 ± 0 2
Ornithine	0 ± 0 6	ND
Leucine	48 ± 5	3 ± 0 9
Isoleucine	43 ± 3	16 ± 2
Glutamine	73 ± 9	15 ± 2
Valine	69 ± 9	40 ± 9

[a] ND, not determined

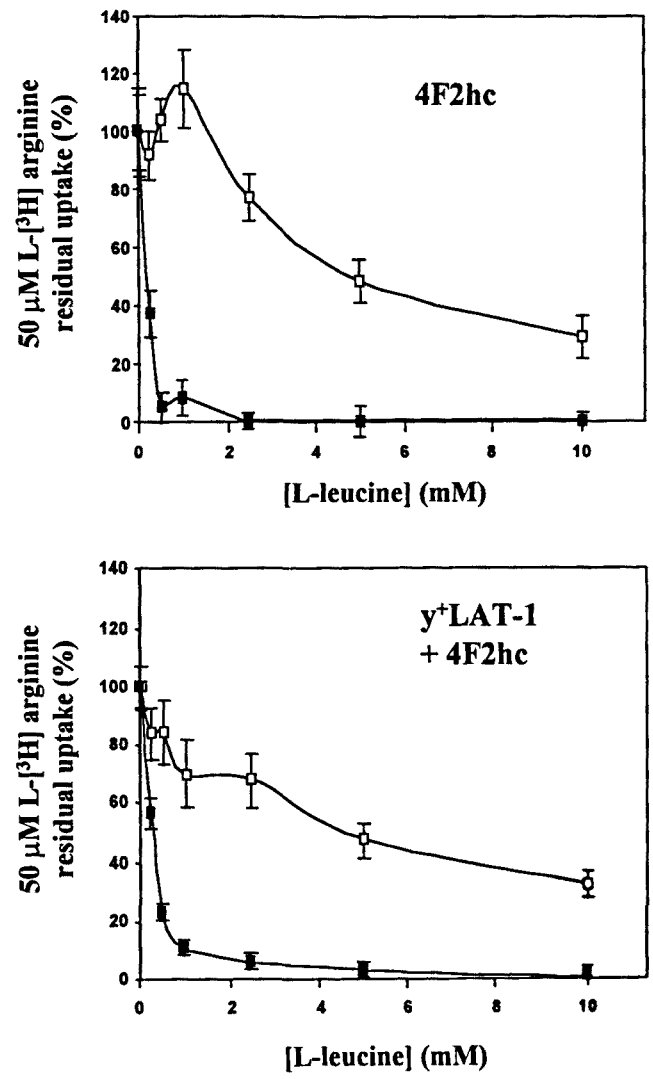

FIG 4 **Inhibition by L-leucine of the y⁺LAT-1 and 4F2hc co-expressed transport activity.** Three days after injection of 10 ng of human 4F2hc alone or human 4F2hc plus 10 ng of y⁺LAT-1 the uptake of 50 μM L-[³H]arginine in the absence (choline medium) (*open squares*) or in the presence (*closed squares*) of 100 mM sodium was measured for 10 min in the presence of different concentrations of leucine (0 250 μM 500 μM, 1 mM, 2 5 mM, 5 mM, and 10 mM) The percentage of the amino acid residual uptake was calculated by subtracting the uptake of uninjected oocytes and dividing by the uptake of 4F2hc alone or 4F2hc plus y⁺LAT-1-injected oocytes without leucine in the medium The basal values of the uptake of 50 μM L-arginine were 3 4 ± 0 2 and 7 8 ± 0 5 pmol/10 min for uninjected oocytes in choline or in sodium medium respectively, 19 5 ± 5 0 and 27 4 ± 3 0 pmol/10 min for 4F2hc injected oocytes in choline or in sodium medium, respectively, and 32 7 ± 2 3 and 43 2 ± 3 3 pmol/10 min for 4F2hc plus y⁺LAT-1 injected oocytes in choline or in sodium medium, respectively

y⁺LAT-1, linked by a disulfide bridge involving cysteine 109 of 4F2hc The 135-kDa band is also visible after very long film exposures in 4F2hc-injected oocytes and might represent the association of 4F2hc with a *Xenopus* y⁺LAT-1 homologous protein (data not shown) It is worth mentioning that this band is the only one that correlates with the induced y⁺L transport activity (see Figs 6 and 7)

DISCUSSION

In this study, we have identified a new member (y⁺LAT-1) of a family of amino acid transporter-related proteins also composed in humans by y⁺LAT-2 (KIAA0245) and E16 We have characterized the human y⁺LAT-1 cDNA sequence, chromosomal location, and pattern of expression of its mRNA and demonstrated that when co-expressed with 4F2hc, it yields y⁺L amino acid transport activity and forms a disulfide bond-dependent complex with 4F2hc through residue Cys-109 in oocytes Therefore, y⁺LAT-1 is a putative light subunit of the surface antigen 4F2hc Moreover, we also present human *y⁺LAT-1* as a strong candidate for the lysinuric protein intolerance (LPI) gene

The surface antigen 4F2 from lymphocytes has been previously immunoprecipitated as a complex of 125 kDa, which upon reduction resulted in two protein bands of 85 kDa (the heavy chain of 4F2 surface antigen, or 4F2hc) and an unidentified light chain with an electrophoretic mobility of 40 kDa, this light chain is known to be nonglycosylated and very hydrophobic (9,

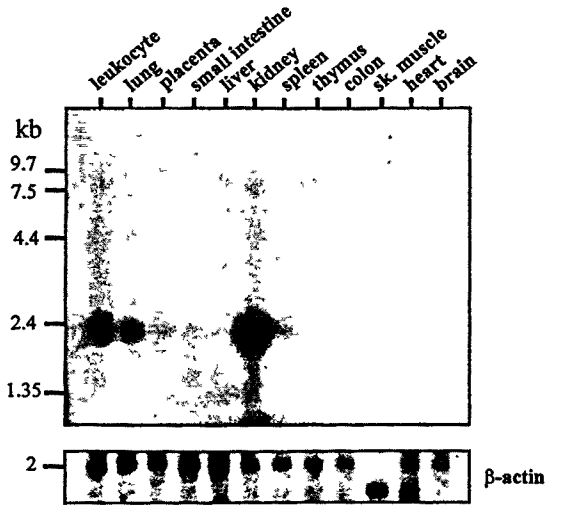

FIG. 5. **Northern blot analysis for y⁺LAT-1 mRNA in human tissues.** A poly(A) RNA (2 μg per lane) membrane containing 12 different human adult tissues was purchased from CLONTECH. Blots were probed with ^{32}P-labeled human IMAGE clone 727811 and washed at high stringency conditions (see under "Experimental Procedures"). Human y⁺LAT-1 cDNA hybridizes to a transcript of ≈2.4 kb and is expressed predominantly in kidney, leukocytes (from peripheral blood), lung, placenta, spleen, and total small intestine. Proper quality and control of loading was substantiated by hybridization with human β-actin cDNA (CLONTECH), used as a control probe.

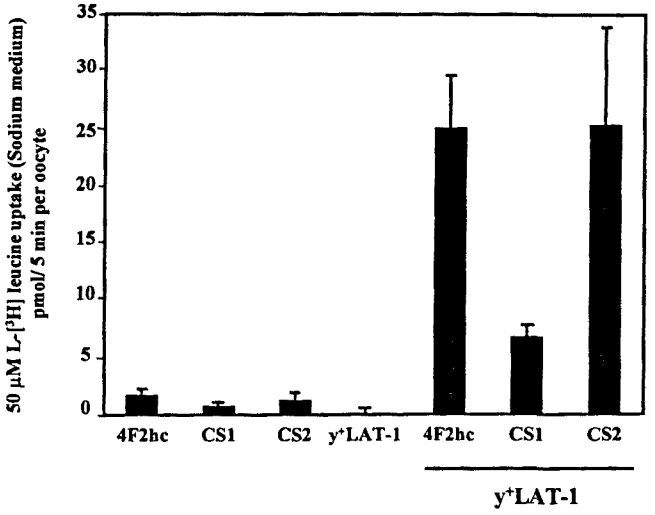

FIG. 6. **Co-expression defect of mutant C109S (CS1) 4F2 but not C330S (CS2) 4F2 with y⁺LAT-1 protein.** Oocytes were injected with 10 ng of human 4F2hc, C109S-human 4F2hc (CS1), C330S-human 4F2hc (CS2), and 10 ng of y⁺LAT-1 cRNA alone or in combination. Three days after the injection, the uptake of 50 μM L-[³H]leucine in the presence of 100 mM sodium was measured in the linear region of the time course. Amino acid uptake was calculated by subtracting the uptake of uninjected oocytes. Data are the mean ± S.E. obtained from the uptake of seven oocytes per group of a representative experiment. Another experiment gave similar results. The rate of 50 μM L-[³H]leucine uptake in the presence of sodium in uninjected oocytes was 2.0 ± 0.1 pmol/5 min.

39). We have recently demonstrated that system y⁺L transport activity induced by 4F2hc in oocytes requires association, most probably by disulfide bridges, with a plasma membrane endogenous protein (11). Here we demonstrated that human y⁺LAT-1 and 4F2hc combine to generate system y⁺L amino acid transport activity in oocytes and form a heterodimeric complex of ≈135 kDa. Moreover, this complex correlates with the induction of y⁺L transport activity by 4F2hc and y⁺LAT-1 co-expression in oocytes. Interestingly, immunoprecipitation of

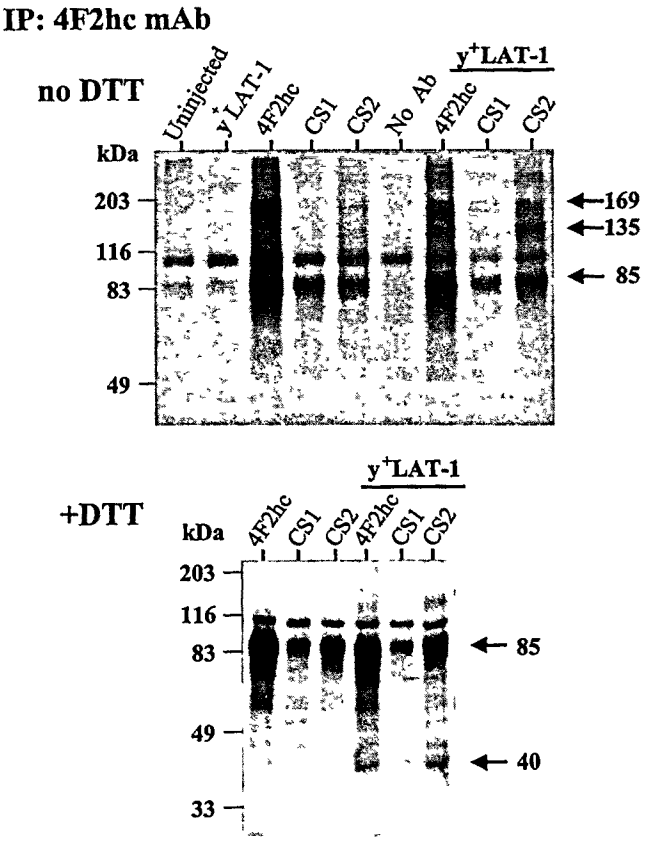

FIG. 7. **4F2hc protein forms a heterodimeric disulfide bond-dependent structure with y⁺LAT-1 through the Cys-109 residue.** Oocytes from the experiment shown in Fig. 6 were injected with 10 ng of each different cRNA as indicated. [^{35}S]Methionine labeling and immunoprecipitation with a monoclonal h4F2hc antibody (*4F2hc mAb*) was performed as described under "Experimental Procedures." Two autoradiographs (under nonreducing conditions (*no DTT*) and under reducing conditions (*+DTT*)) from a representative experiment are shown. Another independent experiment, with higher CS1 expression, gave similar results.

the 135-kDa complex and subsequent reduction results in the appearance of a y⁺LAT-1-specific ≈40-kDa protein band. All of this strongly indicates that human y⁺LAT-1 is a light chain of the surface antigen 4F2hc.

Three proteins, y⁺LAT-1 and y⁺LAT-2 (present study) and IU12 (present study, and for the equivalent protein ASUR4 or the human ortholog E16)[2] induce with 4F2hc several amino acid transport activities in oocytes: system y⁺L activity for y⁺LAT-1 and y⁺LAT-2, or system L-type for IU12 or E16. This suggests that at least these three proteins (human y⁺LAT-1, y⁺LAT-2, and E16) might be light subunits of 4F2hc with associated amino acid transport activities. This is in full agreement with the fact that both y⁺L and L transport activities have been associated with the expression of 4F2hc cRNA or 4F2hc-containing mRNA (2, 40–42, 5–6, 43). Interestingly, y⁺LAT-1 is expressed in tissues where mRNA-induced y⁺L activity has been reported (small intestine, placenta and lung) (41, 42).[4] The final demonstration that y⁺LAT-1, y⁺LAT-2, and E16 are light subunits of the surface antigen 4F2 awaits co-immunoprecipitation studies from tissue or cell samples.

Our data strongly suggest that the 4F2hc and y⁺LAT-1 heterodimeric complex is linked by a disulfide bridge involving 4F2hc residue cysteine 109. Thus, both 4F2hc-induced (present study and Ref. 11) and 4F2hc/y⁺LAT-1-induced system y⁺L transport activity is drastically reduced when the 4F2hc resi-

[4] R. Estévez and M. Palacín, unpublished results.

due cysteine 109 is mutated to serine. In parallel to this, the formation of the 4F2hc/y⁺LAT-1 heterodimer is abolished by this mutation. This suggests that C109S 4F2hc mutant is able to form an active transporter heterodimer with y⁺LAT-1, albeit with lower efficiency than wild type 4F2hc. Most probably, weak protein-protein interactions between C109S 4F2hc and y⁺LAT-1 are destabilized during detergent solubilization prior to immunoprecipitation. In favor of this, the 4F2hc-induced y⁺L transport activity is not sensitive to β-mercaptoethanol treatment, even though this increases sensitivity to inactivation by cysteine-specific reagents (11). Two cysteine residues of y⁺LAT-1 (residues 151 and 174) are conserved among the known full-length protein members of this family. Site-directed mutagenesis studies are currently in progress to identify the y⁺LAT-1 residue involved in the disulfide bridge with the Cys-109 residue of 4F2hc.

One intriguing question is why y⁺LAT-1 does not induce amino acid transport when injected alone in oocytes and why 4F2hc does. One possible explanation is that the exogenous 4F2hc may constitute a functional y⁺L transporter with a homologous protein of the y⁺LAT-1 family already present in the oocyte. The oocyte would synthesize more y⁺LAT-1-like subunits than 4F2hc-like subunits. This would be similar to the activation of the oocyte catalytic α subunit of the Na^+/K^+ ATPase by expression of foreign β subunits (44). By analogy to Na^+/K^+ ATPase (45, 46), the oocyte y⁺LAT-1-like subunits might be present in the endoplasmic reticulum and would be transported to the plasma membrane when exogenous 4F2hc is added. In this sense, the y⁺L activity is already present in the *Xenopus* oocyte (2), and we can visualize an immunoprecipitated 4F2hc antibody protein with the same molecular weight as 4F2hc in uninjected oocytes (Fig. 7).

Structural and functional evidence suggested that rBAT also associates with an oocyte plasma membrane protein to express system $b^{0,+}$-like amino acid transport activity (see under "Introduction," and see Refs. 47 and 48 for recent reviews). Therefore, it will be interesting to determine whether some of the members of the transporter-related family can also interact with the rBAT protein. Preliminary results[5] indicate that y⁺LAT-1, y⁺LAT-2, and IU12 do not cause $b^{0,+}$-like amino acid transport activity with rBAT in oocytes.

LPI is an autosomal recessive disease in which transport of the cationic amino acids lysine, arginine, and ornithine is defective. This defect has been localized at the basolateral membranes of epithelial cells in small intestine (49, 50) and in the renal tubules (51). Simell and co-workers (52) reported that LPI fibroblast showed a reduced *trans*-stimulated efflux of cationic amino acids. Clinical signs of LPI include hyperammonemia and episodes of stupor, immunological abnormalities (53), growth retardation, and muscle hypotonia. Potentially fatal interstitial lung disease and progressive renal failure may occur at any age (54). Recently, Lauteala *et al.* (55) have assigned, through linkage analysis of 20 Finnish LPI families, the LPI gene locus to the proximal long arm of chromosome 14. In this work, recombination studies placed the locus between markers D14S72 and MYH7; the phenotype showed the highest linkage desequilibrium with marker T-cell receptor α chain within this locus. Although functional criteria pointed to the cationic amino acid transporters (hCAT-1 and hCAT-2) as candidate genes, linkage studies, using flanking microsatellite markers, excluded both as the mutated gene in LPI (56) The human *y⁺LAT-1* gene is a good candidate for LPI: (i) 4F2hc is expressed at the basolateral membrane of proximal tubule epithelial cells in the kidney (57). (ii) The y⁺L activity induced by 4F2hc in oocytes is an exchanger activity that mediates the efflux of cationic amino acids and the influx of neutral amino acids plus sodium (58). This would explain why the efflux and not the influx (because of the presence of a member of the CAT family of transporters) is affected. (iii) The expression pattern of this gene is consistent with the tissues in which some defect in LPI has been detected (lung, immune system cells, kidney, and intestine). (iv) Finally, its chromosomal localization is within the locus of the LPI gene (55). Mutational analysis to prove this hypothesis is currently in progress.

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Note Added in Proof—After this paper was accepted for publication the observations by Verrey and co-workers were published (Mastroberardino, L. Spindler, B , Pfeiffer, R., Skelly, P. J., Loffing, J., Shoemaker, C B., and Verrey, F. (1998) *Nature* **395,** 288–291).

[5] M Pineda, D. Torrents, and M Palacín, unpublished observations.

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Treball 5

The C-terminal domain of rBAT determines functional properties of the $b^{0,+}$ amino acid transporter.

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* Aquests autors han contribuit per igual.

El doctorand és autor de les figures 4, 5 i coautor de les figures 1, 3 i 7. Vaig participar en la redacció de l'article, concretament vaig escriure les figures, legend to figures, experimental procedures i results. L'estrategia experimental d'aquest treball va ser proposada pel doctorand.

The C-terminal domain of rBAT determines functional properties of the $b^{0,+}$ amino acid transporter.

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Abstract

Recently, several light subunits that, together with 4F2hc or rBAT, form heterodimeric amino acid transporters have been described. Here we studied the impact of single mutations of the 8 cysteine residues of human rBAT to serine on the properties of amino acid transport. Mutation of cysteine residue 114 (C114S), the putative residue forming a disulfide bridge with the light subunits, abolished the sodium-dependent component of the L-leucine-evoked currents in wild type rBAT injected oocytes. Two activities ($b^{0,+}$ and y^+L) have been associated with the expression of rBAT in oocytes, suggesting interaction of rBAT with the corresponding endogenous light subunits. Here we demonstrate that expression of human rBAT and y^+LAT-1 in oocytes resulted in a mixed amino acid transport activity with characteristics of system $b^{0,+}$ and y^+L, the latter being due to the trafficking of y^+LAT-1 to the oocyte plasma membrane. C114S did not affect the induced $b^{0,+}$ amino acid transport, but abolished functional interaction with y^+LAT-1. Mutation of three other cysteine residues (C571S, C666S and C673S), located near the C-terminus, reduced transport induction. Mutations C571S and C666S abolished induction of transport almost completely, whereas mutation C673S reduced it partially. Kinetic analysis of C673S-induced transport revealed lower apparent K_m values (up to 6-7 fold decrease for neutral amino acids) in comparison to wild type rBAT-induced transport. To address whether the change in affinity was due to modifications of the transport properties associated with endogenous $b^{0,+}$ or y^+L light subunits of the oocyte, we studied the double mutant C114S-C673S. Like C673S, this mutant reduced the apparent K_m values of the induced $b^{0,+}$ activity. These results indicate that the C-terminal domain of rBAT interacts with the $b^{0,+}$ light subunit of the oocyte and determines the properties of the holotransporter. This is the first experimental evidence that a heavy chain (i.e., rBAT) determines functional properties of the corresponding heterodimeric amino acid transporter.

Introduction

Last year, five light subunits of the heavy chain of the cell surface antigen 4F2 (4F2hc, also named CD98) in mammals (LAT-1, LAT-2, y^+LAT-1, y^+LAT-2 and xCT) (1-7) and one subunit ($b^{0,+}$AT) of rBAT were cloned (8). LSHAT (Light Subunits of Heterodimeric Amino acid Transporters) co-express amino acid transport activity with 4F2hc (i.e., system L for LAT-1 and LAT-2, system y^+L for y^+LAT-1 and y^+LAT-2 and system x_c^- for xCT) (1-7) or with rBAT (i.e., system $b^{0,+}$ for $b^{0,+}$AT) (8-9). In contrast to the heavy chains, LSHAT are quite hydrophobic and are expected to have 12 transmembrane domains. The role of LSHAT in amino acid transport has been highlighted by the fact that mutations in *SLC7A7* (y^+LAT-1) (10-11) or in *SLC7A9* ($b^{0,+}$AT) (8) cause the inherited aminoacidurias Lysinuric Protein Intolerance (LPI) or Non-type I cystinuria, respectively.

It is believed that these transporters need both heavy and light subunits to be functional. Thus, inactivation studies of system y^+L with mercurial reagents demonstrate the heterodimeric nature of the functional transporter (12). Moreover, 4F2hc brings LAT-1, LAT-2 and SPRM1 (a *Schistosoma mansoni* member of the LSHAT family) to the cell plasma membrane (2, 6, 13). Similarly, $b^{0,+}$AT brings rBAT to the plasma membrane in COS cells (8). rBAT or 4F2hc are linked by a disulfide bridge with the corresponding LSHAT (3-4, 9, 14-15). Cysteine residues C109 (corresponding to the conserved residue 114 in human rBAT) in 4F2hc and C164 (conserved in all LSHAT family members) in LAT-1 form the disulfide bond (3,14). Furthermore, C-terminal deletion experiments of rBAT additionaly suggested interaction of this domain with its light subunit (16-18).

Expression cloning was used to identify rBAT as a protein which induces amino acid transport system $b^{0,+}$ in oocytes, and homology with rBAT fostered expression of 4F2hc in oocytes, leading to the induction of system y^+L amino acid transport activity (for review see ref. 19). Later, induction of amino acid transport system L was also demonstrated by 4F2hc in oocytes (20). Partial knockout studies of rBAT in the tubular renal OK cells (21) and the fact that mutations in the rBAT gene cause cystinuria (22) demonstrate the role of rBAT in the high-affinity re-absorption system $b^{0,+}$. Similarly, there is evidence for the role of 4F2hc in the transport system y+L in the basolateral plasma membrane of epithelial cells. Thus, 4F2hc has a basolateral location in these cells (13, 23) and mutations in y+LAT-1 cause LPI (10-11), an aminoaciduria due to a basolateral transport defect of dibasic amino acids. In addition to these transport activities, rBAT and 4F2hc induce other amino acid transport systems in oocytes (i.e.

y^+L for rBAT and $b^{0,+}$ for 4F2hc) (18, 24-26). Most probably, these latter transport activities are a consequence of the overexpression of rBAT or 4F2hc. Furthermore, co-expression of amino acid transport function by 4F2hc and $b^{0,+}$AT has been described (27).

Here we examine the role of the eight cysteine residues of rBAT in the amino acid transport activity, and in the interaction between rBAT and y^+LAT-1 in oocytes. Two groups of cysteine residues were relevant for amino acid transport: i) induction of system y^+L, but not system $b^{0,+}$, depends on residue C114. ii) System $b^{0,+}$ activity is affected by mutation to serine of three cysteine residues located near the C-terminus of rBAT (C571, C666, C673). Interestingly, mutant C673S reduced the apparent K_m of the induced system $b^{0,+}$. This is the first evidence that a heavy chain (i.e., rBAT) determines functional properties of the corresponding heteromeric amino acid transporter.

Experimental Procedures

Oocytes, Injections and Uptake Measurements. Oocytes origin, management, and injections were as described elsewhere (28-29). Defolliculated stage VI *Xenopus laevis* oocytes were injected with different amounts of cRNA of human rBAT, cysteine mutants of rBAT (see next section *Site-directed mutagenesis and plasmids construction*), human 4F2hc, human y^+LAT-1 or N-myc-y^+LAT-1 alone or in combination. Synthesis of these cRNAs was as described (3, 28-29). Influx rates of L-[^{3}H]arginine, L-[^{3}H]leucine and L-[^{35}S]cystine (Amersham Pharmacia Biotech) were measured in 100 mM NaCl or 100 mM CholineCl medium at the indicated number of days after injection and under linear conditions as described (28). The cRNA-induced uptake was calculated by subtracting uptake values in uninjected oocytes from those of the corresponding cRNA-injected oocytes. Amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those of uninjected oocytes (data not shown).

Electrophysiology. *Xenopus* oocytes were injected with 4 ng of the respective cRNA. After 1-7 days of expression two electrode voltage clamps recordings were performed at room temperature. The holding potential was –50 mV unless otherwise stated. The data were filtered at 10 Hz and recorded using a MacLab D/A converter and software for data acquisition and analysis. The external control solution (ND 96) contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.4. For experiments examining the Na^+-dependence, Na^+ was substituted by NMDG (N-methyl-D-glucamine). The final solutions were titrated to the indicated pH using KOH or HCl. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath was reached within 10 s. The currents given are the maximal values measured during 30 s of substrate superfusion. All chemicals were obtained from Sigma.

Curves were obtained by using the Hill equation $I = I_{max} * [S]^n/([S]^n + K_m^n)$ where n and [S] are the Hill coeficient and the amino acid concentration, respectively, I_{max} is the extrapolated maximal current, and K_m is the apparent concentration needed for half-maximal current. Curves were fitted for data from each oocyte and the values obtained for K_m and V_{max} were used for statistical analysis. All data are means ± SEM and *n* represents the number of experiments performed. The magnitude of the currents varied depending on the time after cRNA injection and on the batch of oocytes (from different animals). Therefore, throughout the paper we show experimental data obtained on the same day and from the same batch of oocytes for each specific experiment. All

experiments were repeated with at least two or three batches of oocytes; in all repetitions qualitatively similar data were obtained. All data were tested for significance using the paired or unpaired Student's *t*-test, and only results with $P < 0.05$ were considered significant.

Site-directed mutagenesis and plasmids construction. For construction of the cysteine to serine human rBAT mutants, we used the QuickChange Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's protocol. The mutagenic oligonucleotides were (sense strand, the mutated nucleotide is indicated in parentheses): 5'-AGTATGAAGGGAT(C)CCAGACA-3' for the C18S, 5'-TCCAAAGT(C)CCTAGACTG-3' for the C114S, 5'-ATCTGGCATGACT(C)TACCCAT-3' for the C242S, 5'-GACGAAGTGCGAAACCAAT(C)TTATTTTCATCAG-3' for the C273S, 5'-GCTGGTTTT(C)CCATTTGAGG-3' for the C571S, 5'-GAGATAGAT(C)CTTTGTTTCCAA-3' for the C666S, 5'-GAGCAT(C)CTATTCCAGTGTAC-3' for the C673S and 5'-ATACCTCGT(C)TTAGGCACCTT-3' for the C685S. Standard techniques of *in vitro* recombination were used to obtain the double mutant C114S/C673S. Mutants were checked by sequencing, to ensure no changes in the nucleotide sequence, as described (6).

To obtain N-*myc*-y$^+$LAT-1, the IMAGE clone 727811 was cut with *Nco*I, filled in with Klenow, cut again with *Not*I, and ligated into pNKS2-*myc Not*I (30). Addition of the *myc*-tag to y$^+$LAT-1 did not affect the activity of the transporter. Three days after the injection, the net cRNA-induced uptake of L-[^{3}H] leucine (50 μM in the presence of Na^+), expressed in pmol/5 min per oocyte, was: 12.8 ± 4.6 for 4F2hc-injected oocytes, 29.5 ± 3.8 for 4F2hc plus y$^+$LAT-1-injected oocytes and 33.1 ± 5.7 for 4F2hc plus N-*myc*-y$^+$LAT-1-injected oocytes.

Confocal immunofluorescence microscopy. Groups of five oocytes were prepared for immunofluorescence 3 days after injection with 10 ng/oocyte human 4F2hc, human rBAT, C114S and N-*myc*-y$^+$LAT-1 cRNA alone or in combination. Oocytes were processed for immunohistochemistry with monoclonal antibody 9E10 anti-*myc* (ATCC, Manassas, VA) as described elsewhere (6). Background signal was negligible in 4F2hc-, rBAT- or C114S-injected oocytes.

Results

The human rBAT protein contains eight cysteine residues (Fig. 1a). To study the impact of the eight cysteine residues on the amino acid transport associated with rBAT, each of these residues was changed to serine. Every mutant RNA was injected into oocytes and assayed functionally. As illustrated in Fig.1b, L-arginine induced transport (choline medium) was only clearly reduced in mutants C571S, C666S and C673S. Similar results were obtained when measuring L-arginine uptake in the presence of sodium, and L-leucine uptake in the presence or in the absence of sodium (data not shown). These three cysteine residues are located near the C-terminal domain of the protein. According to the topological model of rBAT described by Tate and co-workers (31), C571 residue is located extracellularly before the last transmembrane domain, whereas C666 and C673 are located intracellularly, close to the C-terminal end (residue C685) (Fig. 1a).

Cysteine residue 114 (C114; Fig. 1a) is conserved in the homologous protein 4F2hc, where it is involved in the formation of a disulfide bond with members of the LSHAT family of proteins. (3, 14). In contrast to this proposed role for C114, mutations C114S did not affect the induced amino acid transport (Fig. 1b), in agreement with a previous report (17).

Mutation C114S and functional interaction of rBAT with y$^+$LAT-1

The amino acid transporter $b^{0,+}$, associated with the expression of rBAT in oocytes, has been characterized as an electrogenic hetero-exchanger for dibasic (influx) and neutral (efflux) amino acids (32-33). Several rBAT mutations found in type-I cystinuria patients have been described as trafficking mutants (34-35). All these mutant proteins share the property that the extent of the defect depends on the expression period or the amount of cRNA injected. This effect has been related to the level of saturation of the quality control machinery in the oocyte (34). A deeper analysis of the C114S-induced amino acid transport revealed that neither the time-course of expression nor the voltage dependence of L-arginine- and L-leucine-induced currents showed differences in wild type- and C114S-injected oocytes (Fig. 2a,b). In agreement with this, preloading of C114S expressing oocytes with L-leucine increased L-arginine-induced inward currents, whereas preloading with L-arginine reduced these currents (data not shown). For the L-leucine-induced outward currents preloading with these amino acids had the reverse effect (data not shown). This is the same electrogenic exchange behavior described for wild type rBAT expressing oocytes (32-33). All this suggests that C114S has no defects in trafficking or in the exchange mechanism of transport. In contrast, kinetics of the L-leucine transport induced by C114S showed differences with that of

wild type rBAT (Fig. 2c): i) For wild type rBAT, V_{max} was greater in the presence of 100 mM sodium than in its absence, whereas for C114S, V_{max} remained unchanged irrespectively of the presence of sodium. ii) In comparison to the wild type, C114S-induced transport showed a small reduction (~50%) in the apparent K_m values both in the absence and presence of sodium. This suggested that in contrast to C114S, wild type rBAT induces more than one amino acid transport activity in oocytes.

Two activities have been associated with the expression of rBAT in oocytes: $b^{0,+}$ and y^+L, possibly due to the interaction with the corresponding endogenous light subunits (18). In order to analyse the interaction between rBAT and a y^+L subunit, we coexpressed in oocytes human rBAT with human y^+LAT-1, one of the members of the LSHAT family (3-4,10) that expresses y^+L activity with 4F2hc. We compared the uptake of L-arginine and L-leucine in the presence or in the absence of sodium and the uptake of L-cystine in the absence of sodium for human rBAT, rBAT plus human y^+LAT-1 and human 4F2hc plus y^+LAT-1 (Fig. 3a). The uptake of L-leucine is sodium-independent for oocytes injected with rBAT, as reported for $b^{0,+}$ activity (36), whereas it is sodium-dependent for oocytes injected with 4F2hc plus y^+LAT-1, as reported for y^+L activity (37). Interestingly, the oocytes injected with rBAT plus y^+LAT-1 showed a sodium dependence for L-leucine. To further characterize this sodium-dependence, the uptake of L-arginine was measured in the presence or in the absence of sodium and in the presence of different concentrations of L-leucine (Fig. 3b). These results showed clearly that sodium increased the affinity for L-leucine in oocytes injected with rBAT plus y^+LAT-1, whereas it had no effect in oocytes injected with rBAT. L-cystine is not transported by 4F2hc plus y^+LAT-1 (Fig. 3a) or by the erythrocyte system y^+L (37). Because there is some cystine uptake in the oocytes expressing rBAT and y^+LAT-1, we can conclude that there is expression of a combination of $b^{0,+}$ and y^+L activities.

In full agreement with the expression of system y^+L activity by rBAT and y^+LAT-1, rBAT guides an N-tagged y^+LAT-1 (N-*myc*-y^+LAT-1) to the oocyte plasma membrane as 4F2hc does (Fig. 4). 4F2hc interacts with y^+LAT-1 through cysteine residue 109 (3), which is conserved in rBAT (C114). To test whether this is also the case with rBAT and y^+LAT-1, we performed co-injection experiments with C114S and y^+LAT-1 (Fig. 5). rBAT plus y^+LAT-1 showed a sodium-dependence for L-leucine uptake, whereas rBAT, C114S and C114S plus y^+LAT-1 did not. In agreement with this, C114S did not bring y+LAT-1 to the oocyte plasma membrane (Fig. 4). In all, this demonstrates that C114S does not interact functionally with y^+LAT-1, although it does interact with the $b^{0,+}$ endogenous subunit.

Mutation C673S and change in the kinetic properties of system $b^{0,+}$

As indicated above, several cystinuria-specific rBAT mutations that show a defect in the trafficking to the plasma membrane (34-35) share the property that the transport they induce in oocytes increases slowly with time after cRNA injection or with the amount of cRNA injected. To check whether the transport function of C571S, C666S and C673S mutants could be increased, we injected different amounts of cRNA (1 ng, 10 ng and 30 ng) in oocytes and measured their induced amino acid transport activity (Fig. 6). The wild type rBAT-induced uptake was similar for the three doses of cRNA injected, which may be related to the limited endogenous level of y^+LAT and $b^{0,+}$AT *Xenopus* transporters (34). The uptake induced by the C666S and C673S mutants could be partially recovered by increasing amounts of injected cRNA, whereas no effect was found for the C571S mutant. These data suggest that C666S and C673S are mutants that affect either trafficking to the plasma membrane or the stability of the transporter protein.

To assess whether the decrease in function of these mutations was due to a defect in the electrogenic exchange mechanism or to changes in the K_m for the different substrates, we analyzed the induced transport by electrophysiological techniques. For the C571S and C666S mutants, these analyses were not possible because the low induced uptake precluded accurate estimations. The voltage dependence of L-arginine- and L-leucine-induced currents showed no difference between wild type- and C673S-injected oocytes (data not shown), suggesting that the exchange mechanism of transport was not altered. For the C673S mutant, the first kinetic experiment showed a clear reduction in V_{max} (around 50%) and a small reduction in the K_m value (around 50%) for L-arginine uptake (data not shown). Therefore, a more detailed kinetic analysis for a series of L-amino acids was performed in the same batch of oocytes: arginine, lysine, ornithine, leucine, phenylalanine, methionine and alanine. Table I compares the K_m and V_{max} values for these aminoacids in the wild type and the C673S mutant in sodium medium. Again, a small reduction in the K_m values for dibasic amino acids was found in the C673S mutant (1.8 to 2.3 fold), but a much bigger reduction for neutral amino acids (up to 7.4 fold). The strongest decrease of the K_m value was found for methionine. For all the amino acids, V_{max} was lower in the C673S mutant (from 9% to 39% residual activity). Similar results were obtained in the absence of sodium (NMDG medium, data not shown).

To examine whether the change in the functional properties of the C673S-induced transport was due to changes in the interaction with the endogenous $b^{0,+}$ or y^+L light subunits a C114S/C673S double mutant was constructed. The fact that the rBAT

mutant C114S cannot interact with y^+LAT-1 (Figs. 4 and 5) suggests that it cannot interact with the endogenous y^+L subunit. Thus, functional analysis of the double rBAT mutant C114S/C673S, should disclose the effect of C673S on the properties of the $b^{0,+}$ transporter. In figure 7 the kinetics of L-arginine and L-leucine in the presence of sodium for the wild type rBAT and the C114S/C673S double mutant are shown. As demonstrated before for the C673S mutant (Table I), a reduction in the K_m values for L-arginine and L-leucine was observed (see inset in Fig. 7), which was more pronounced for L-leucine (aprox. 8-fold). Similar results were obtained in the absence of sodium. The voltage-dependence of the double mutant was the same as for the wild type (data not shown). These results demonstrate that the rBAT subunit plays a role in the amino acid transport characteristics of system $b^{0,+}$.

Discussion

In this study we have demonstrated that rBAT interacts with y^+LAT-1 in oocytes, and that this interaction is abolished by the mutation of the cysteine residue 114 (C114) to serine. Moreover, we have identified a cysteine residue of rBAT (C673), located in the C-terminal domain of the protein, that determines functional properties of the *Xenopus* oocyte $b^{0,+}$ amino acid transporter. Replacement of cysteine residue 673 with serine (C673S) resulted in a reduced apparent K_m of the rBAT-induced $b^{0,+}$ amino acid transport activity. This suggests that the C-terminal domain of rBAT participates directly in the transport mechanism or alternatively, mutant C673S produces conformational changes in the holotransporter that alter its functional properties. In any case, these results demonstrate that rBAT plays an important role in the amino acid transport characteristics of system $b^{0,+}$.

The homologous proteins rBAT (~90 kDa) and 4F2hc (~85 kDa) are linked by disulfide bridges to a light subunit of ~40 kDa constituting a heterodimer of ~125 kDa (38-40). The first functional evidence for heteromeric amino acid transporters in mammals was obtained from the study of the most common Type I cystinuria rBAT mutation M467T (34) and with mercury-agent-inactivation of the y^+L amino acid transport activity induced by 4F2hc in oocytes (12). These studies strongly suggested that induction of systems $b^{0,+}$ or y^+L in oocytes by the expression of rBAT or 4F2hc, respectively, relies on the interaction with oocyte endogenous subunits of these transporters. Very recently, light subunits that when coexpressed with 4F2hc constitute systems y^+L, L and x_c^- (1-7) and a putative light subunit ($b^{0,+}$AT) that constitutes system $b^{0,+}$ with rBAT (8-9) have been cloned. Immunoprecipitation studies demonstrated the formation of a heterodimer between 4F2hc and LAT1 or y^+LAT-1 (2-4, 13-14). Similarly, Western blot studies suggested association between rBAT and $b^{0,+}$AT in membranes from rat kidney (9). Immunofluorescence studies have shown that 4F2hc drives the corresponding LSHAT to the cell plasma membrane (2, 6, 13, 14 and present study). Alternatively, expression of $b^{0,+}$AT increases the expression of rBAT at the plasma membrane in COS cells (8). For the 4F2hc protein, no other function related to transport beside this trafficking role has been demonstrated. For the rBAT protein a dual role is suggested by our experiments. On the one hand, all analyzed cystinuria-rBAT mutants in oocytes seem to affect the trafficking of the protein to the plasma membrane (41). On the other hand, mutant C673S (or C114S/C673S) reduces the apparent K_m for the $b^{0,+}$ transport activity induced by rBAT in oocytes. This demonstrates that either rBAT is directly involved in the mechanism of transport of system $b^{0,+}$ or that changes in the structure of rBAT produce conformational changes in the $b^{0,+}$ light subunit ($b^{0,+}$AT), which affects the kinetics of the transporter.

The C-terminal domain of rBAT seems to determine the type of amino acid transport activity induced in oocytes (16-18). The above mentioned rBAT cysteine residue (C673) is located in this domain. Moreover, C-terminal deletions of rBAT result in changes in the amino acid transport activity induced in oocytes. Tate and co-workers (17) reported that elimination of residues 658-C-terminus abolishes induction of amino acid transport in oocytes by rat rBAT. An extended deletion (residues 588-C-terminus) recovered $b^{0,+}$ amino acid transport induction. Expression in oocytes of a larger C-terminal deletion (511-C-terminus) of human rBAT resulted in the induction of transport activity with characteristics of system y^+L rather than $b^{0,+}$ (16). Similarly, a smaller C-terminal deletion (671-C-terminus) results in the induction of system y^+L activity in oocytes (18). These studies showed artificial promiscuity between the heavy chains and LSHAT. Indeed, it has recently been reported that 4F2hc coexpresses with $b^{0,+}$AT (named 4F2lc6 in this study) system $b^{0,+}$ in transfected cells (27). Whereas 4F2hc has a basolateral location in epithelial cells (13, 23), $b^{0,+}$AT has an apical location (9). Therefore, this interaction most likely results from the artificial overexpression of both proteins. On the other hand, we have shown here that rBAT (apical; refs. 9, 42-43) drives y^+LAT-1 (basolateral; refs. 10-11 and Fernández, Torrents and Palacín, unpublished results) to the oocyte plasma membrane and that together they coexpress y^+L transport activity. Interestingly, mutation of the cysteine residue C114 of rBAT to serine (i.e., the residue analogous to the one (C109) in 4F2hc implicated in the disulfide bond with LSHAT; refs. 12, 14) abolished the functional interaction between rBAT and y^+LAT-1. Interaction between the heavy chains and the corresponding LSHAT depends not only on this disulfide bridge. Thus, mutation C114S in rBAT or C109S in 4F2hc does not affect ($b^{0,+}$), or only partially reduces (y^+L or L) the induced transport activity respectively (3, 12, 14, 17; and present study). The fact that C114 is necessary for the artificial rBAT/y^+LAT-1 association suggests that non-covalent interactions between these two subunits are weaker than those participating in the natural holotransporters rBAT/$b^{0,+}$, 4F2hc/y^+L or 4F2hc/L.

These results are the first evidence that a heavy chain (i.e., rBAT) determines functional properties of the corresponding heteromeric amino acid transporter (i.e., $b^{0,+}$). A deeper insight into the role of rBAT in the structure/function relationship of the $b^{0,+}$ holotransporter could not be acquired by coexpression of rBAT and its available putative light subunit ($b^{0,+}$AT) because the low induction over background of the transport activity elicited by the expression of these two proteins in oocytes and transfected cells (8-9). Purification and reconstitution studies will be necessary to gain further information about the role of the heavy and light subunits in the function of the holotransporter.

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Legend to figures

Figure 1. Transport activity induced by cysteine to serine mutants of rBAT. a) Schematic representation of the human rBAT protein. This four transmembrane (amino acid residue numbers indicate the limits of these segments) topology model is based on studies by Tate and co-workers (31). The position of the eight cysteine residues of the protein is indicated by circles. Of these residues, only C114 is conserved in 4F2hc. (Y) indicates six potential N-glycosylation sites. **b)** Three days after injection of 10 ng of wild type human rBAT or each of the cysteine to serine mutants of rBAT, the uptake of 50 μM L-arginine (choline medium) was measured for 5 min. The expressed transport (i.e. subtracting the transport activity of uninjected oocytes) in rBAT-injected oocytes was 78 ± 8 pmol/5min per oocyte. Data (mean ± S.E.) represent percentages of the amino acid uptake compared with those oocytes injected with wild-type rBAT. Data correspond to three independent experiments, in which 7 oocytes were used per group in each experiment.

Figure 2. Analysis of L-amino acid induced currents for wild type rBAT- or C114S-injected oocytes. Three days after injection of human wild-type rBAT (squares) or C114S (circles) cRNA, two electrode voltage clamp recordings were performed in oocytes. Unless otherwise stated, the holding potential was –50 mV. The currents shown are the resulting currents obtained by subtraction of control currents (in the absence of amino acids) from the currents in the presence of amino acids. **a)** Time course of expression of 1 mM L-arginine- (closed symbols) or L-leucine-induced (open

symbols) currents. In agreement with the electrogenic heteroexchange mechanism of the rBAT-induced transport in oocytes (32), L-arginine evoked inward currents (negative) and L-leucine evoked outward currents (positive). Data (mean ± S.E.) represent currents (nA) from a representative experiment with 5 oocytes per group. **b)** Voltage dependence of 1 mM L-arginine- (closed symbols) or L-leucine-induced (open symbols) currents. Data (mean ± S.E.) represent relative L-amino acid-induced currents (I_{max} corresponds to the maximal measured current in each IV curve) from a representative experiment with 5 oocytes per curve. **c)** Kinetics of L-leucine-induced currents in the presence (closed symbols) or in the absence (open symbols) of 100 mM sodium. Data (mean ± S.E.) represent relative L-amino acid-induced currents (I_{max} corresponds to the maximal measured current in the presence of sodium recorded in wild type rBAT- or C114S-injected oocytes) from 4-5 oocytes per curve. Curves were fitted to data from each oocyte and the values obtained for K_m and V_{max} were used for statistical analysis. The estimated V_{max} values were: for wild type rBAT, -104 ± 3 nA, and -68 ± 4 nA in the presence or absence of sodium respectively ($p \leq 0.01$); for C114S, -53 ± 1 nA, and -59 ± 4 nA in the presence or absence of sodium respectively (non significant difference). The estimated apparent K_m values were: for wild type rBAT, 380 ± 20 μM, and 360 ± 20 μM in the presence or absence of sodium, respectively (non significant difference); for C114S, 190 ± 30 μM, and 160 ± 20 μM in the presence or absence of sodium, respectively (non significant difference).

Figure 3. a) Expressed amino acid transport activity by rBAT and y$^+$LAT-1. Oocytes were injected with 10 ng of human rBAT cRNA, or with 10 ng of human y$^+$LAT-1 cRNA together with 10 ng of rBAT or 10 ng of human 4F2hc cRNA. Three days after the injection, the uptake of 50 μM L-[^{3}H] arginine (Arg) or 50 μM L-[^{3}H] leucine (Leu) in the presence or in the absence of 100 mM NaCl, and the uptake of 50 μM L-[^{35}S] cystine (CSSC) in the absence of sodium was determined for 5 min. Amino acid uptake rates (pmol/5 min per oocyte) were calculated by subtracting the uptake of the uninjected group from that of the cRNA injected groups. The amino acid uptake activity of uninjected oocytes was as follows: L-[^{3}H] arginine uptake: 1.2 ± 0.2 (choline medium) and 3.4 ± 0.5 (sodium medium); L-[^{3}H] leucine uptake: 4.6 ± 1.0 (choline medium) and 5.6 ± 0.6 (sodium medium); L-[^{35}S] cystine uptake: 0.4 ± 0.1 (choline medium). Data (mean ± S.E.) correspond to a representative experiment with 7-8 oocytes per group. **b) Inhibition by L-leucine of the rBAT and rBAT**

plus y⁺LAT-1 expressed arginine transport activity. Three days after the injection of 10 ng of human rBAT alone or rBAT plus 10 ng of human y⁺LAT-1, the uptake of 50 μM L-[^{3}H] arginine in the absence (choline medium, closed squares) or in the presence (open squares) of 100 mM sodium was measured for 5 min in the presence of different concentrations of L-leucine (0, 250 μM, 500 μM, 1 mM, 2.5 mM, 5 mM and 10 mM). The percentage of the induced amino acid residual uptake was calculated versus basal uptake values (i.e., in the absence of L-leucine): 59 ± 4 and 43 ± 5 pmol/5 min for rBAT-injected oocytes in choline or in sodium medium, respectively, and 20 ± 2 and 31 ± 5 pmol/5 min for rBAT plus y⁺LAT-1 injected oocytes in choline or in sodium medium, respectively. Induced uptakes were calculated by subtracting the uptake of uninjected oocytes (1.7 ± 0.4 and 1.2 ± 0.2 pmol/5 min in choline or in sodium medium, respectively). Data (mean ± S.E.) correspond to a representative experiment with 7-8 oocytes per group.

Figure 4. Localization of N-*myc*-y⁺LAT-1 in oocytes. Oocytes were injected with *myc*-tagged human y⁺LAT-1 cRNA alone or in combination with human 4F2hc, human rBAT or C114S cRNAs. Three days later oocytes were processed for immunocytochemistry with mAb 9E10 anti-*myc* as a primary antibody and Texas red-conjugated goat anti-mouse as secondary antibody. Micrographs show that 4F2hc and rBAT drive N-*myc*-y⁺LAT-1 to the oocyte plasma membrane, whereas C114S does not. Background staining was negligible in the negative controls (i.e., oocytes injected with 4F2hc, rBAT or C114S-rBAT) (data not shown). The induced uptake of 50 μM L-leucine (choline medium) expressed in pmol/5 min per oocyte was 0.3 ± 0.5 for N-myc-y⁺LAT-1, 24 ± 3 for rBAT, 35 ± 2 for C114S, 9 ± 1 for rBAT plus y⁺LAT-1 and 34 ± 2 for C114S plus y⁺LAT-1.

Figure 5. C114S-rBAT does not interact functionally with y⁺LAT-1. Oocytes were injected with human rBAT cRNA or C114S alone, and human y⁺LAT-1 together with rBAT or C114S cRNA. Three days after the injection, the uptake of 50 μM L-[^{3}H] arginine (Arg) and 50 μM L-[^{3}H] leucine (Leu) in the presence or in the absence of 100 mM NaCl, and the uptake of 50 μM L-[^{35}S] cystine (CSSC) in the absence of sodium was determined for 5 min. Amino acid uptake rates (pmol/5 min per oocyte) were calculated by subtracting the uptake of the uninjected group from that of

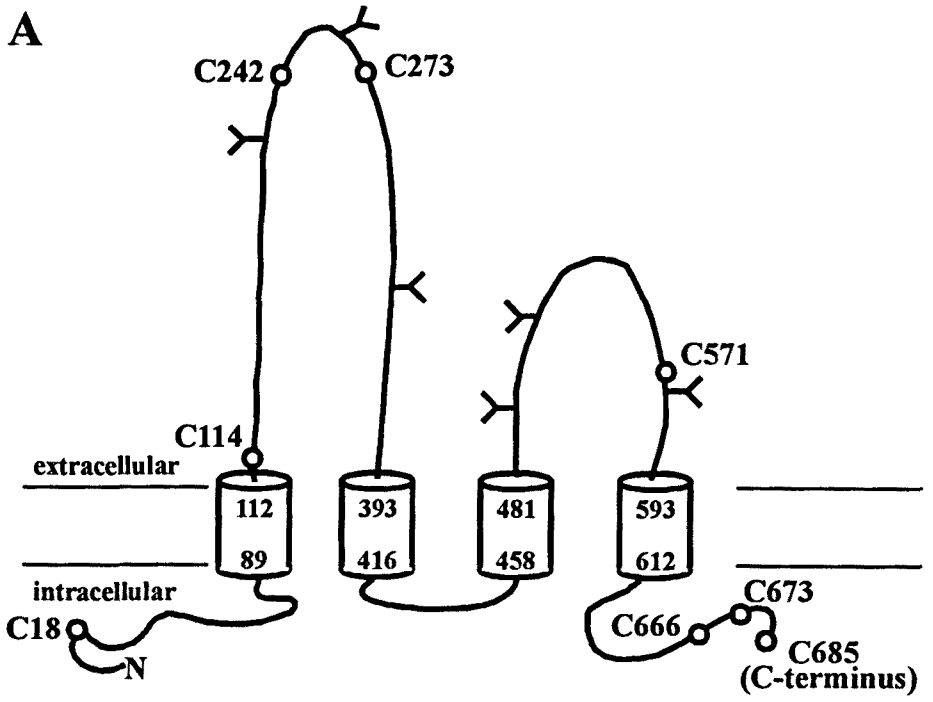
the cRNA injected groups. The amino acid uptake activity of uninjected oocytes was as follows: L-[^{3}H] arginine uptake: 1.4 ± 0.2 (choline medium) and 2.1 ± 0.2 (sodium medium); L-[^{3}H] leucine uptake: 6.1 ± 1.4 (choline medium) and 14.5 ± 1.3 (sodium medium); L-[^{35}S] cystine uptake: 0.3 ± 0.1 (choline medium).

Figure 6. cRNA dose-dependence of the induced amino acid transport in wild type and mutant rBAT-injected oocytes. Oocytes were injected with the indicated doses of cRNA of the wild type human rBAT or the indicated rBAT mutants (C571S, C666S or C673S). The uptake of 50 μM L-arginine was determined for 5 min 3 days after the injection. Each bar is the mean ± S.E. of the induced uptake values measured in 6-8 oocytes from a representative experiment. The arginine uptake activity of uninjected oocytes was 0.9 ± 0.2 pmol/5 min per oocyte.

Figure 7. Kinetic analysis of L-leucine and L-arginine induced currents for wild type rBAT- and C114S/C673S-injected oocytes. Three days after injection of human wild-type rBAT or C673S mutant cRNA, two electrode voltage clamp recordings were performed in oocytes. The holding potential was –50 mV. Data are the mean ± S.E. from 4-5 oocytes. Curves were fitted for data from each oocyte and the obtained values for K_m (see inset) and V_{max} were used for statistical analysis. The V_{max} of the L-amino acid-induced currents were -104 ± 3 nA for wild type rBAT, and 15 ± 0.6 for C114S/C673S ($p \leq 0.01$). The apparent K_m values for C114S/C673S were statistically different from those for wild type rBAT ($p \leq 0.001$).

	K_m (μM)		V_{max} (nA)	
L-amino acid	**rBAT**	**C673S**	**rBAT**	**C673S**
Arginine	36 ± 1	18 ± 2	-105 ± 3	-22 ± 1
Lysine	41 ± 1	23 ± 5	-82 ± 4	-32 ± 2
Ornithine	29 ± 3	13 ± 3	-56 ± 1	-19 ± 1
Leucine	378 ± 17	59 ± 1	84 ± 3	18 ± 1
Phenylalanine	674 ± 180	149 ± 44	62 ± 4	16 ± 1
Methionine	460 ± 72	62 ± 15	69 ± 8	8 ± 1
Alanine	2184 ± 114	1152 ± 107	260 ± 32	22 ± 2

Table I. Kinetic parameters of L-amino acid-induced transport currents in wild-type or C673S rBAT cRNA-injected oocytes. 3 days after injections, oocyte expressing human wild type rBAT or C673S-rBAT were voltage clamped at –50 mV and subjected to increasing concentrations of amino acid ranging from 1 mM to 10 mM in the presence of 100 mM NaCl. Two electrode-voltage clamp recording was performed as described in Experimental procedures. Data from individual oocytes were fit to the Hill equation and K_m (μM) and Vmax (nA) values are presented (mean ± S.E.; n = 3-4 oocytes). Kinetic parameters in C673S-injected oocytes were statistically lower than in wild type rBAT-injected oocytes (at least $p \leq 0.05$).


A
C242
C273
C571
C114
extracellular
112
89
393
416
481
458
593
612
intracellular
C18
N
C673
C666
C685
(C-terminus)

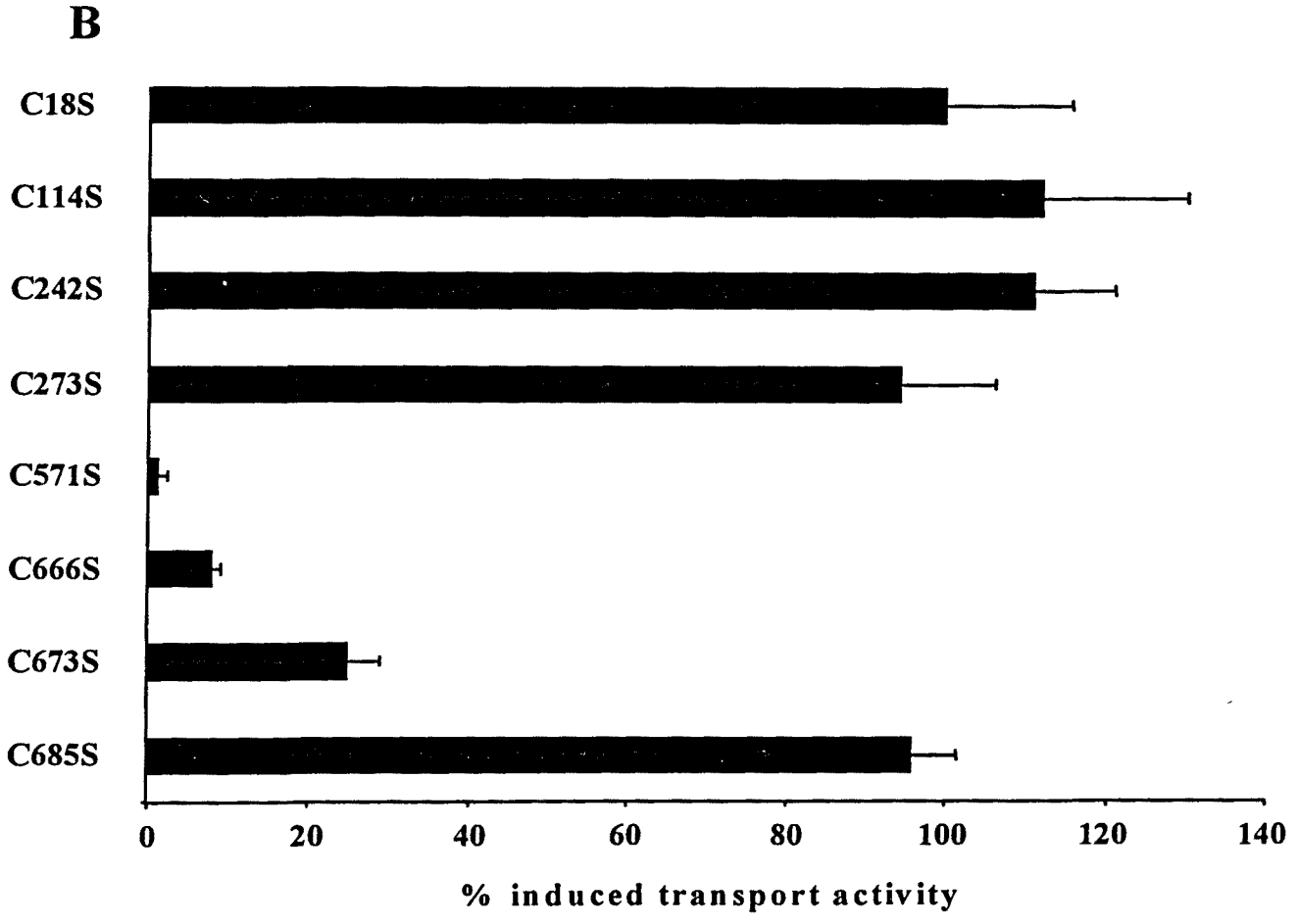

B
C18S
C114S
C242S
C273S
C571S
C666S
C673S
C685S
0
20
40
60
80
100
120
140
% induced transport activity

Fig. 1.

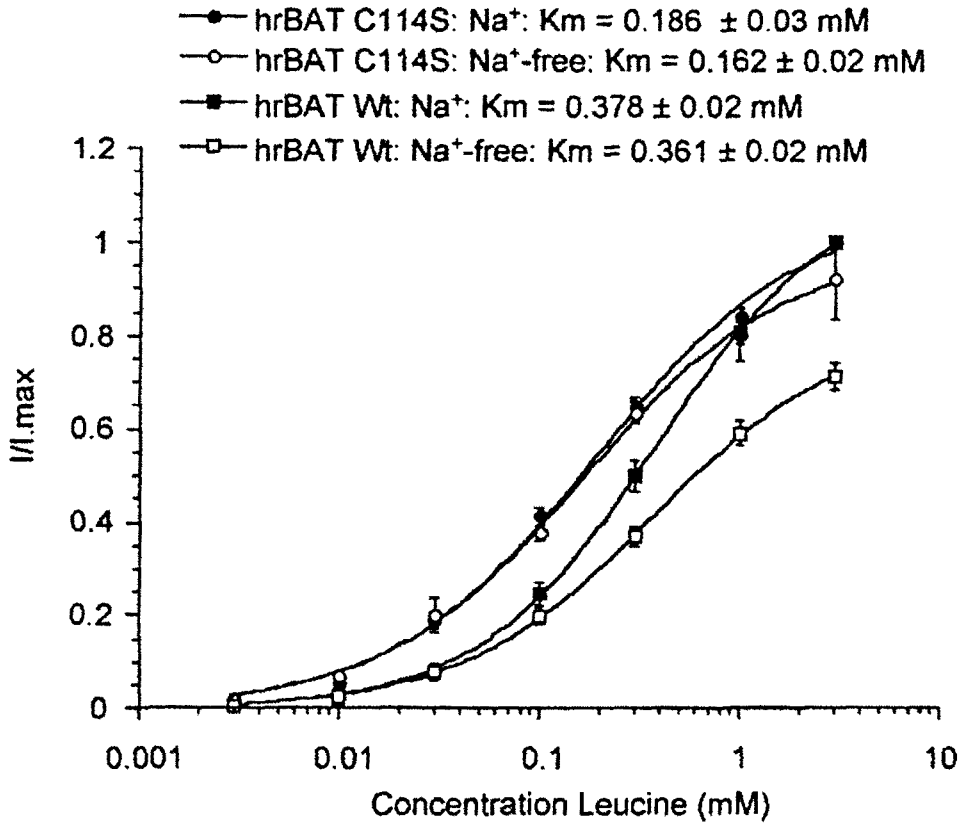

hrBAT C114S: Na+: Km = 0.186 ± 0.03 mM
hrBAT C114S: Na+-free: Km = 0.162 ± 0.02 mM
hrBAT Wt: Na+: Km = 0.378 ± 0.02 mM
hrBAT Wt: Na+-free: Km = 0.361 ± 0.02 mM
I/I.max
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
Concentration Leucine (mM)

Fig. 2

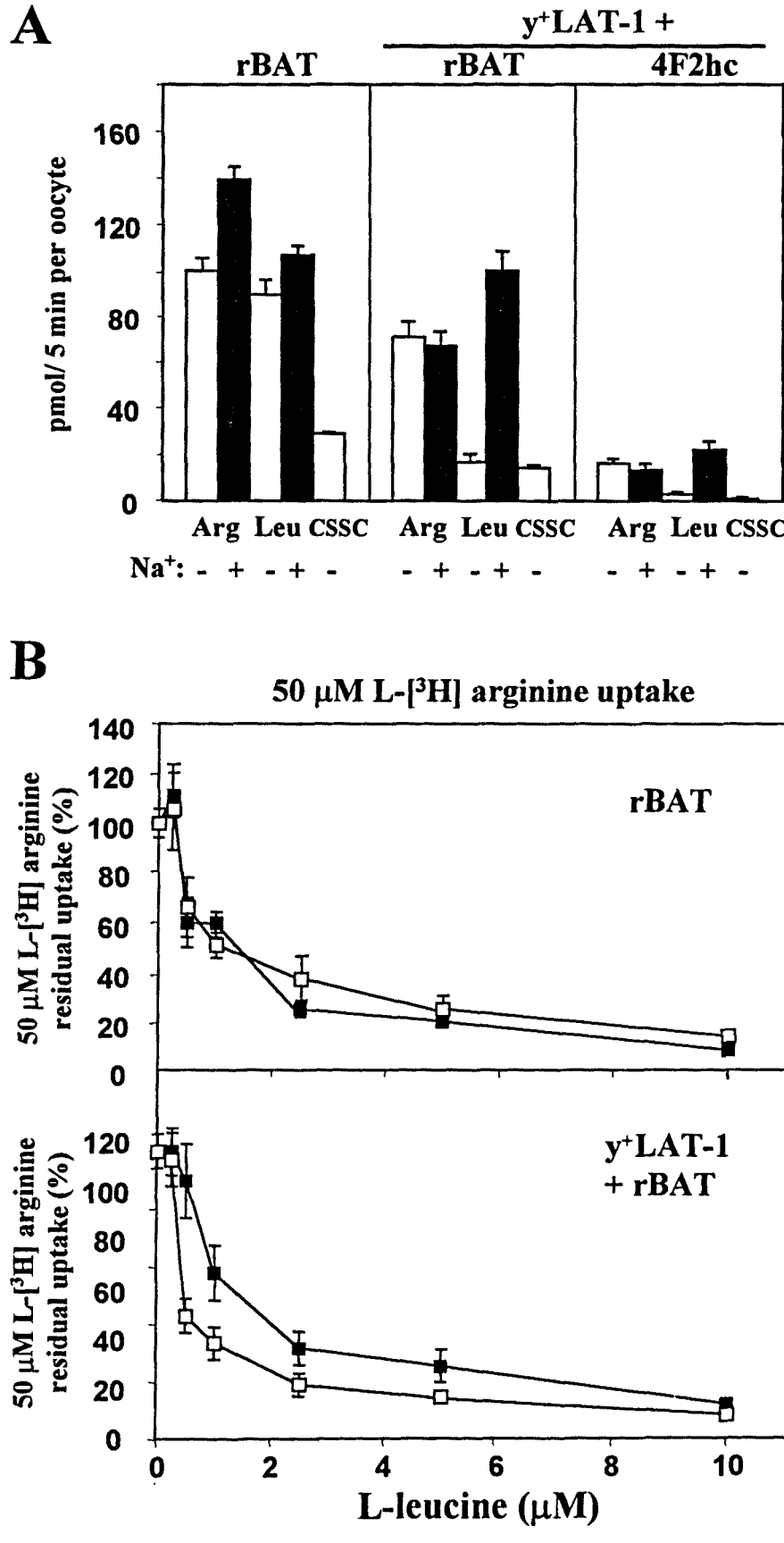
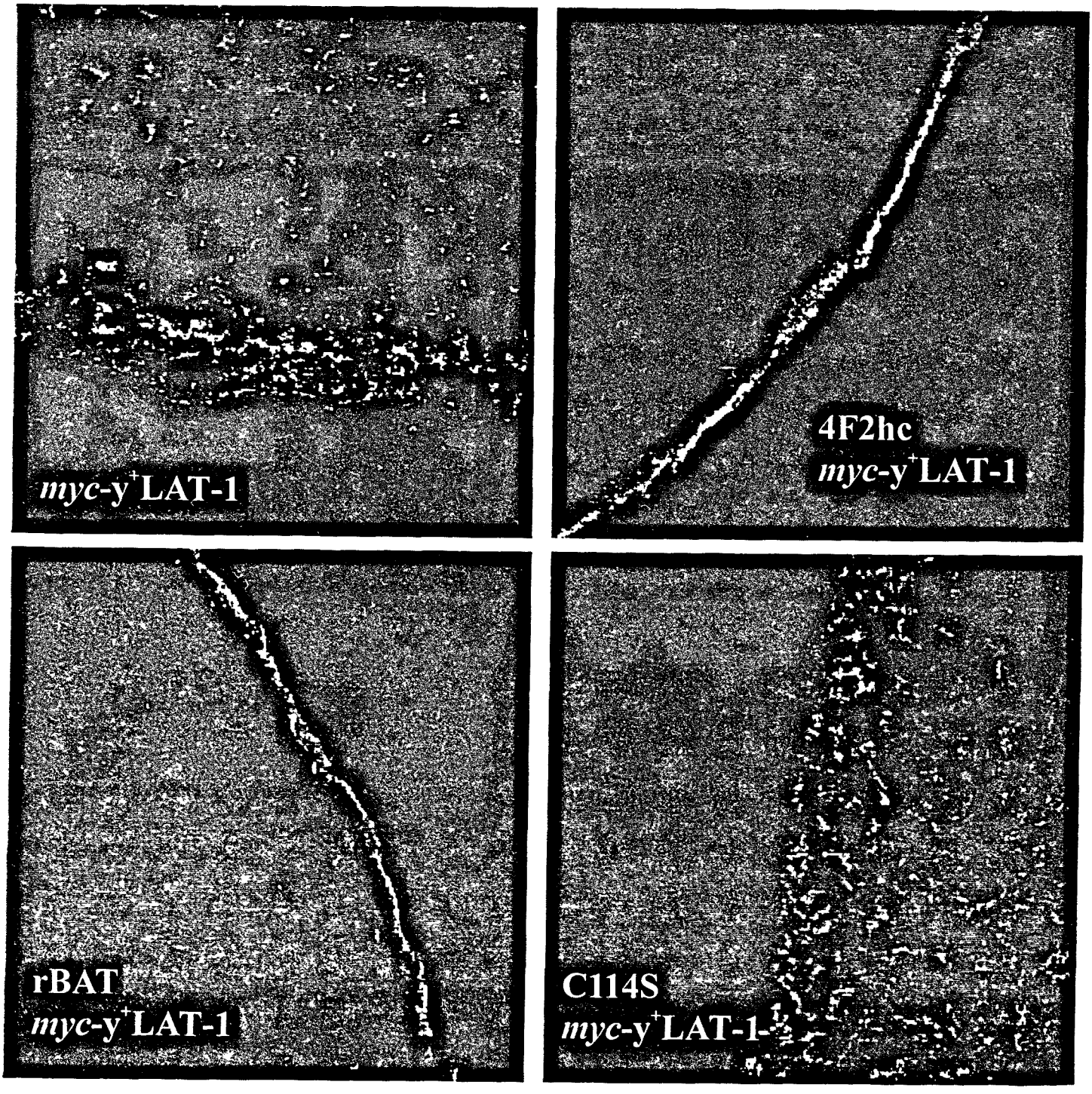
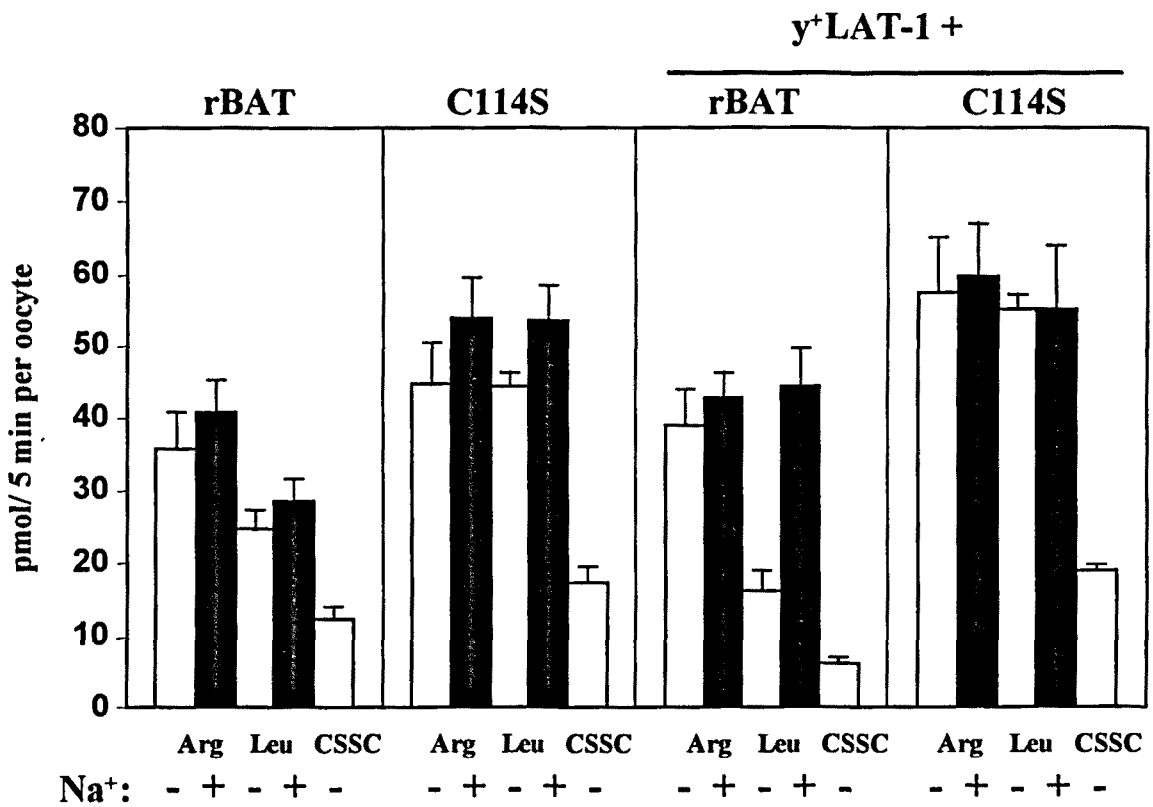

A
y+LAT-1 +
rBAT
rBAT
4F2hc
pmol/ 5 min per oocyte
160
120
80
40
0
Arg Leu CSSC Arg Leu CSSC Arg Leu CSSC
Na+: - + - + - - + - + - - + - + -
B
50 μM L-[3H] arginine uptake
50 μM L-[3H] arginine residual uptake (%)
140
120
100
80
60
40
20
0
rBAT
y+LAT-1 + rBAT
0
2
4
6
8
10
L-leucine (μM)

Fig. 3


myc-y$^+$LAT-1
4F2hc
myc-y$^+$LAT-1
rBAT
myc-y$^+$LAT-1
C114S
myc-y$^+$LAT-1


y+LAT-1 +
rBAT
C114S
rBAT
C114S
pmol/ 5 min per oocyte
80
70
60
50
40
30
20
10
0
Arg Leu CSSC Arg Leu CSSC Arg Leu CSSC Arg Leu CSSC
Na+: - + - + - - + - + - - + - + - - + - + -

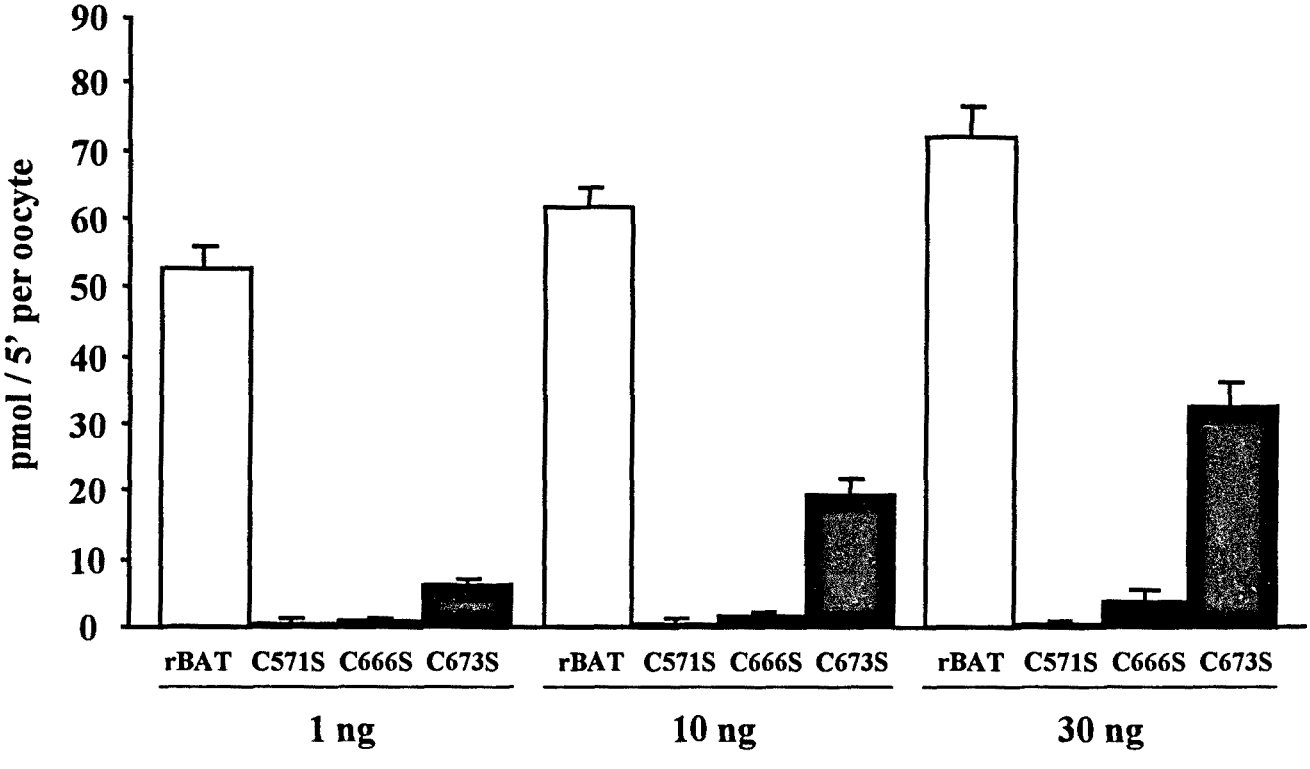
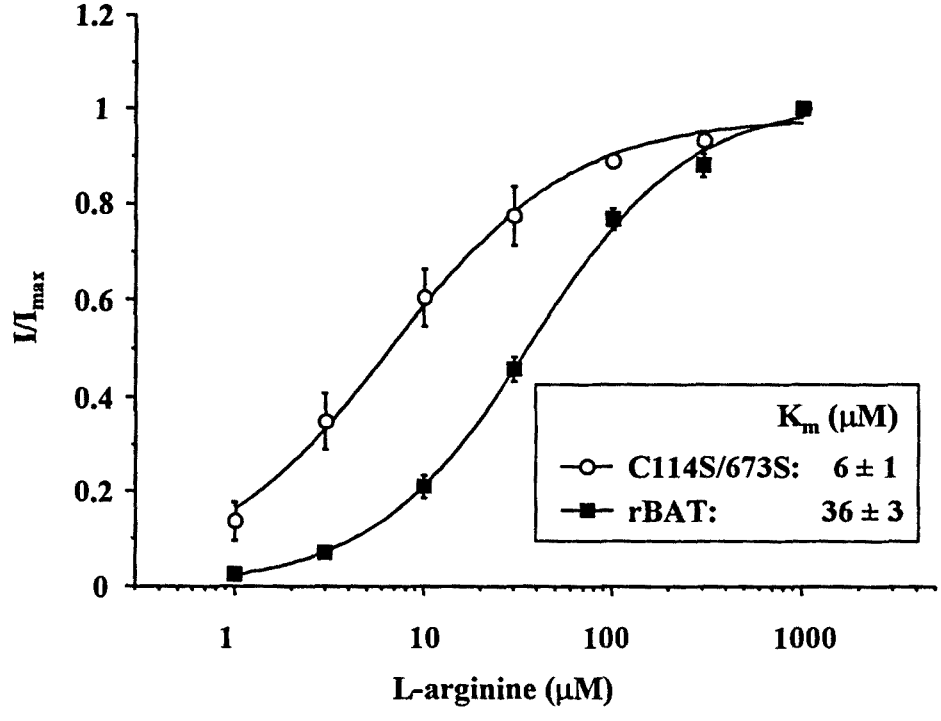

90
80
70
60
50
40
30
20
10
0
pmol / 5' per oocyte
rBAT C571S C666S C673S
rBAT C571S C666S C673S
rBAT C571S C666S C673S
1 ng
10 ng
30 ng

Fig 6


1.2
1
0.8
0.6
0.4
0.2
0
I/I_max
K_m (μM)
C114S/673S: 6 ± 1
rBAT: 36 ± 3
1
10
100
1000
L-arginine (μM)

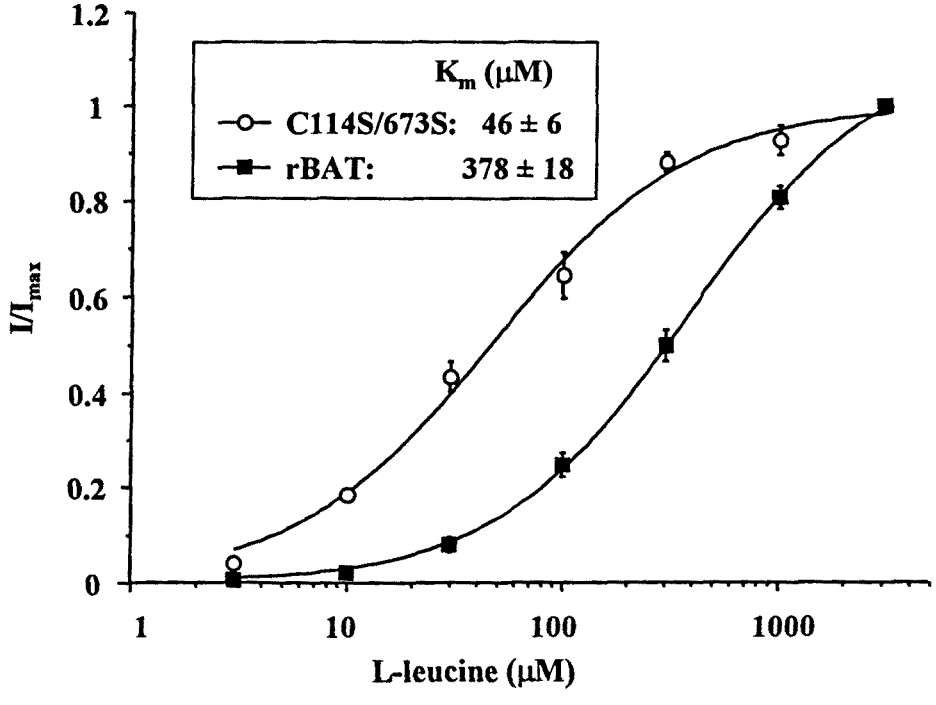

1.2
1
0.8
0.6
0.4
0.2
0
I/I_max
K_m (μM)
C114S/673S: 46 ± 6
rBAT: 378 ± 18
1
10
100
1000
L-leucine (μM)

Fig. 7

Treball de col.laboració 1

Identification of SLC7A7, encoding y^+LAT-1, as the lysinuric protein intolerance gene.

David Torrents*, Juha Mykkänen*, Marta Pineda, Lidia Feliubadaló, Raúl Estévez, Rafael de Cid, Pablo Sanjurjo, Antonio Zorzano, Virginia Nunes, Kirsi Huoponen, Arja Reinikainen, Olli Simell, Marja-Liisa Savontaus, Pertti Aula, Manuel Palacín

Nature Genetics (1999) **21**, 293-296.

* Aquests autors han contribuit per igual.

El doctorand és coautor de la figura 5.

Identification of *SLC7A7*, encoding y+LAT-1, as the lysinuric protein intolerance gene

David Torrents[1*], Juha Mykkänen[2*], Marta Pineda[1], Lidia Feliubadaló[1,3], Raúl Estévez[1], Rafael de Cid[3], Pablo Sanjurjo[4], Antonio Zorzano[1], Virginia Nunes[3], Kirsi Huoponen[2,5], Arja Reinikainen[2], Olli Simell[2], Marja-Liisa Savontaus[2,5], Pertti Aula[2,7] & Manuel Palacín[1]

These authors contributed equally to this work.

Lysinuric protein intolerance (LPI; OMIM 222700) is a rare, recessive disorder with a worldwide distribution, but with a high prevalence in the Finnish population[1]; symptoms include failure to thrive, growth retardation, muscle hypotonia and hepatosplenomegaly. A defect in the plasma membrane transport of dibasic amino acids has been demonstrated at the basolateral membrane of epithelial cells in small intestine and in renal tubules[2–4] and in plasma membrane of cultured skin fibroblasts[5] from LPI patients. The gene causing LPI has been assigned by linkage analysis to 14q11–13. Here we report mutations in *SLC7A7* cDNA (encoding y+L amino acid transporter-1, y+LAT-1), which expresses dibasic amino-acid transport activity and is located in the LPI region, in 31 Finnish LPI patients and 1 Spanish patient. The Finnish patients are homozygous for a founder missense mutation leading to a premature stop codon. The Spanish patient is a compound heterozygote with a missense mutation in one allele and a frameshift mutation in the other. The frameshift mutation generates a premature stop codon, eliminating the last one-third of the protein. The missense mutation abolishes y+LAT-1 amino-acid transport activity when co-expressed with the heavy chain of the cell-surface antigen 4F2 (4F2hc, also known as CD98) in *Xenopus laevis* oocytes. Our data establish that mutations in *SLC7A7* cause LPI.

A linkage study in 20 Finnish LPI families assigned the locus of LPI to the proximal long arm of chromosome 14 (ref. 6). A strong linkage disequilibrium was seen between the disease locus and alleles of the markers *TCRA* (T-cell receptor, α, V, D, J, C) and *D14S283* at 14q11.2. A common haplotype, present in all but one of the Finnish LPI chromosomes, indicated a founder mutation in this population. LPI in non-Finnish families, mainly of Italian origin, was later linked to the same locus[7].

We previously identified a human cDNA, *SLC7A7* (ref. 8), as encoding a member of the growing family of amino acid transporters that combine with 4F2hc and thus constitute functional heterodimeric transporters[8–11]. The expression of human y+LAT-1, the co-expressed transport activity with 4F2hc (refs 8,12–14), the chromosomal location in the LPI locus[8] and the basolateral localization of 4F2hc in epithelial cells[15] suggested *SLC7A7* as a candidate for LPI (ref. 8).

We searched for sequence alterations in *SLC7A7* in 31 well-documented Finnish LPI patients presenting typical clinical findings together with increased urinary excretion and low plasma concentration of lysine, arginine and ornithine[1,6]. Sequence analysis of the RT-PCR–amplified cDNA from seven Finnish LPI patients revealed a 10-bp deletion (1181delACTTTTGCAG) in both alleles from all patients (Fig. 1). We identified a 258-bp intron (data not shown) by amplifying a 441-bp fragment of genomic DNA encompassing the deletion site. The sequence of this fragment showed that the penultimate nucleotide of the intron carried a base change, A→T, in all cases (Fig. 1). This mutation (1181–2 A→T) destroys an acceptor AG splice site at the end of the intron and leads to cryptic splicing at the next AG site, 10 bp downstream (Fig. 1). The A→T substitution also abolishes a unique cutting site for *Dde*I in the amplified 441-bp PCR fragment. We studied 31 patients, 10 obligate heterozygotes and 53 control individuals of Finnish origin using *Dde*I digestion of genomic DNA. DNA from all Finnish LPI patients produced only

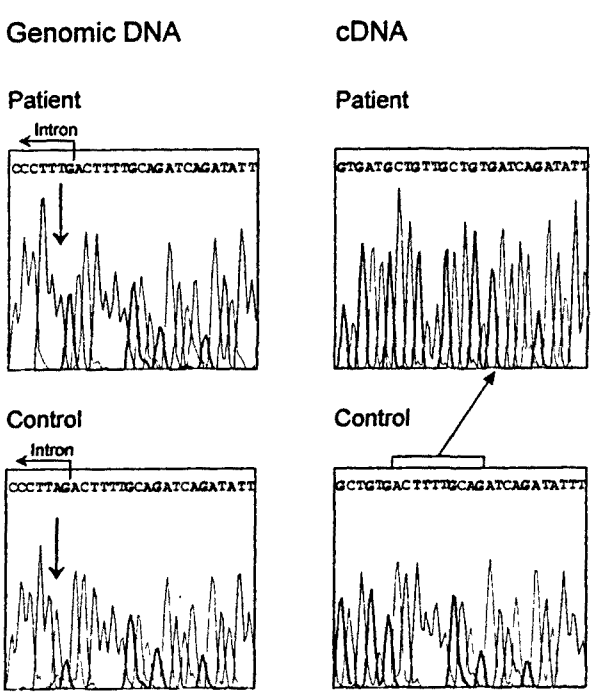


Fig. 1 Sequence chromatograms of a Finnish LPI patient and a control showing the frameshift mutation at the genomic and cDNA level. At the genomic level, the A before the last nucleotide of the intron is mutated to T (arrows, 1181–2A→T), causing the splice site to shift. As a result, the cDNA from the LPI patient lacks a 10-bp sequence (outlined from control) starting from cDNA position 1,181 (arrow).

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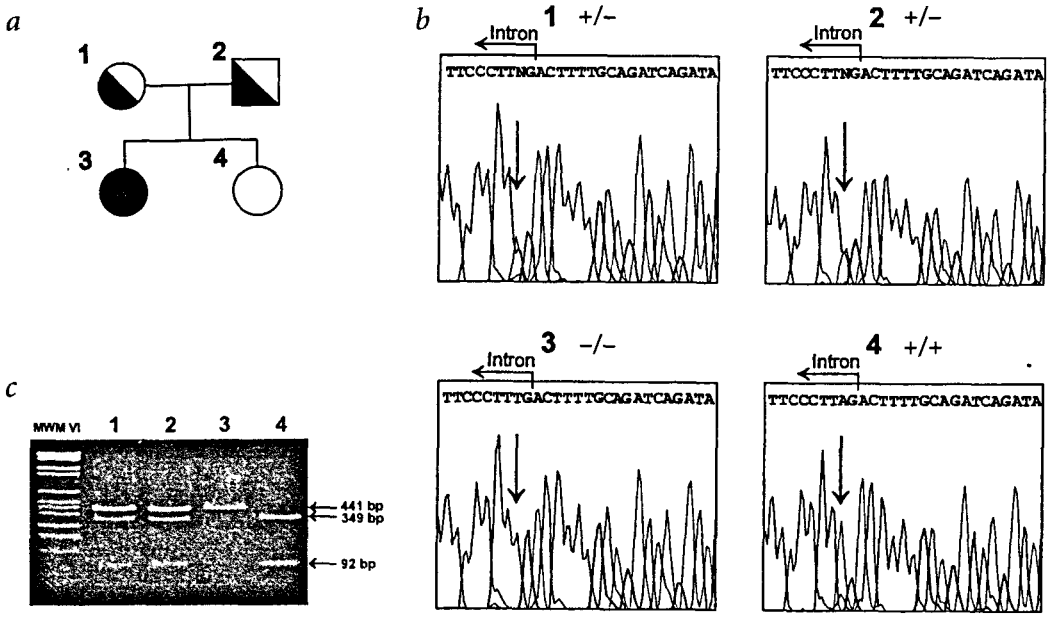

Fig. 2 Segregation of the Finnish LPI (1181–2 A→T) mutation. ***a***, Pedigree of a Finnish LPI family. ***b***, Sequence chromatograms of the family members showing the mutated nucleotide (arrows). ***c***, The mutation-specific *Dde*I analysis. In the control sample, the 349-bp and 92-bp bands are seen. In the patient, the mutation destroys the *Dde*I site and the intact 441-bp band is seen. MWM VI, molecular weight marker. Heterozygotes show all three bands.

the 441-bp fragment, indicating the presence of the mutation in a homozygous state (Fig. 2). This is compatible with the assumption of a founder mutation in the Finnish population. LPI thus provides a further example of founder effect in the genetically isolated population of Finland[16,17]. The only known Finnish LPI patient without the 6-4 haplotype was also homozygous for the splice mutation. The different haplotype must therefore be due to recombination between the LPI locus and the markers.

We also investigated a 15-year-old Spanish boy with clinical and laboratory findings compatible with LPI (ref. 18) for mutations in *SLC7A7*. We detected a missense mutation (L334R; c.1287T→G) and a frameshift mutation (1291delCTTT) by sequencing a 1,665-bp cDNA fragment amplified from his RNA (data not shown). His mother, but not his father, carries the L334R mutation (data not shown). Deletion 1291delCTTT was present in one allele of his father, but not in his mother (data not

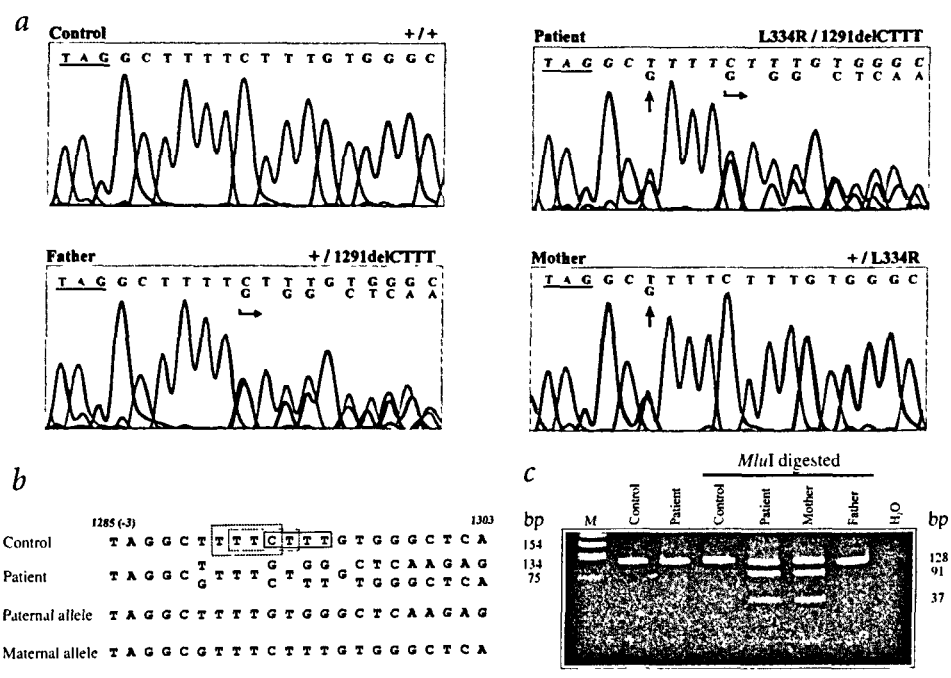

Fig. 3 Characterization of the two *SLC7A7* mutations found in the Spanish family. ***a***, Sequence comparison of genomic *SLC7A7* DNA from the patient, his parents and a control. Sequences correspond to the sense strand between positions 1,285 (–3; intron) and c.1,300 (exon) in the control sequence. The T at position c.1,287 in the coding region changes to G (arrows) in one allele of the patient and his mother, but not in his father; this mutation changes Leu 334 to Arg (L334R). A double sequence appears after position c.1,291 (horizontal arrows) in the patient and his father, but not in his mother. These sequences were confirmed by sequencing the antisense strand. ***b***, Genomic *SLC7A7* DNA sequence from a control and the patient between positions 1,285 (–3; intron) and c.1,303 (exon). The sequence of the mutated maternal and paternal alleles is indicated below. The mother carries mutation L334R (c.1287T→G; mutated maternal allele). Subtraction of this sequence from the patient genomic sequence and of the control sequence from the genomic paternal sequence gave a unique sequence (mutated paternal allele) with a deletion of four nucleotides. Due to the specific sequence around base c.1,291, it cannot be determined whether this mutation results from deletion of nt c.1,289–1,292, c.1,288–1,291 (dashed boxes in the control sequence) or c.1,291–1,294 (solid box in the control sequence). This deletion was confirmed by direct sequencing of the paternal allele amplified by an allele-specific PCR from genomic DNA using the specific-allele primer PI3D and primer P18R. ***c***, Analysis of mutation L334R in amplified genomic DNA by restriction endonuclease digestion. PMuta-P18R amplified a fragment from control genomic DNA (128 bp) and two bands (128 and 124 bp; these two bands are not resolved in the gel shown) from genomic DNA of the Spanish LPI patient. The presence of *Mlu*I-digested products (37-bp and 91-bp fragments) and the undigested fragments shows that the patient and his mother are heterozygous for mutation L334R. *Mlu*I-digestion fragments were not detected in the father or control samples, confirming that they do not carry the L334R mutation. H_2O, without template DNA; M, 1-kb DNA ladder.

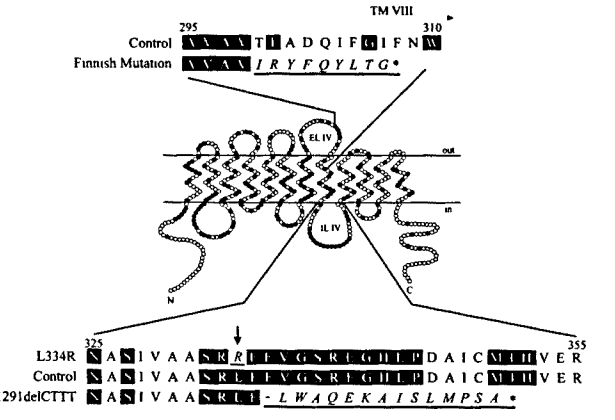

Fig. 4 Schematic representation of the three LPI-specific mutations identified in the y+LAT-1 amino-acid transporter. Membrane topology prediction algorithms suggest that y+LAT-1 contains 12 transmembrane domains with the amino- and carboxy-termini located intracellularly[8]. The missense mutation L334R and the frameshift mutation 1291delCTTT modify the protein in intracellular loop 4 (IL IV), whereas the Finnish frameshift mutation (1181–2A→T) modifies extracellular loop 4 (EL IV). Mutation L334R changes a leucine residue (vertical arrow) conserved in this family of amino acid transporters. Amino acid changes due to frameshift mutations 1291del CTTT and (1181–2A→T, Finnish mutation) are underlined and in italics. Control, wild-type sequence, *, premature stop codon. White characters on black background denote amino acids conserved in human y+LAT-1, y+LAT-2 and LAT-1 amino acid transporters. TM VIII, putative transmembrane domain VIII. Amino acid numbers are indicated above the alignments.

shown). The sequence of an approximately 1.5-kb *SLC7A7* genomic DNA fragment encompassing both mutations confirmed that the patient is a compound heterozygote for mutations L334R and 1291delCTTT, and that his mother and father carry mutations L334R and 1291delCTTT, respectively (Fig. 3*a*,*b*). Restriction endonuclease analysis (Fig. 3*c*) of *SLC7A7* genomic DNA from the patient and his parents further confirmed mutation L334R in the patient and his mother, but not in his father or in 100 chromosomes from healthy Spanish subjects (data not shown).

Two (1181–2A→T and 1291delCTTT) of three identified *SLC7A7* mutations in LPI patients are frameshift mutations and produce truncated proteins (Fig. 4). The third (L334R) is a missense mutation and corresponds to a leucine that is conserved in the amino-acid transporters of this family (Fig. 4). We analysed y+L-induced amino-acid transport after co-injection of wild-type or L334R mutated synthetic RNA (cRNA) with 4F2hc cRNA in *X. laevis* oocytes. The L334R substitution nearly abolished (approximately 10% residual activity) sodium-independent L-arginine and sodium-dependent L-leucine transport activity (that is, y+L activity) associated with the co-injection of 4F2hc (Fig. 5).

Plasma membrane transport of dibasic amino acids lysine, arginine and ornithine at the basolateral membranes of epithelial cells in small intestine and in renal tubules is defective in LPI (refs 2–4). Systems y+ (human CAT1–4 amino-acid transporters; ref. 19) and y+L (4F2hc/y+LAT-1 and 4F2hc/y+LAT-2 amino-acid transporters; refs 8,20) may explain the defect in LPI amino-acid transport activity. System y+ amino-acid transporters, y+LAT-2 and 4F2hc were excluded as LPI genes by linkage studies, chromosomal location or tissue expression distribution[21–23] (*SLC7A6*, encoding y+LAT-2, localizes to chromosome 16; G25418, Whitehead Institute/MIT Center for Genome research).

Here we have shown that mutations in *SLC7A7* segregate with

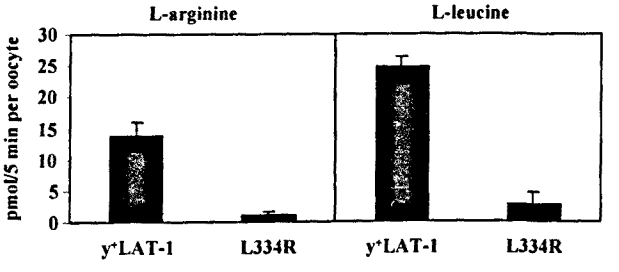

Fig. 5 Analysis of L334R-y+LAT-1 amino-acid transport activity in *X. laevis* oocytes. The uptake of L-arginine in the absence of sodium and the uptake of L-leucine in the presence of sodium were measured two and three days after cRNA injections, respectively. The co-expressed transport activity (that is, transport in wild-type or L334R *SLC7A7* cRNA co-injected with 4F2hc cRNA minus that of 4F2hc cRNA-injected oocytes) is shown. Data are the mean±s.e.m. from 7–8 oocytes per group. Transport of L-arginine and L-leucine in uninjected oocytes were 2.3±0.4 and 2.9±0.4 pmol/5 min per oocyte, respectively. Transport of L-arginine and L-leucine in 4F2hc cRNA-injected oocytes were 4.0±0.1 and 3.3±0.6 pmol/5 min per oocyte, respectively. Identical results were obtained with another preparation of cRNAs.

the LPI phenotype in one Spanish family and in the Finnish population. The three mutations described produce truncated proteins or a defect in amino-acid transport activity (L334R mutation) when expressed in oocytes. These results demonstrate that mutations in *SLC7A7* cause LPI. Our study represents only the second defect of amino-acid membrane transport associated with hereditary disease, following the $b^{0,+}$-like transporter (rBAT) in cystinuria type I/I (refs 24,25).

The evidence that *SLC7A7* is the LPI gene illustrates the role of system y+L in renal reabsorption and intestinal absorption of dibasic amino acids. System y+L was first described in human erythrocytes and placenta[12,26]; its transport activity shows *trans*-stimulated efflux of dibasic amino acids caused by external neutral amino acids in the presence of sodium[12]. It was suggested that system y+L might explain the efflux of dibasic amino acids against the membrane potential by the exchange of neutral amino acids plus sodium ion[12]. This counter-transport mechanism via system y+L was demonstrated in oocytes expressing 4F2hc (ref. 13). This, together with the basolateral location of 4F2hc in proximal tubule epithelial cells[15] and the leucine-stimulated efflux of lysine at the basolateral plasma membrane of the epithelial cells of the small intestine[27], allows us to propose a model in which system y+L is responsible for the renal reabsorption and intestinal absorption of dibasic amino acids at the basolateral plasma membrane[13]. The role reported here for the amino-acid transporter y+LAT-1 in LPI further supports this model.

Methods

RT-PCR amplification and sequencing of *SLC7A7* cDNA. For the Finnish samples, total RNA extraction from control and patient cultured fibroblasts and lymphoblasts was performed using RNeasy Midi Kit (Qiagen) according to the manufacturer's protocol. We generated first-strand cDNA using total RNA (2 μg), mouse moloney leukaemia virus reverse transcriptase (MuMLV RT; 200 U; Promega), 1×buffer (Promega), Rnasin (20 U; Promega) and antisense primer P6R (20 pmol) in a reaction volume (20 μl). The PCR-I reaction mixture (80 μl) contained dNTPs (200 μM of each), forward (P11D) and reverse (P6R) primers (20 pmol), *Taq* DNA polymerase (2 U; Promega) and 1×PCR buffer (Dynazyme). PCR-I conditions were 95 °C for 5 min; 39 cycles of 95 °C for 1 min, 55 °C for 1.5 min, 72 °C for 1.5 min; and 72 °C for 10 min. The subsequent PCR-II (nested with primers P12D and P1R) reaction mixture (50 μl) contained PCR-I product (1 μl), 1×PCR buffer (Dynazyme) and *Taq* DNA polymerase (2 U). PCR-II conditions were as in PCR-I. All PCR products were run on 0.8% agarose gel, purified and sequenced. For the Spanish samples, RNA was isolated from total blood, cultured skin fibroblast and EBV-transformed lymphoblastoid cell lines, as described[24]. We synthesized first-strand cDNA from total RNA (5 μg) using random primers and SuperScript II kit (Life Technologies). PCR-I reaction cocktail was as described above, using primers (0.13 μM of each), *Taq* DNA polymerase and PCR buffer (1.5 mM $MgCl_2$ final concentration; Boehringer). For PCR-II, primers (0.3 μM of each) and PCR-I (2 μl) were used. Conditions for PCR-I and PCR-II were:

hot start of 5 min at 94 °C, 10 cycles of denaturing (94 °C 25 s), annealing (56 °C, 25 s) and extension (74 °C, 120 s), 25 cycles of denaturing (90 °C, 25 s), annealing (55 °C 30 s) and extension (74 °C, 120 s), and a final extension of 4 min at 74 °C All PCR products were purified using QIAquick PCR purification kit (Qiagen) and sequenced with d Rhodamine dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer) The 1 665 bp fragment was sequenced completely using primers P15D, P4D, P13D, P2D, P3R, P4R, P6R and P8R (primer sequences are available on request)

Genomic DNA amplification sequencing and mutation analysis The PCR amplification of the 440 bp fragment of genomic DNA containing the critical intron exon region of the Finnish LPI mutation was carried out with primers LPI 1087F and LPI 1268R Otherwise the PCR reaction mixture was the same as in PCR II The PCR cycles were 95 °C for 4 min, 39 cycles of 94 °C for 35 s, 55 °C for 35 s, and 72 °C for 6 min The samples for sequencing were run on 1 5% agarose gel, purified and sequenced as described above The samples for 1181–2A→T mutation analysis were digested with *DdeI* and run on 2% agarose gel We amplified genomic DNA of the patient, his parents and a control using primers P10D and P2R The reaction cocktail consisted of dNTPs (200 µM of each), primers (0 13 µM of each), RT reaction (2 µl), *Taq* DNA polymerase (0 025 units/µl) and PCR buffer containing $MgCl_2$ (1 5 mM, final concentration) PCR conditions were as for PCR I and PCR II A major band of approximately 1 5 kb was purified from a 1% agarose gel and sequenced with primers 10D and 18R Specific amplification of 1291delCTTT allele from the patient was performed using primers PI3D (5 –TCTCCTATCT TACCTAACATAGGCT–3 , the base at the 3 end corresponds to the wild type sequence at position c 1,287, which is the nucleotide mutated in L334R, the base underlined corresponds to the first nucleotide of this exon) and P18R PCR reaction was carried out using template genomic DNA (200 ng) dNTPs (200 µM of each), primers (0 35 µM of each), *Taq* DNA polymerase (0 025 units/µl) and PCR buffer containing $MgCl_2$ (1 5 mM) PCR conditions were hot start of 5 min at 94 °C, 10 cycles of denaturing (94 °C, 25 s), annealing (56 °C, 25 s) and extension (74 °C, 20 s), 25 cycles of denaturing (90 °C, 25 s), annealing (56 °C, 30 s) and extension (74 °C, 20 s), and a final extension of 5 min at 74 °C The PCR product was purified and sequenced with primer P18R, as described above To detect the presence of mutation L334R, genomic DNA isolated from the patient, his parents and 50 non related Spanish controls was amplified with a mutagenesis primer PMuta (5 – CCAATTTTCTCAGCTTCTCCTATCTTACCTAACATA[C]GC–3 , underlined character corresponds to the first nucleotide of an exon at position 1,282 of *SLC7A7* cDNA) and P18R The addition of a C (in brackets) in position 37 in the PMuta primer (instead of a G present in the original sequence) together with the L334R mutation generates a *MluI* (Boehringer) site We carried out PCR reactions using template DNA (200 ng), dNTPs (200 µM of each), primers (0 35 µM of each), *Taq* DNA polymerase (0 025 units/µl) and PCR buffer containing $MgCl_2$ (1 5 mM) PCR conditions were hot start of 5 min at 94 °C, 10 cycles of denaturing (94 °C, 25s) annealing (58 °C, 25 s) and extension (74 °C, 20 s), 25 cycles of denaturing (90 °C, 25 s), annealing (58 °C, 30 s) and extension (74 °C, 20 s), and a final extension of 5 min at 74 °C The resulting fragments were run on 10% polyacrylamide gel

Sequence contig assembly and comparison We constructed the contigs and sequence comparisons of all automated sequences with Sequencher 3 0 program (Gene Codes) and performed cDNA genomic DNA comparisons using the BLAST program

Construction of L334R cDNA Site directed mutagenesis for the construction of the L334R human y^+LAT-1 we used the QuickChange™ site directed mutagenesis kit (Stratagene) according to the manufacturer s protocol The mutagenic oligonucleotide was 5 –CTGCTTCTAGGC(G)TTTCT TTGTGGGCTC–3 (sense strand, the mutated nt 1,287 is indicated by parentheses) Mutants were identified by sequencing Proper construction of the mutated cDNA was confirmed by complete sequencing

Oocytes, injections and uptake measurements *X laevis* (H Kahler) oocyte management, cRNA synthesis (human 4F2hc y^+LAT 1 and L334R y^+LAT 1) and injections (10 ng cRNA per oocyte) were as described[8,13–14] The uptake of L [2,3,4,5 ^{3}H] arginine (Amersham) or L [4 5 ^{3}H] leucine (Amersham) was measured as described[8] Amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those of uninjected oocytes (data not shown)

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Treball de col.laboració 2

Identification of a membrane protein, LAT-2, that co-expresses with 4F2 heavy chain, an L-type amino acid transport with broad specificity for small and large zwiitterionic amino acids

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* Aquests autors han contribuit per igual.

El doctorand és coautor de les figures 4, 6 i 7.

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RELACIÓ ESTRUCTURA-FUNCIÓ EN LA FAMÍLIA DE TRANSPORTADORS D'AMINOÀCIDS HETEROMULTIMÈRICS IDENTIFICACIÓ D'UNA NOVA FAMÍLIA DE TRANSPORTADORS LISOSOMALS

RAÚL ESTÉVEZ POVEDANO

Barcelona, desembre de 1999

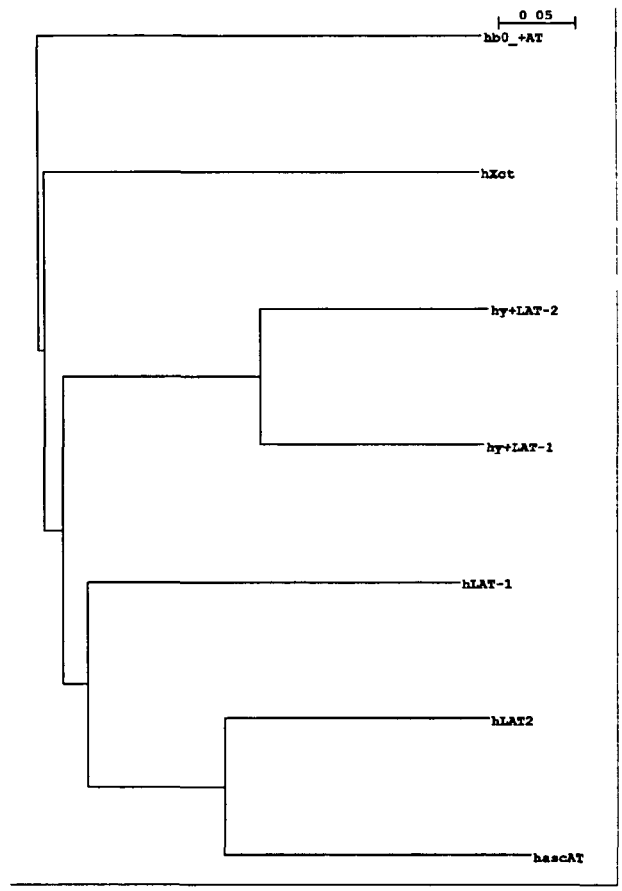

Figura 22. Arbre filogenètic dels membres coneguts de la família HAT.

5. INTERACCIONS CREUADES ENTRE SUBUNITATS. PAPER DE LA CADENA PESADA EN LA FUNCIÓ DEL TRANSPORTADOR (TREBALL 5)

En l'apartat 3 de "Resultats i discussió" he comentat que una de les evidències indirectes que les proteïnes 4F2hc i rBAT no constituïen per si soles un transportador era que s'havia observat que, en expressar-les en oòcits, induïen diferents activitats de transport. En el cas de 4F2hc podem entendre ara aquest resultat: com que hi ha diferents subunitats lleugeres que comparteixen la mateixa subunitat pesada, les diferents activitats associades a 4F2hc es deuen a l'expressió en l'oòcit de la subunitat lleugera corresponent. Com podem interpretar els resultats de les diferents activitats associats a l'expressió de la proteïna rBAT? En el nostre laboratori sempre hem detectat una lleugera component dependent de sodi, més visible a concentracions altes de substrat.

Per comprovar si podien haver-hi interaccions creuades entre subunitats vaig coexpressar en els oòcits les proteïnes rBAT i y^+LAT-1 i vaig mesurar el transport induït d'arginina i leucina en presència o no de sodi, i de cistina en absència de sodi. Vaig observar dos fenòmens: una reducció del total de substrat transportat, però, molt més important, un increment notable de la dependència de sodi per a la leucina. És a dir, podríem dir que havíem expressat dos sistemes de transport: el sistema $b^{0,+}$, ja que

observem una inducció de transport de cistina (la cistina no es transporta pel sistema y^+L), i el sistema y^+L, ja que observem una dependència de sodi pel transport de leucina.

Per estar segurs que estàvem expressant el sistema y^+L van mesurar el transport d'arginina en presència de diferents concentracions de leucina en presència o en absència de sodi. L'experiment es mostra en la **figura 23.**

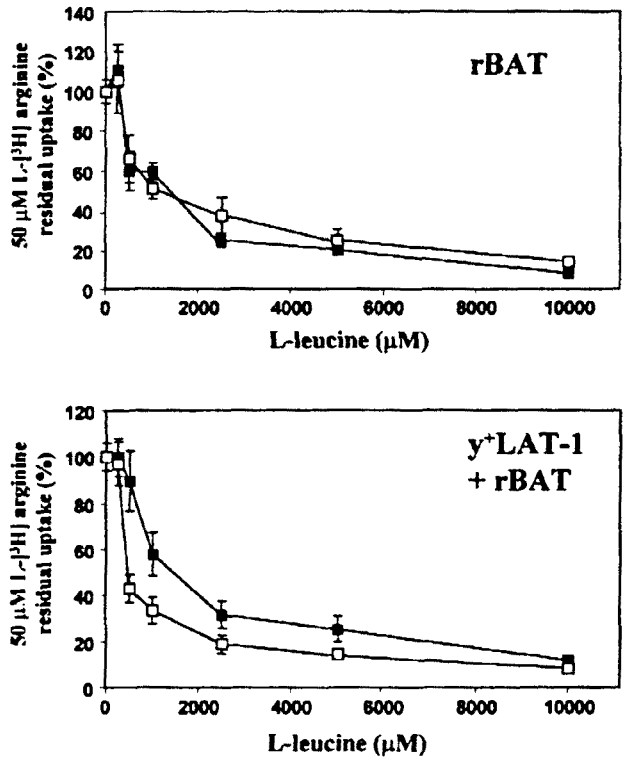

Figura 23. Expressió del sistema y^+L en coexpressar rBAT i y^+LAT-1 en oòcits de *Xenopus*. Els quadrats negres són en absència de sodi i els blancs, en presència.

Com es pot veure en aquesta figura la corba d'inhibició per leucina en el cas d'rBAT és independent de si és present o no l'ió de sodi, mentre que en coexpressar amb y^+LAT-1 observem que la leucina inhibeix més en presència de sodi.

Vam voler estudiar llavors si les dues proteïnes interaccionaven físicament. Per això vaig fer un experiment d'immunocitoquímica expressant N-*myc*-y^+LAT-1 sol, amb 4F2hc o amb rBAT. Els experiments van mostrar que igual que 4F2hc, rBAT porta y^+LAT-1 a la membrana plasmàtica (vegeu **figura 25**). Com interaccionen aquestes dues proteïnes? El més lògic és pensar que la interacció es donaria a través de la cisteïna homóloga a la proteïna 4F2hc. Vaig construir el mutant de rBAT cisteïna114serina i, en primer lloc, el vam voler caracteritzar. Carsten A. Wagner, del grup del professor Florian Lang, a Tübingen, va analitzar el mutant mitjançant tècniques d'electrofisiologia. Va analitzar la dosi de cRNA injectat-funció, la dependència de voltatge de la intensitat de corrent, l'afinitat del mutant per arginina i leucina en presència o no de sodi, i les propietats funcionals de l'intercanvi. Només va detectar un canvi en comparar les cinètiques en presència o no de sodi, experiment que es mostra en la **figura 24.** Es pot observar que el mutant C114S-hrBAT té una activitat que és 100%

$b^{0,+}$, és a dir, el transport d'aminoàcids bàsics i neutres és completament independent de sodi.

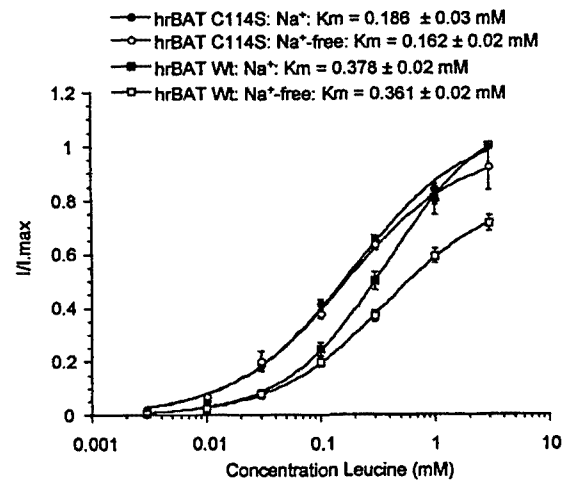

Figura 24. El mutant C114S-hrBAT funciona com un transportador "pur" $b^{0,+}$ en oòcits de *Xenopus*.

Aquest resultat suggeria que aquest mutant seria incapaç d'interaccionar amb y^+LAT-1, ja que suposàvem que aquesta dependència de sodi que presentava rBAT es devia a la interacció amb altres subunitats que no serien la subunitat lleugera $b^{0,+}$.

Vaig coexpressar llavors el mutant C114S-hrBAT amb la proteïna y^+LAT-1 en oòcits, ho vaig analitzar funcionalment i per immunocitoquímica, i ho vaig comparar amb el mutant CS1 de 4F2hc. Vam observar que el mutant C114S no presentava cap tipus de dependència de sodi en el transport dels aminoàcids neutres, encara que es coexpressés amb y^+LAT-1. En analitzar per immunocitoquímica veiem que aquest mutant és incapaç de portar y^+LAT-1 a la membrana plasmàtica, a diferència del mutant CS1 de 4F2hc **(figura 25).**

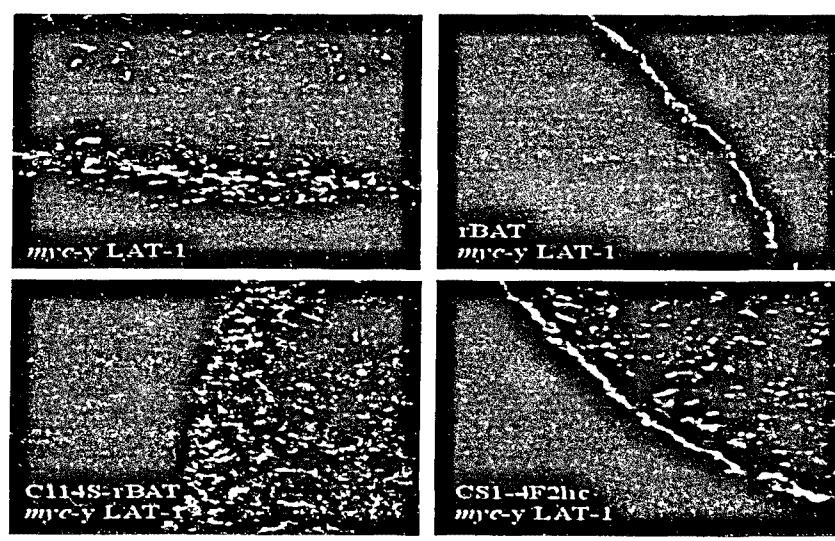

Figura 25. El mutant C114S-hrBAT no porta y^+LAT-1 a la membrana plasmàtica a diferència del mutant CS1 de 4F2hc.

Amb aquest resultat podem extreure la conclusió que la cisteïna 114, en el cas d'rBAT o la cisteïna 109, en el cas de 4F2hc no tenen cap paper per determinar l'especificitat de la subunitat. El fet que el mutant CS1 de 4F2hc però no el mutant C114S de rBAT, pugui portar y^+LAT-1 a la superfície indica que 4F2hc presenta un domini no present en rBAT que pot interaccionar (probablement de forma no covalent) amb y^+LAT-1. La formació d'aquest pont disulfur entre 4F2hc i y^+LAT-1 no és imprescindible però és necessària per tenir la mateixa funció que la proteïna normal. En canvi, el fet que no observem cap tipus d'inhibició en el transport induït pel mutant C114S d'rBAT respecte a la proteïna salvatge, indica que la formació del pont disulfur entre rBAT i $b^{0,+}$AT té un paper poc important respecte a altres interaccions entre altres dominis d'ambdues proteïnes.

Quins són aquests dominis? Quan es va identificar el cDNA d'rBAT, es va suggerir que l'extrem C-terminal tindria un possible paper com a domini que permetria la interacció amb altres proteïnes (Wells *et al.*, 1992), ja que semblava que hi hagués un domini conegut com *cremallera de leucina.* El grup de Takeda va construir i expressar constructes en oòcits on havia suprimit alguns aminoàcids de l'extrem C-terminal (Miyamoto *et al.*, 1996). En concret, el mutant Δ(511-685)hrBAT induïa una activitat en oòcits que era compatible amb l'activitat y^+L excepte per una característica: podia detectar corrents en afegir aminoàcids. Únicament es detecten corrents de sortida en expressar 4F2hc en oòcits quan s'afegien aminoàcids neutres en absència de sodi, perquè funciona com un intercanviador asimètric (veure apartat 2 de "Resultats i discussió"). De totes formes aquest treball suggeria que l'extrem C-terminal podria jugar un paper en la interacció amb la subunitat endògena. El grup de Tate va aportar una altra peça d'informació. També va expressar diferents delecions de l'extrem C-terminal. Les primeres deleccions (625-683) provocaven una pèrdua total de la funció. En canvi, la delecció Δ(588-683)rBAT no presentava cap defecte respecte a la proteïna salvatge, però era inactivat per la mutació de la cisteïna homòloga a 4F2hc. Igual que els nostres resultats, la mutació d'aquesta cisteïna per si sola no afectava la funció de la proteïna, encara que feia desaparèixer els complexos de pes molecular alt que es detectaven en els oòcits, i que podrien correspondre a la unió d'rBAT amb la subunitat endògena.

Per entendre millor la importància del domini C terminal vaig construir una quimera (kc4F2) on intercanviava els dominis C-terminal (**figura 26**).

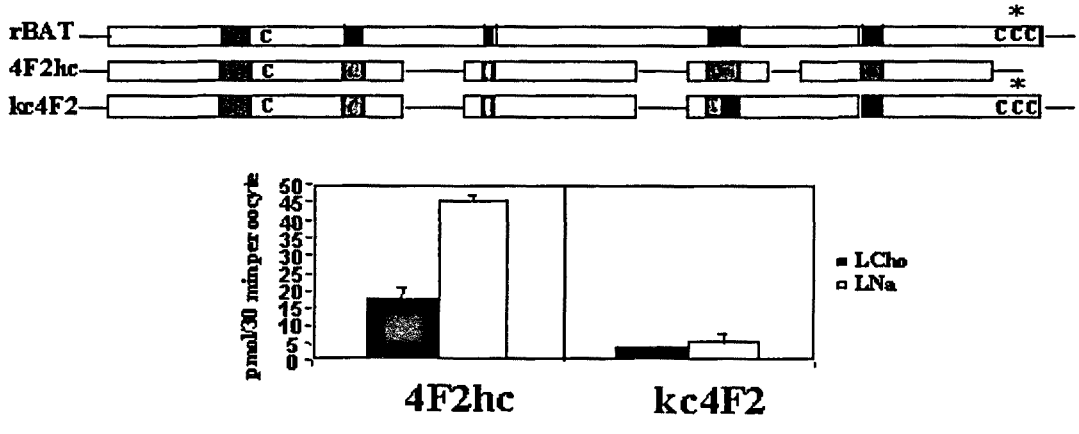

Figura 26. El domini C-terminal d'rBAT és important per a la interacció amb la subunitat lleugera $b^{0,+}$. El (*) representa la cisteïna 673, la qual hem vist que té un paper funcional important (vegeu més endavant).

El constructe kc4F2, com es pot veure en la **figura 26**, s'expressa a nivells inferiors que la proteïna 4F2hc. En canvi, l'activitat induïda és completament diferent; mentre que el transport de leucina depèn de l'ió de sodi en 4F2hc, en el cas de kc4F2, aquest transport és independent de sodi. Aquest resultat, juntament amb els abans citats suggereix que l'extrem C-terminal d'rBAT és necessari per interaccionar amb la seva subunitat.

Si analitzem en detall l'extrem C-terminal de rBAT observem que en l'extrem final hi ha 3 residus de cisteïna molt propers, i l'últim residu de la proteïna és una cisteïna (vegeu **figura 24**). Marta Pineda, del nostre grup, va mutagenitzar aquests residus (cisteïna 666, cisteïna 673 i cisteïna 685) a serina, per intentar comprendre quina seria la seva funció. Els mutants C666S i C673S són mutants de tràfic (vegeu apartat 1 "Resultats i discussió"), ja que es recuperen en expressar més proteïna, mentre que el mutant C685S és completament funcional. El mutant C673S es recupera totalment respecte a la proteïna salvatge, mentre que el mutant C666S no presenta uns nivells d'expressió alts.

C. Wagner, el nostre col·laborador a la Universitat de Tübingen, va analitzar el mutant C673S, ja que era l'únic que presentava defecte i per això tenia suficient sensibilitat per estudiar-lo. Va comprovar que la dependència de voltatge era la mateixa que la de la proteïna salvatge, però sorprenentment, en fer cinètiques d'arginina i leucina, va observar que mentre la K_m per arginina es mantenia constant, la K_m de leucina disminuïa clarament en el mutant. Va fer llavors cinètiques per altres aminoàcids: lisina, ornitina, histidina, fenilalanina, alanina i metionina. En tots ells va trobar que el mutant presentava clarament una K_m disminuïda per aminoàcids neutres.

Per comprovar que aquest defecte era específic del sistema $b^{0,+}$ i no d'altres possibles interaccions amb altres subunitats presents en l'oòcit, vaig construir el doble mutant C114S-C673S, ja que el mutant C114S no pot interaccionar amb les altres subunitats. Aquest doble mutant perd la dependència de sodi per aminoàcids neutres i bàsics i continua tenint una Km reduïda respecte a la proteïna salvatge.

Per tant, tenim un mutant que afecta propietats funcionals del transportador. Així, la proteïna rBAT no és únicament un activador que portaria la subunitat lleugera a la membrana, sinó que a més pot estar modulant propietats intrínsiques del transport. Quin és el sentit fisiològic d'aquesta modificació? El transportador $b^{0,+}$ tindria la funció de reabsorbir aminoàcids bàsics i cistina, encara que també transporta aminoàcids neutres. A més de l'asimetria creada pel potencial de membrana i la diferent composició d'aminoàcids neutres a l'interior de la cèl·lula (vegeu apartat 2 de "Resultats i discussió"), la proteïna rBAT podria provocar canvis conformacionals en el transportador, que farien que presentés una K_m superior pels aminoàcids neutres. Evidentment, són necessaris altres tipus d'experiments que estudiïn les dues proteïnes aïllades i juntes mitjançant purificació i reconstitució.

Amb aquest treball també expliquem els resultats anteriors on s'observaba que la proteïna rBAT induïa múltiples sistemes de transport en oòcits. Creiem que aquestes interaccions no són específiques, sinò que depenen del sistema d'expressió. Amb aquesta línia, Bröer va observar que la proteïna 4F2hc també induïa activitat $b^{0,+}$ en els oòcits (Bröer, *et al.*, 1998). Més tard, Rajan va obtenir resultats similars expressant $b^{0,+}$AT amb el sistema d'expressió del virus *vaccinia*. (Rajan *et al.*, 1999). En canvi, en el ronyó 4F2hc presenta una localització basolateral mentre que $b^{0,+}$AT és apical. Per

tant, s'han de demostrar que les interaccions són específiques en el teixit on trobem aquestes proteïnes per immunoprecipitació. Això ja s'ha fet per a rBAT i $b^{0,+}$AT, 4F2hc i LAT-1 (Mannion *et al.,* 1998). Podríem postular també que el mutant de la primera cisteïna de 4F2hc (C109S) seria incapaç d'induir l'activitat $b^{0,+}$ o interaccionar amb $b^{0,+}$AT en el sistema del virus *vaccinia.*

6. BUSCANT RELACIONS ESTRUCTURA-FUNCIÓ: MUTAGÈNESI DE LES CISTEÏNES DE LAT-1 (TREBALL EN PREPARACIÓ)

En l'apartat 3 de "Resultats i discussió" he explicat com a partir de l'efecte dels reactius sulfhidril sobre l'activitat y^+L induïda en oòcits per 4F2hc, deduïem que la cisteïna C109 formava un pont disulfur amb la subunitat lleugera que ja seria present en l'oòcit. Com que ara tenim les subunitats lleugeres, podem intentar trobar quina és la cisteïna diana d'aquests agents mitjançant mutagènesi dirigida de les cisteïnes presents en la subunitat.

Però, és aquest l'únic objectiu? Diferents grups d'investigació en el camp de les proteïnes de membrana (revisió de Frillingos *et al.,* 1998) fan servir l'estratègia de la mutagènesi dirigida en cisteïnes combinat després amb *cysteine-scanning mutagenesis* i la utilització de diferents reactius específics de cisteïnes, per obtenir informació estructural de la proteïna com ara topologia, lloc d'unió de substrat, zones de la proteïna conformacionalment actives, distàncies entre parts de la proteïna,etc. El fet de que sigui quasi impossible obtenir cristalls de proteïnes de membrana impedeix l'anàlisi detallada per difracció de raigs X, cosa que fa que siguin necessàries estratègies indirectes com ara la mutagènesi dirigida. De manera relativament més fàcil (encara que requereix molt esforç) es poden obtenir cristalls en dues dimensions (Wang *et al.,* 1993), que permeten l'anàlisi mitjançant criomicroscòpia electrònica, encara que la resolució no és tan elevada com en el cas de la difracció de raigs X.

Per totes aquestes raons vaig decidir estudiar una d'aquestes subunitats mitjançant mutagènesi dirigida en residus de cisteïna. Vaig escollir la proteïna xLAT-1 (IU12) per dues raons: *a*) a diferència de les altres subunitats, el nivell de transport d'aminoàcids neutres en absència de sodi és quasi zero en els oòcits injectats amb 4F2hc; pensem que aquesta subunitat no seria present en els oòcits com les subunitats y^+L o $b^{0,+}$; *b*) La proteïna s'expressa sempre a nivells alts, probablement pel fet de ser un gen de *Xenopus laevis*.

El primer que van comprovar va ser si l'activitat L induïda era sensible a reactius sulfhidril. Vam comprovar que l'activitat era sensible a pCMBS (reactiu poc permeable, vegeu apartat 3 "Resultats i discussió") i que aquesta activitat era reversible en tractar-se després amb β-mercaptoetanol, fet que indicava que la inhibició es devia a inhibició sobre residus de cisteïna. Si coexpressem el mutant CS1 de 4F2hc amb LAT-1, l'activitat és més sensible a reactius sulfhidril que si el coexpressem amb 4F2hc, igual que passava amb 4F2hc i la subunitat endògena. Això suggereix que aquest efecte dels reactius sulfhidril es conserva en tots els membres de la família.

Vam començar llavors a mutagenitzar cada residu de cisteïna de la proteïna LAT-1. Així vaig canviar cadascuna de les 11 cisteïnes a serina (excepte les mutacions C164S i C187S, que les va fer Marta Pineda) i vaig analitzar com era la funció induïda i la

sensibilitat a reactius mercurials (pCMBS) respecte a la proteïna salvatge. Vaig observar que únicament les mutacions C164S (la implicada en la formació del pont disulfur) i C443S afectaven a la funció, però no de forma total, cosa que indicava que no eren residus imprescindibles per a la funció.

Vaig observar també que la mutació C178S era més resistent als reactius sulfhidril que la proteïna salvatge, però sense arribar a tenir una protecció total. Això indica que hi deuen haver altres residus que també han de conferir resistència, o que la inhibició parcial es deu a una inhibició de l'activitat endògena. Podríem postular que la formació del pont disulfur provoca un canvi conformacional en el domini transmembrana on es troba aquesta cisteïna (vegeu **figura 27**). En la **figura 28** mostro el resultat de l'experiment de l'anàlisi de cada mutant únic de cisteïna respecte a la funció i la sensibilitat a reactius sulfhidril i en la **figura 27** mostro un esquema dels possibles dominis transmembrana de la proteïna LAT-1, on es marquen en negre els residus conservats en tota la famíla de proteïnes HAT i en vermell, la localització de les cisteïnes.

Per saber llavors quins eren els residus implicats vaig construir el mutant *Cysless* (sense cisteïnes) de LAT-1. Aquest mutant funciona entre un 40-50% respecte a la proteïna salvatge. Aquest reactiu que he construït servirà com a base per a estudis futurs en el camp de l'estructura d'aquesta família de proteïnes. En primer lloc, seria interesant comprovar que aquest mutant és completament resistent a agents mercurials. Un dels experiments interesants a realitzar seria fer *cysteine-scanning mutagenesis* (substitució de residus individuals per cisteïnes) en la proteïna sense cisteïnes o en la proteïna que pugui formar el pont disulfur. D'aquesta forma podríem entendre els canvis topològics que indueix la formació del pont disulfur sobre la proteïna LAT-1.

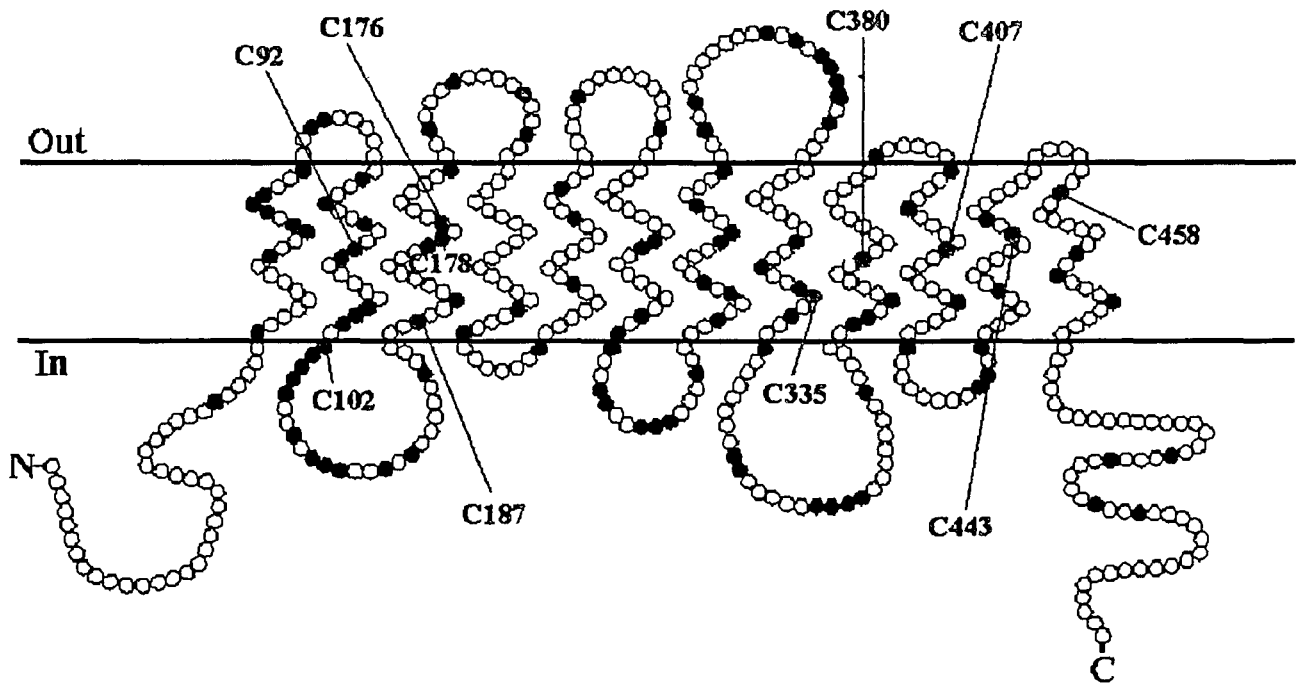

Figura 27. Esquema de la localització de les cisteïnes en la proteïna LAT-1 (vermell) i dels residus conservats en tota la família.

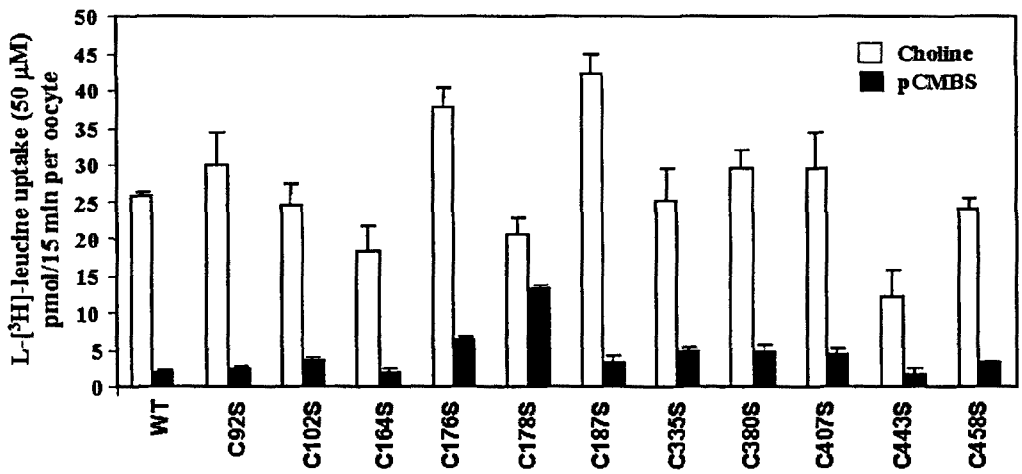

Figura 28. Anàlisi de la funció i de la sensibilitat a pCMBS dels diferents mutants individuals de la proteïna LAT-1.

7. DESCOBRIMENT D'UNA NOVA FAMÍLIA DE TRANSPORTADORS LISOSOMALS (TREBALL EN PREPARACIÓ)

En l'apartat 4 de "Resultats i discussió" explicava que una de les estratègies que vaig dur a terme per intentar aïllar la subunitat y^+L de 4F2hc va ser la coexpressió funcional de 4F2hc amb mRNA de pulmó. Així teníem un grup de 300 clons que era positiu en el nostre assaig funcional (vegeu **figura 14**). En seguir subfraccionant aquest conjunt de clons teníem grans problemes per seguir el senyal funcional de coexpressió, ja que el nivell basal induït per 4F2hc era molt variable i l'increment no era gaire consistent. Finalment, després de molts intents sense resultats, vaig decidir analitzar clons aïllats que continguéssin fragments de cDNA. Vam ser molt afortunats, ja que únicament vaig haver de coexpressar 23 clons fins a aïllar un clon, que vam anomenar posteriorment LyCAT, que incrementava la funció induïda per 4F2hc en els oòcits.

No podem saber si aquest és l'únic gen responsable de l'increment que vèiem en coexpressar-lo amb mRNA de pulmó, però almenys és un resultat positiu del cribellatge. En aquest punt vaig decidir que caracteritzaria aquest gen i no seguiria buscant altres possibles candidats. Val a dir que en aquell moment també estàvem caracteritzant els gens y^+LAT-1 i y^+LAT-2, qué també provocaven un increment en la funció induïda per 4F2hc, igual que LyCAT: no calia buscar gens que ja teníem.

Quina és l'activitat coexpressada per LyCAT? L'activitat coexpressada era també y^+L, cosa que estava d'acord amb tots els passos que havíem seguit fins a aquell moment durant l'*screening* funcional. Vaig seqüenciar llavors tot el cDNA i vaig comprovar que es tractava de l'ortòleg d'un gen que ja s'havia clonat en humans i en ratolí en un procés d'hibridació substractiva. En la **figura 29** presento un esquema d'aquesta proteïna.

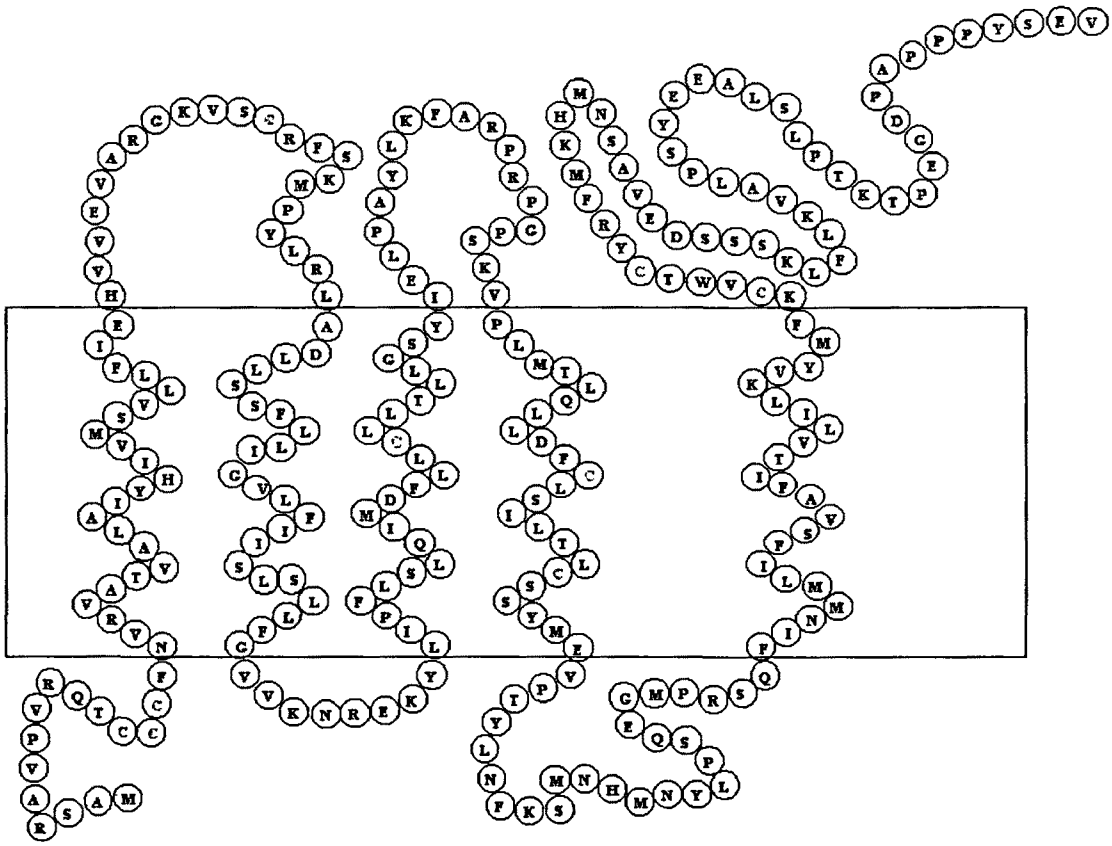

Figura 29. Possible esquema en 2-D de la proteïna LyCAT. En color apareixen marcats alguns aminoàcids conservats en aquesta família (vegeu més endavant).

A simple vista, veiem que no hi ha cap similitud entre aquesta proteïna i les subunitats lleugeres que he descriit en els capítols anteriors. Què és el que se sabia d'aquest gen? Va ser clonat per dos grups independents i de dues formes diferents: mitjançant hibridació subtractiva entre cèl·lules hematopoètiques i no hematopoètiques (Adra *et al.*, 1996) i com un gen induïble ràpidament per àcid retinoic en cèl·lules mieloides (Scott *et al.*, 1996). S'havia analitzat la seva expressió i s'havia detectat en línies cèl·lulars de llinatge eritroide, limfoide i mieloide, amb nivells alts en melsa, medul·la òssia, timus i nòduls limfoides i també en pulmó, placenta, fetge i ronyó. També es va analitzar la seva expressió en teixits embrionaris, i es va detectar en tots els teixits a nivells similars, però amb nivells superiors en cèl·lules ES. En el promotor del gen hi ha múltiples elements reguladors: E2A, TCF2α, AP2, IRF1, IRF2 i la família de factors de transcripció GATA en la regió proximal, i PEBP2, NFκB i SRF localitzats més cap a 5'. El promotor respon a àcid retinoic.

El grup dirigit per Lim (Adra *et al.*, 1996) va produir un anticòs policlonal dirigit contra l'extrem C-terminal, i va detectar una proteïna de 29 kD. En fer servir fraccions cel·lulars van detectar la proteïna en fraccions lisosomals i, en quantitats més petites en l'homogenat i en les fraccions microsomals. En fer immunocitoquímica van observar que estava distribuïda per l'aparell de Golgi i els lisosomes, però ells mateixos van afirmar no estar molt segurs de la seva localització. Van observar, fent transferència

Southern que era un gen que estava altament conservat durant l'evolució, ja que fins i tot es detectava en espècies inferiors com ara peixos.

En l'extrem C-terminal hi ha molts residus de prolina (vegeu **figura 26**), fet poc inusual per a una proteïna. Aquest mateix grup comenta en l'article com a dades no publicades que havien fet servir l'extrem C-terminal en un assaig de doble híbrid i van observar que interaccionava amb ubiqüitina. Això els va fer suggerir que aquesta proteïna tindria una funció en la degradació de proteïnes en el lisosoma.

Hi ha algun gen homòleg? El mateix any, però uns mesos després va aparèixer publicat a *Journal of Biological Chemistry* pel grup de C. Cass (Hogue *et al.*, 1996) la identificació d'un gen que presenta un cert grau d'homologia amb LyCAT. Feien servir una soca del llevat *S. cerevisiae,* on van inhibir l'enzim timidilat-sintasa per metotrexat (amb la qual cosa no pot sintetitzar desoxitimidina-fosfat i no pot créixer), com una eina per trobar transportadors de nucleòsids: en transfectar-se amb una genoteca, únicament aquelles soques que poguessin transportar nucleòsids sobreviurien en un medi amb metotrexat. D'aquesta forma van aïllar un cDNA que oferia resistència al metotrexat. Sorprenentment, en sequenciar-lo van veure que es tractava d'una proteïna truncada sense uns 80 aminoàcids de l'extrem N-terminal. Finalment, van aïllar el cDNA complet; era una proteïna de 233 aminoàcids amb una predicció d'estructura de 4 dominis transmembrana, però, en expressar-lo en llevat veien que no rescatava el fenotip.

Quina era la raó? Van observar que la proteïna era present en membranes intracel·lulars enriquides en membranes del complex de Golgi, mitjançant fraccionament subcel·lular i detecció amb un anticòs. En estudiar la seqüència de la proteïna van construir delecions més petites de l'extrem N i C-terminal i van observar en expressar-les en oòcits de *Xenopus*, que la delecció de l'extrem C-terminal (33 aminoàcids) induïa transport de timidina a nivells més alts que la delecció de 80 aminoàcids trobada inicialment. En canvi, ni la proteïna salvatge (que van anomenar MTP, per *mouse transporter protein*) ni la deleció de l'extrem N-terminal (22 aminoàcids) induïen cap tipus de transport.

No van poder caracteritzar en detall l'activitat induïda pel constructe MTPΔC (MTP amb l'extrem C-terminal delecionat), ja que els oòcits que expressaven aquesta proteïna eren especialment fràgils, però el transport induït semblava altament selectiu per nucleòsids. Posteriorment, per estudis d'immunocitoquímica en cèl·lules transfectades s'ha confirmat que la proteïna es localitza en lisosomes i en endosomes tardans (Cabrita *et al.*, 1999).

Hi ha altres gens homòlegs en les bases de seqüències? Vaig trobar l'homòleg humà del gen MTP que s'anomenava KIAA0108 i un grup d'EST que presentava alta homologia amb MTP. Vaig demanar diferents clons a IMAGE però cap d'ells tenia l'extrem N-terminal complet. Fent servir el clon 546789 vaig cribellar una genoteca de cor (ja que aquest nou gen s'expressava en aquest teixit a nivells alts, vegeu més endavant) i amb això vaig poder aïllar el cDNA complet. Aquest nou gen, LyMAT, es va seqüenciar i en la **figura 30** presento un multialineament d'aquestes 3 proteïnes.

```
mMTP          MVSMTFKRSRS-DRFYSTRCCGCFHVRTGTIILGTWYMVVNLLMAILLTVEVTHPNSMPA 59
hMTP          MVSMSFKRNRS-DRFYSTRCCGCCHVRTGTIILGTWYMVVNLLMAILLTVEVTHPNSMPA 59
hLyMAT        -----MNMVAPWTRFYSNSCCLCCHVRTGTILLGVWYLIINAVVLLILLSALADPDQY-- 53
hLyCAT        MDPRLSTVRQT--------CC-CFNVRIATTALAIYHVIMSVLLFIEHSVEVAHG----- 46
mLyCAT        MASRAAPVRQT--------CC-CFNIRVATIALAIYHIVMSVLLFIEHVVEVARG----- 46

mMTP          VNIQYEVIGNYYSSERMAD-NACVLFAVSVLMFIISSMLVYGAISYQVGWLIPFFCYRLF 118
hMTP          VNIQYEVIGNYYSSERMAD-NACVLFAVSVLMFIISSMLVYGAISYQVGWLIPFFCYRLF 118
hLyMAT        NFSSSELGGDF---EFMDDANMCIAIAISLLMILICAMATYGAYKQRAAWIIPFFCYQIF 110
hLyCAT        KASCKLSQMGY---LRIAD--LISSFLLITMLFIISLSLLIGVVKNREKYLLPFLSLQIM 101
mLyCAT        KVSCRFFKMPY---LRMAD--LLSSFLLIGVLFIISISLLFGVVKNREKYLIPFLSLQIM 101

mMTP          DFVLSCLVAISSLTYLPRIKEYL---DQLP-DFPYK-----DDLLALDSSC--------- 160
hMTP          DFVLSCLVAISSLTYLPRIKEYL---DQLP-DFPYK-----DDLLALDSSC--------- 160
hLyMAT        DFALNMLVAITVLIYPNSIQEYI---RQLPPNFPYR-----DDVMSVNPTC--------- 153
hLyCAT        DYLLCLLTLLGSYIELPAYLKLASRSRASSSKFPLMTLQLLDFCLSILTLCSSYMEVPTY 161
mLyCAT        DFLLCLLTLLGSYIELPAYLKLA-RPRPGPSKVPLMTLQLLDFCLSILTLCSSYMEVPTY 160

mmtp          -------------------------LLFIVLVFFVVFIIFKAYLINCVWNCYKYINNRNV 195
hMTP          -------------------------LLFIVLVFFALFIIFKAYLINCVWNCYKYINNRNV 195
hLyMAT        -------------------------LVLIILLFISIILTFKGYLISCVWNCYRYINGRNS 188
hLyCAT        LNFKSMNHMNYLPSQEDMPHNQFIKMMIIFSIAFITVLIFKVVMFKCVWRCYRLIKCMNS 221
mLyCAT        LNFKSMNHMNYLPSQEGVPHSQFINMMLIFSVAFITVLILKVVMFKCVYTCYKFLKHMNS 220

mmtp          PEIAVYPAFETPPQYVLPTYEMA-VKIPEKEP-----PPPYLPA 233
hMTP          PEIAVYPAFEAPPQYVLPTYEMA-VKMPEKEP-----PPPYLPA 233
hLyMAT        SDVLVY-VTSNDTTVLLPPYDDATVNGAAKEP-----PPPYVSA 226
hLyCAT        VEEKRN--SKMLQKVVLPSYEEA-LSLPSKTPEGGPAPPPYSEV 262
mLyCAT        AMEDSS--SKMFLKVALPSYEEA-LSLPPKTPEGDPAPPPYSEV 261
```

Figura 30. Multialineament d'MTP humà i de ratolí, LyCAT humà i de ratolí i LyMAT humà.

Amb totes aquestes dades vaig plantejar la hipòtesi següent: LyCAT i LyMAT serien també transportadors intracel·lulars d'algun substrat, i l'extrem C-terminal seria el domini que determinaria la localització subcel·lular. En primer lloc vaig construir la proteïna LyCAT amb l'extrem C-terminal delecionat (LyCATΔC) i vaig posar un tag *myc* a l'extrem N-terminal, tant a LyCAT com a LyCATΔC. Vaig expressar aquests constructes en cèl·lules COS i en oòcits de *Xenopus*, i vaig visualitzar la localització subcel·lular mitjançant immunocitoquímica. En el cas dels oòcits vaig també coexpressar-los amb la proteïna 4F2hc, per veure si hi havia algun canvi en la localització subcel·lular, com succeïa en el cas de y^+LAT-1 (vegeu **figura 16**). En la **figura 31** i en la **figura 32** mostro el resultat d'aquests experiments.

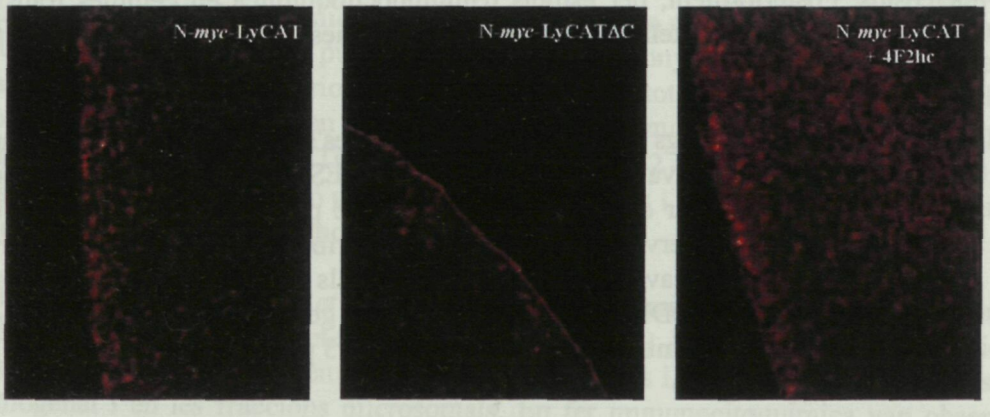

Figura 31. Immunocitoquímica de la proteïna N-*myc*-LyCAT i N-*myc*-LyCATΔC en oòcits de *Xenopus*. Efecte de l'expressió de 4F2hc sobre la localització subcel·lular de N-*myc*-LyCAT.

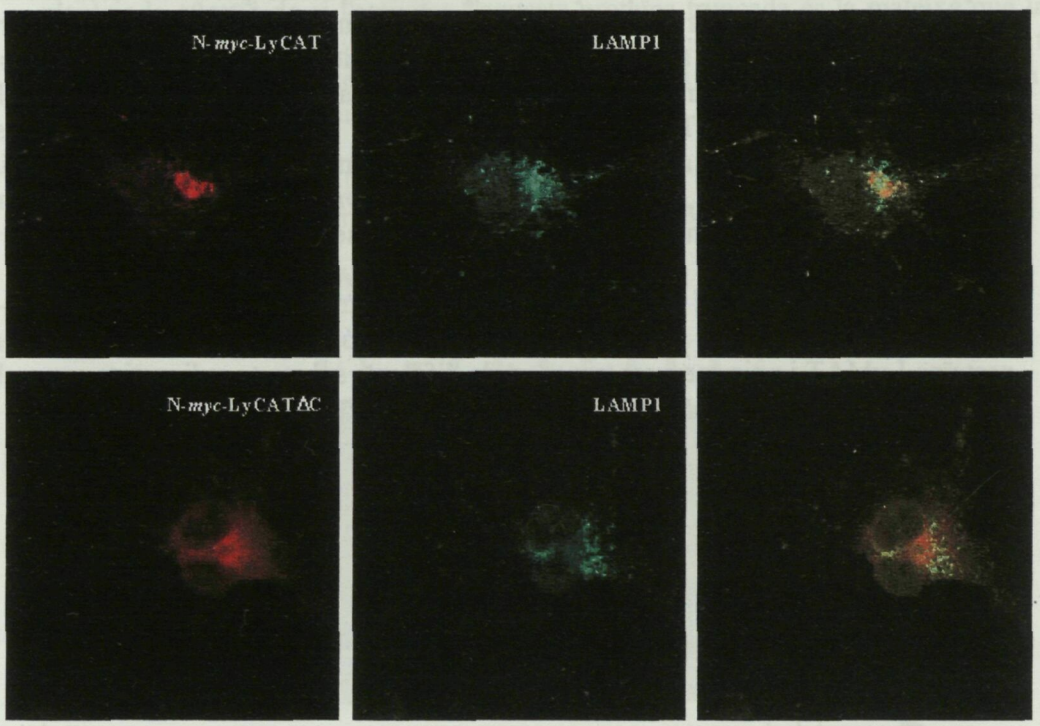

Figura 32. Immunocitoquímica de la proteïna N-*myc*-LyCAT i N-*myc*-LyCATΔC en cèl·lules COS transfectades. Col·localització amb LAMP-1. Efecte de la deleció C-terminal.

En expressar-la en oòcits (**Figura 31**) podem constatar que la proteïna presenta una localització intracel·lular. No podem afirmar que aquest marcatge correspongui als lisosomes de l'oòcit, ja que no disposem d'altres marcadors lisosomals. També veiem que en coexpressar-la amb la proteïna 4F2hc, a diferència del que passava amb y^+LAT-1, la proteïna N-*myc*-LyCAT no modifica la seva localització intracel·lular. Experiments de marcatge metabòlic i immunoprecipitació fent servir un anticòs dirigit contra 4F2hc mostren que no hi ha cap grau d'immunoprecipitació. Quin és el mecanisme pel qual LyCAT incrementa l'activitat y^+L? Més endavant intentaré donar una possible explicació.

Com es pot veure en la **figura 32**, la proteïna N-*myc*-LyCAT presenta una localització subcel·lular en el compartiment lisosomal, ja que es col·localitza completament amb la proteïna LAMP-1. Observem també que en delecionar l'extrem C-terminal la proteïna perd aquesta localitació, probablement queda en el reticle endoplasmàtic. Podem suggerir llavors que l'extrem C-terminal estaria implicat en la determinació de la localització subcel·lular de la proteïna LyCAT.

De forma molt important, en expressar en oòcits la proteïna N-*myc*-LyCATΔC, veiem que es localitza a la membrana plasmàtica. Com podem explicar aquest fet? Els oòcits són cèl·lules que es conserven a 17ºC on el control de qualitat del reticle no funciona de forma tan eficient. A més, estem expressant una gran quantitat de proteïna, cosa que pot saturar aquests mecanismes de retenció. Seria una situació similar a la descrita en l'apartat 1 de "Resultats i discussió" per als mutants d'rBAT M467T i M467K.

Disposem llavors d'una eina per poder expressar aquestes proteïnes a la membrana plasmàtica si suprimim una part de l'extrem C-terminal. En aquest domini apareixen dues senyals de tràfic cap a lisosomes: tirosina-X-X-hidrofòbic, concretament un senyal apareix en els 4 últims aminoàcids de la proteïna; també hi ha una quantitat alta de prolines, fet poc usual. Tots aquests residus estan altament conservats en els tres membres de la família aquí descrits. Desconeixem quin és el senyal implicat a determinar la localització: estudis com *alanine-scanning mutagenesis*, on es canvia cada residu per alanina, serien necessaris per trobar aquest senyal de localització.

Indueixen alguna activitat de transport aquestes proteïnes lisosomals en expressar-se en la membrana plasmàtica? Vaig fer la mateixa deleció en l'extrem C-terminal per a la proteïna LyMAT i vaig mesurar transport de diferents substrats radioactius per ambdues proteïnes (LyCATΔC i LyMATΔC) com es veu en la **figura 33.**

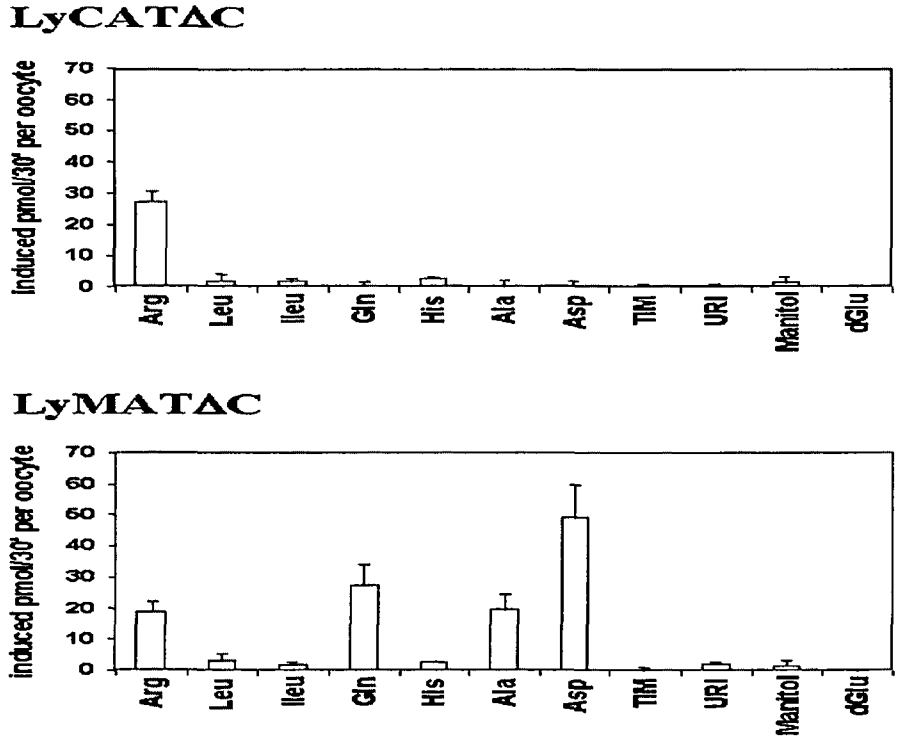

Figura 33. LyCATΔC i LyMATΔC indueixen diferents activitats de transport d'aminoàcids.

Podem observar que LyCATΔC indueix transport d'aminoàcids bàsics (arginina) i LyMATΔC indueix transport de diferents aminoàcids sense tenir en compte la càrrega. Podem assumir llavors que aquestes dues proteïnes funcionaran com a transportadors lisosomals d'aquests aminoàcids. D'aquest fet ve el nom d'aquestes dues proteïnes: LyCAT, de *lysosomal cationic amino acid transporter* i LyMAT, de *Lysosomal multiple amino acid transporter.*

Van voler caracteritzar més en detall el transport induït per LyCATΔC en els oòcits de *Xenopus.* Així, vam veure que presentava una afinitat molt alta per arginina; la K_m era de 13 μM. El transportador era molt específic per aminoàcids similars a l'arginina (evidenciat per experiments de competició amb altres aminoàcids bàsics). El transport era independent del pH, i per experiments d'acumulació veiem que acumula en els mateixos nivells que la proteïna MCAT-1 (sistema de transport de difusió facilitada).

Podem postular que la direcció de transport dependrà del gradient electroquímic. Es planteja la qüestió de si l'activitat d'aquesta proteïna truncada és la mateixa que la que tindria la proteïna nativa: totes les proteïnes que funcionen com a transportadors tenen els residus que determinen la selectivitat en dominis transmembrana o propers a ells, dominis que no s'han alterat en aquests constructes.

Quina és la funció fisiològica d'aquests dos gens?. En primer lloc vam analitzar-ne l'expressió mitjançant transferència Northern (**figura 34**).

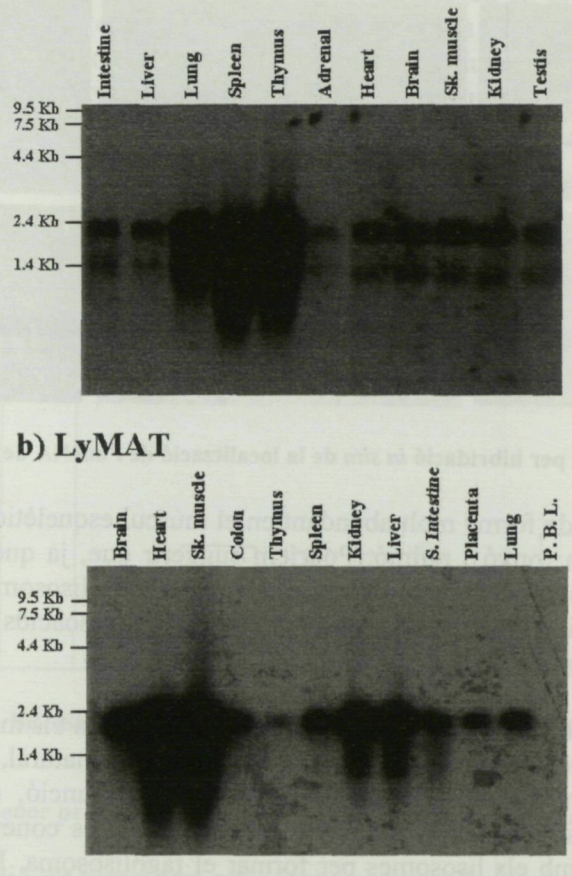

Figura 34. Anàlisi per transferència Northern de l'expressió tissular de LyCAT i LyMAT.

L'expressió de LyCAT és ubiqua però amb nivells més alts en melsa, pulmó i leucòcits. Aquest fet feia sospitar que l'expressió del gen estaria restringida a cèl·lules del sistema immune. Urs Berger, del grup del professor Hediger, va fer experiments d'hibridació *in situ* (**figura 35**). Tres dades experimentals apunten cap a aquesta idea: 1) els llocs d'expressió en els diferents teixits analitzats són patrons clars de cèl·lules immunes (**figura 35ª**, pulmó; **figura 35c,** timus; **figura 35b** i **35d,** controls amb la sonda amb sentit); 2) el marcatge en cervell s'incrementa en els lloc on s'ha produït una lesió amb quisquelat. (compareu **figura 35f** amb **figura 35e**); 3) el marcatge de LyCAT es col·localitza amb el marcatge d'M-CSF-R (*macrophage-colony stimulating factor-receptor*), un marcador típic dels macròfags (**figura 35g** amb **figura 35i**; **figura 35h** mostra la col·localització en melsa). Aquest fet suggeria que LyCAT s'expressava majoritàriament en cèl·lules del sistema immune, però especialment en els macròfags.

De fet, es va analitzar l'expressió en la línia cel·lular RAW 264.7 de macròfags i es van detectanr nivells alts d'expressió del missatger.

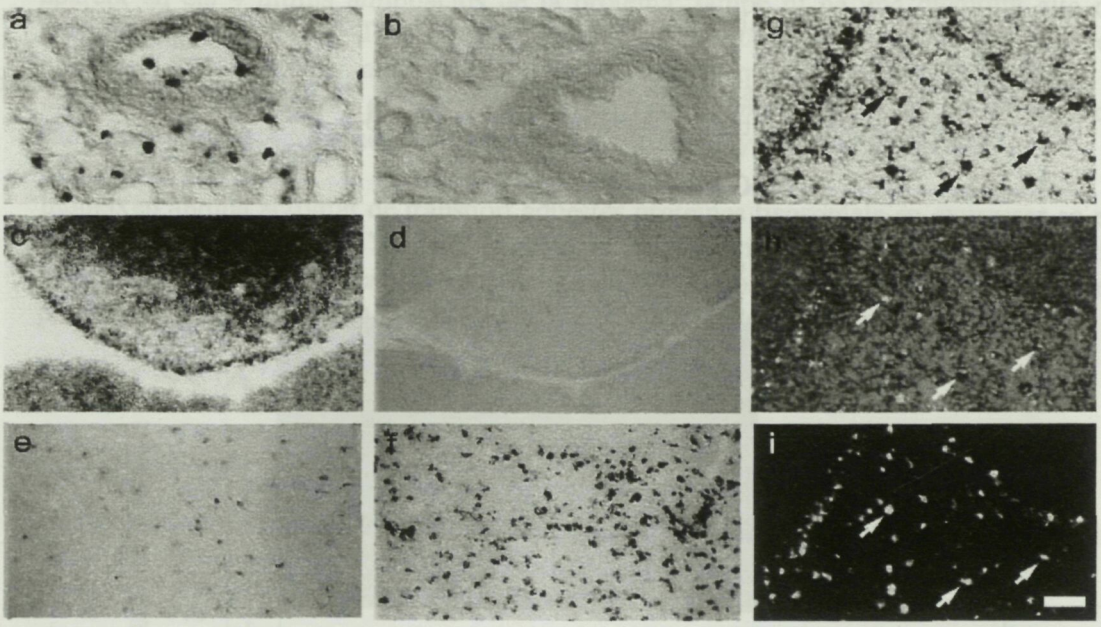

Figura 35. Anàlisi per hibridació *in situ* de la localització de l'mRNA de LyCAT.

LyMAT s'expressa de forma molt abundant en el múscul esquelètic i el cor, encara que també és present en ronyó i pulmó. Podríem suggerir que, ja que aquests teixits són teixits on la proteòlisi de proteïnes en el compartiment lisosomal és molt elevada, LyMAT podria tenir la funció de transportar aquests aminoàcids cap a l'exterior del compartiment lisosomal.

Quina seria la funció fisiològica del transportador LyCAT en els macròfags? Una de les funcions dels macròfags, en el context de la immunitat natural, és la destrucció de bacteris, virus, paràsits, etc. Per dur a terme aquesta funció, el primer pas és la fagocitosi d'aquests organismes extranys formant el que es coneix com fagosoma, el qual es fusionarà amb els lisosomes per formar el fagolisosoma. En aquesta estructura vesícular es produeix la destrucció del patògen, principalment per dos mecanismes: *a*) la producció de radicals derivats de l'oxigen (anió superòxid, radicals hidroxil, etc) generats a partir de l'acció de l'enzim NADPH-oxidasa sobre l'oxigen; *b*) la producció d'òxid nítric obtingut per l'acció de l'enzim òxid nítric-sintasa sobre l'L-arginina. Podríem hipotetitzar que la funció del transportador LyCAT seria la de regular les concentracions d'arginina en el compartiment lisosomal per mitjà del'enzim NO-sintasa.

Ja s'ha descrit que el transportador CAT2 (de la familia CAT, sistema y^+) està implicat en la introducció de l'arginina des de l'exterior de la cèl·lula per la síntesi de NO. Així, un ratolí *knockout* per CAT2 presenta una reducció dràstica dels nivells de NO produïts (Carol MacLeod, treball no publicat, Congrés de Colorado sobre transport d'aminoàcids). Encara no és clar en quin compartiment té lloc la síntesi de l'òxid nítric. Hi ha autors que diuen que la síntesi es produeix en el citosol, mentre que altres diuen

que l'enzim NO-sintasa estaria associat a unes vesícules de membrana que es fusionarien amb el compartiment lisosomal.

De totes formes, per provar aquesta hipòtesi vaig construir clons estables de la línia cel·lular de macròfags que expressaven establement una part de l'extrem 5' del gen en la orientació amb sentit i altres en la orientació sense sentit, per reduïr la concentració del gen endogen (vegeu "Materials i mètodes"). Amb quatre d'aquests clons *antisense* i dos *sense* vaig estudiar la producció d'òxid nítric davant d'estímuls que n'incrementen la seva producció, l'interferó γ (IFN-γ) i el lipopolisacàrid (LPS), de dues formes: mesurant la producció de nitrats i nitrits que apareixen en el medi de cultiu i mesurant per citometria de fluxe la fluorescència associada a la molècula DAF-2DA, que ens dóna una idea de la producció de NO intracel·lular durant una hora (vegeu "Materials i mètodes"). Vam observar una disminució de la producció de NO, com es mostra en la **figura 36.**

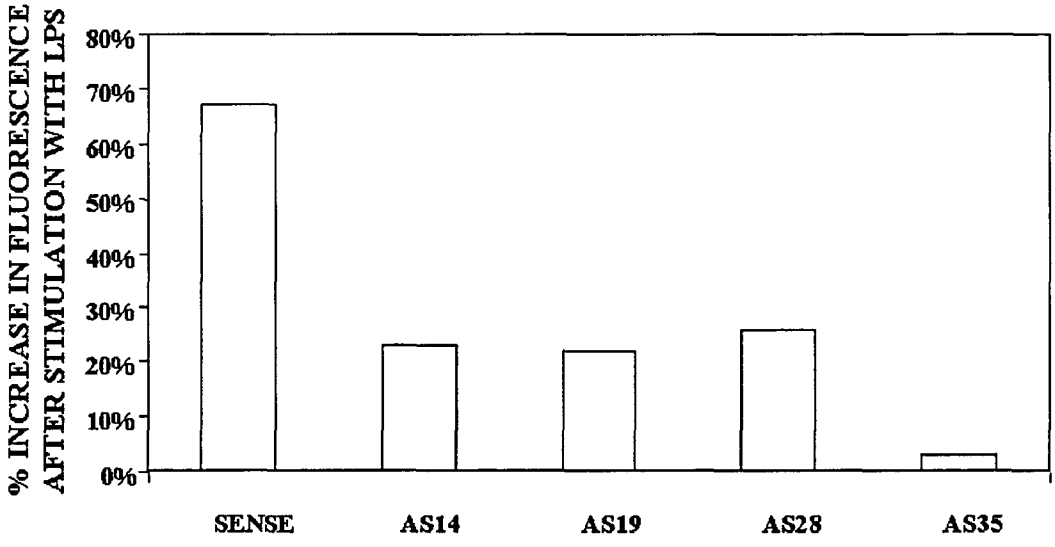

Figura 36. Menor producció de nitrats i nitrits en els macròfags *antisense* per LyCAT.

Durant la redacció d'aquesta tesi estic fent tres tipus d'experiments: 1) comprovar que la menor producció de NO no es deu a canvis en la síntesi o en l'activitat de l'enzim que produeix el NO (òxid nítric-sintasa induïble, iNOS); 2) demostrar que els clons *antisense* degraden en menor proporció microorganismes com ara la *leishmània major*, degradació que depèn sobretot de la producció de NO (així podríen donar un paper fisiològic a aquest transportador); 3) comprovar que els clons *antisense* presenten un defecte en el transport d'arginina en el compartiment fagolisosomal mitjançant la purificació d'aquests. 4) Determinar mitjançant la purificació de fagolisosomes o per immunocitoquímica de macròfags que estan fagocitant quina és la localització de l'enzim iNOS, informació que encara es desconeix.

Si aquests resultats fossin positius podrien plantejar un model en el que davant d'un estímul exterior el macròfag produiria òxid nítric amb la síntesi de l'enzim iNOS. Aquest NO es produiria de forma majoritària dins del fagolisosoma, que és el compartiment on té lloc la degradació dels agents exteriors. L'arginina, necessària per la síntesi de NO, s'obtindria desde l' exterior a través del transportador MCAT-2 (veure

"Introducció") i es transportaria cap al lisosoma a través del transportador LyCAT, el qual funcionaria en una direcció o un altre en funció del gradient de concentracions d'arginina.

Finalment, voldria proposar un mecanisme que expliqués com l'expressió de la proteïna LyCAT pot incrementar l'activitat y^+L induïda per 4F2hc. Com ja vaig explicar en l'apartat 2 de "Resultats i discussió", el transportador y^+L funciona com un intercanviador asimètric: permet la sortida d'aminoàcids bàsics mitjançant l'intercanvi amb aminoàcids neutres més sodi. A causa d'aquest mecanisme, una conseqüència que en podem extreure és que si no hi ha aminoàcids bàsics a l'interior el transportador no podrà funcionar.

Això és realment així; si es fa una preincubació amb leucina 1 mM més sodi durant 6 hores, i després es mesura el transport pel sistema y^+L, no s'observa cap inducció. Per tant, podem concloure que si modulem la concentració d'aminoàcids bàsics interna podem també modular el transportador y^+L. Podem hipotetitzar llavors que el transportador LyCAT estaria incrementaria la concentració d'aminoàcids bàsics assequible pel transportador, amb la qual cosa incrementaria la seva activitat. Aquest model està esquematitzat en la **figura 37**.

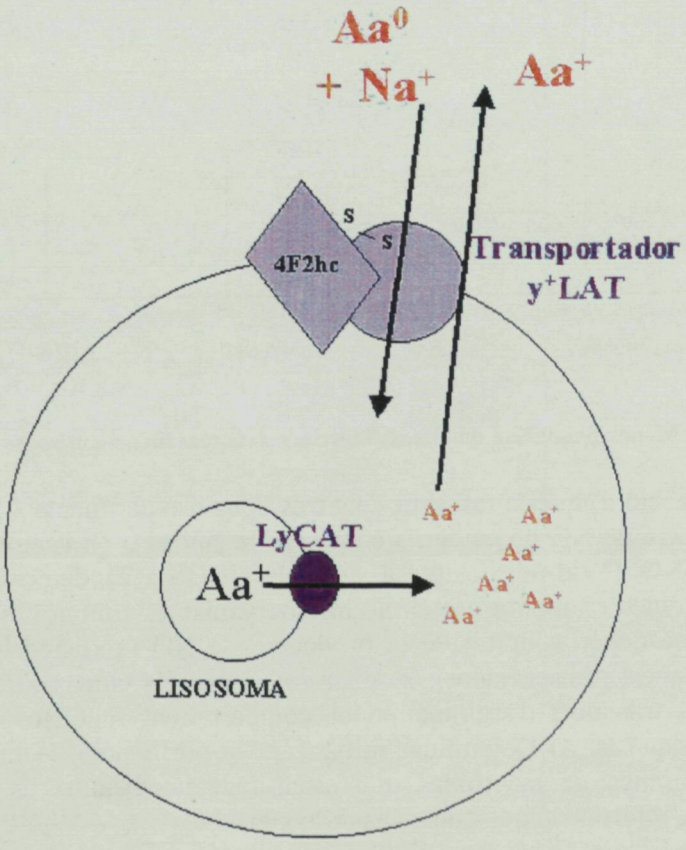

Figura 37. Model especulatiu que intenta explicar per què la coexpressió de LyCAT i 4F2hc incrementa l'activitat y^+L en oòcits de *Xenopus*.

Conclusions

CONCLUSIONS

1. Es reafirma la implicació del gen rBAT en la cistinúria de tipus I. Dues de les mutacions trobades en pacients cistinúrics, Met467Lys i Met467Thr, no són completament funcionals a causa d'un problema en el trànsit cap a la membrana plasmàtica.

2. El transportador $b^{0,+}$ funciona com un intercanviador obligatori d'aminoàcids amb estequiometria 1:1. En aquesta tesi, hem vist que el potencial de membrana i l'intercanvi amb aminoàcids neutres són les principals forçes que permeten acumular aminoàcids. Aquest mecanisme explica completament el fenotip de la cistinúria de tipus I. El transportador y^+L també funciona com un intercanviador d'aminoàcids, però de forma asimètrica ja que únicament permet la sortida d'aminoàcids bàsics, a causa probablement de les baixes concentracions de sodi intracel·lulars.

3. Els transportadors $b^{0,+}$ i y^+L formen part d'una gran família de transportadors heteromultimèrics constituïts per dues subunitats: una subunitat lleugera (LAT-1, LAT-2, ascAT, y^+LAT-1, y^+LAT-2, XCAT, $b^{0,+}$AT, etc) responsable de l'especificitat de substracte, i una subunitat pesada (4F2hc, rBAT), necessària per a l'expressió en superfície de la subunitat lleugera. Aquestes dues proteïnes interaccionen a través d'un pont disulfur entre 2 residus de cisteïna que estan altament conservats. A part d'aquesta interacció covalent, són necessàries altres interaccions entre altres dominis com ara l'extrem C-terminal d'rBAT. Així, rBAT determina propietats funcionals del transportador $b^{0,+}$.

4. La mutació L334R trobada en un pacient espanyol amb LPI i la mutació V170M trobada en pacients joeus amb cistinúria de tipus no-I, provoquen un defecte en la funció de la proteïna y^+LAT-1 i $b^{0,+}$AT, respectivament

5. S'ha identificat una nova família de transportadors lisosomals formada per 3 membres, MTP, LyCAT i LyMAT. LyCAT funciona com un transportador lisosomal d'aminoàcids bàsics, mentre que LyMAT funciona com un transportador lisosomal de múltiples aminoàcids. El domini C-terminal d'aquestes proteïnes en determina la localització subcel·lular. Estudis posteriors permetran implicar el transportador lisosomal LyCAT en la regulació de la síntesi de NO en macròfags, com permeten suggerir les dades obtingudes en la present tesi.

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Articles

Treball 1

An intracellular trafficking defect in type I cystinuria mutants M467T and M467K.

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An Intracellular Trafficking Defect in Type I Cystinuria rBAT Mutants M467T and M467K*

(Received for publication, May 29, 1996, and in revised form, January 22, 1997)

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The human rBAT protein elicits sodium-independent, high affinity obligatory exchange of cystine, dibasic amino acids, and some neutral amino acids in *Xenopus* oocytes (Chillarón, J., Estévez, R., Mora, C., Wagner, C. A., Suessbrich, H., Lang, F., Gelpí, J. L., Testar, X., Busch, A. E., Zorzano, A., and Palacín, M. (1996) *J. Biol. Chem.* 271, 17761–17770). Mutations in rBAT have been found to cause cystinuria (Calonge, M. J., Gasparini, P., Chillarón, J., Chillón, M., Galluci, M., Rousaud, 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). We have performed functional studies with the most common point mutation, M467T, and its relative, M467K, using the oocyte system. The K_m and the voltage dependence for transport of the different substrates were the same in both M467T and wild type-injected oocytes. However, the time course of transport was delayed in the M467T mutant: maximal activity was accomplished 3–4 days later than in the wild type. This delay was cRNA dose-dependent: at cRNA levels below 0.5 ng the M467T failed to achieve the wild type transport level. The M467K mutant displayed a normal K_m, but the V_{max} was between 5 and 35% of the wild type. The amount of rBAT protein was similar in normal and mutant-injected oocytes. In contrast to the wild type, the mutant proteins remained endoglycosidase H-sensitive, suggesting a longer residence time in the endoplasmic reticulum. We quantified the amount of rBAT protein in the plasma membrane by surface labeling with biotin 2 and 6 days after injection. Most of the M467T and M467K protein was located in an intracellular compartment. The converse situation was found in the wild type. Despite the low amount of M467T protein reaching the plasma membrane, the transport activity at 6 days was the same as in the wild type-injected oocytes. The increase in plasma membrane rBAT protein between 2 and 6 days was completely dissociated from the rise in transport activity. These data indicate impaired maturation and transport to the plasma membrane of the M467T and M467K mutant, and suggest that rBAT alone is unable to support the transport function.

The heterologous expression of rBAT in *Xenopus* oocytes elicits the sodium-independent, high affinity transport of cystine, dibasic amino acids, and some neutral amino acids ($b^{0,+}$-like activity) (1–3) by an obligatory exchange mechanism that accounts for the accumulation of such substrates in the oocytes (4, 5). The rBAT protein is located in the brush border of the epithelial cells of the proximal straight tubule and the small intestine (6, 7). Very recently, our group has demonstrated that rBAT is expressed in the "proximal tubule" cell line OK. An antisense sequence against rBAT abolishes *in vivo* the $b^{0,+}$-like activity expressed apically in these cells (8). All these data indicate a role of rBAT in cystinuria. This common inheritable disorder is due to the defective transport of cystine and dibasic amino acids through the epithelial cells of the renal tubule and the intestinal tract (9–11): the low solubility of cystine leads to the development of cystine calculi in the kidney. Three types of classic cystinuria have been described, but rBAT is responsible only for type I, as has been demonstrated by mutational and genetic analysis (12, 13).

Type I individuals who inherit one mutant rBAT gene are completely normal and do not hyperexcrete cystine or dibasic amino acids. Only the homozygotes for mutant rBAT genes display the above mentioned phenotype. Several mutations of rBAT have been described (12, 14–16). Among them, the most common point mutation is M467T, found in 26% of type I cystinuria chromosomes so far analyzed. This is also the only mutation that has been found in homozygosis in one Spanish family (12). Its relative, M467K, has been found as a compound heterozogous with the L678P mutant in one Italian patient (14). The methionine at position 467 is completely conserved among all the species in which rBAT sequences are known (1–3, 8). In the 4-transmembrane domain model proposed by Tate and co-workers (17) this residue lies in the third transmembrane domain, very close to the cytosol. There is little information regarding the dysfunctions provoked by rBAT mutations. Calonge and co-workers (12) reported preliminary experiments on the M467T mutant, showing a decrease in transport activity. Miyamoto and co-workers (18) obtained similar results with the E268K and T341A mutants. However, they do not provide any explanation for the reduced function.

The exact role of rBAT in the amino acid transport of system $b^{0,+}$-like is unknown. This is largely due to its unusual topology. Experimental evidence obtained using site-directed antibodies suggests a 4-transmembrane domain model for rBAT (17). This contrasts with common metabolite transporters, which appear to contain 8–12 transmembrane domains (19). Thus, it has been suggested that rBAT is only part of the

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functional transport unit (2, 3) rBAT is expressed in kidney and intestine as a 94-kDa protein in reducing conditions and as a 125-kDa complex in nonreducing conditions, suggesting a disulfide-linked heterodimer with another protein of 35–50 kDa (20, 21) This complex has also been detected in total membranes of oocytes injected with rBAT cRNA (20)

Here, we report the molecular basis for the defect seen in the M467T mutation and its relative M467K There is no significant difference in the functional properties of the M467T mutant compared with the wild type, but the M467K mutant shows a clear V_{max} defect The two mutants are expressed only as an endo H[1]-sensitive band that remains mostly inside the cell The lack of correlation between the rBAT protein in the plasma membrane and the expressed transport activity suggests that rBAT is necessary but not sufficient for the amino acid transport activity of system $b^{0,+}$-like

EXPERIMENTAL PROCEDURES

Uptake Experiments and Electrophysiological Studies—Oocyte management, injections, uptake measurements, and electrophysiological studies were as described elsewhere (1, 22) *Xenopus laevis* (H Kahler, Institut fur Entwicklungsbiologie, Hamburg, Germany) oocytes were defolliculated by collagenase (Boehringer Mannheim) treatment Only healthy looking stage VI oocytes were used The uptake of the labeled amino acids (DuPont NEN) was measured in 7–8 oocytes per individual data point at 25 °C for 5 min when initial rates were measured or for 3 h for accumulation experiments The data are expressed as the difference between the uptake in rBAT-injected oocytes and the uptake in uninjected oocytes (induced uptake) Uptake values in uninjected oocytes are the same as in water-injected oocytes For electrophysiological measurements the oocytes were each injected with 1 ng of cRNA, and two electrode voltage and current clamp recordings were performed 3–8 days later Recordings were performed at 22 °C using a Geneclamp amplifier (Axon Instruments, Foster City, CA) and MacLab D/A converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia) Amino acid induced voltage changes or currents were filtered at 10 Hz Experiments were repeated with two batches of oocytes, in all repetitions, qualitatively similar data were obtained 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 The holding potential was −50 mV, but this was varied in one set of experiments to analyze the voltage dependence of amino acid induced currents The solution flow was adjusted to 20 ml/min, which guaranteed a complete solution exchange in the recording chamber within 10–15 s The maximal current amplitude induced during amino acid superfusion was measured Inward currents are shown with the prefix − All data are given as means ± S E where n is the number of oocyte measurements

Site directed Mutagenesis, cRNA Synthesis, and Injection—The construction of the M467T mutant in pSPORT 1 has been described elsewhere (12) For the construction of the M467K mutant we used the general procedure of Kunkel and co-workers (23) with additional steps described by Yan and Maloney (24) The mutagenic oligonucleotide was 5 -AAGC[T]TGTTCATCACGTT-3 (antisense strand, the mutated nucleotide (position 1400, Ref 1) is indicated between brackets) Mutants were identified by their acquisition of an *Alu*I restriction site (12), and a cassette between *Nco*I and *Bst*EII sites was completely sequenced Then, this cassette was ligated into pGEM4Z containing the cDNA from human rBAT that had been cut with the same enzymes Finally, the whole cassette was sequenced The three plasmids were isolated using a miniprep kit (Promega), linearized by *Xba*I restriction endonuclease digestion, and transcribed *in vitro* using T7 RNA polymerase (Promega) for the wild type and the M467T mutant and SP6 RNA polymerase (Pharmacia Biotech Inc) for the M467K in the presence of ^{7}mGpppG (NEB) as described elsewhere (1) Oocytes were injected (Inject+Matic-System, J A Gabay, Geneva, Switzerland) with 50 nl of cRNA at the concentrations given in the figures

Kidney Brush Border and Oocyte Membranes—Brush border membranes from rat kidney cortex were obtained as described (6) Total oocyte membranes were purified as described elsewhere (25) Briefly 30–50 oocytes were homogenized in 10 μl/oocyte buffer A (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7 5, plus 5 μg/ml leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) by 20 strokes of an Eppendorf Teflon-glass homogenizer The homogenate was centrifuged twice at 1,000 × g for 10 min at 4 °C to eliminate the yolk, and the supernatant was pelleted (100,000 × g, 90 min, 4 °C) and resuspended in 2 μl/oocyte buffer A Aliquots were used to quantify proteins by the method of Bradford (26) The membranes were stored at −20 °C until use

Endoglycosidase H Treatment—50–100 μg of total membranes or rat kidney brush borders was denatured by boiling for 5 min in the presence of 100 mM DTT and 0 54% SDS, and incubated in a NaP_i buffer (50 mM NaP_i, pH 5 5, 0 36% SDS, 0 5 mM phenylmethylsulfonyl fluoride) 10 milliunits of endo H (Boehringer Mannheim) was added The mixture was incubated for 18 h at 37 °C, and the reaction was stopped by the addition of Laemmli sample buffer (27) with DTT to 100 mM Samples were boiled for 5 min and stored at −20 °C until use

Surface Biotin Labeling and SDS PAGE—The procedure for biotin labeling of oocytes was adapted from that described in Ref 28 50–75 oocytes, which were injected with wild type or M467T rBAT cRNA, were washed five times in OR-2 medium (82 5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES adjusted to pH 7 5 with NaOH) and then placed into an agarose coated Petri dish filled with 2 ml of OR-2 medium The fresh membrane-impermeant reagent NHS LC-biotin stock (2 mg/500 μl of OR 2) was carefully added to the dish After 10 min at room temperature the reaction was stopped by adding 1 ml of 500 mM glycine, pH 7 4 The oocytes were then washed three times in 500 mM glycine, pH 7 4, followed by two washes in OR-2 The oocytes were then transferred to a microcentrifuge tube, and 0 5 ml of lysis buffer (2% Nonidet P-40, 150 mM NaCl, 2 mM $CaCl_2$, 20 mM Tris, pH 7 4, plus 2 μM leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) was added The oocytes were passed 20 times through a 200-μl pipette, and the homogenate was centrifuged twice (1,000 × g, 10 min, 4 °C) to remove the yolk The supernatant was sonicated for 1 min and then centrifuged again at 1,000 × g (10 min, 4 °C) The supernatant was dialyzed overnight at 4 °C against streptavidin buffer (SAv buffer) (0 3% Nonidet P 40, 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris, pH 8, plus 2 μM leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) and then centrifuged for 30 min at 14,000 rpm at 4 °C to remove insoluble material The supernatant was incubated overnight with 75 μl of streptavidin-agarose bead suspension (previously washed three times in SAv buffer) Supernatant and pellet were separated by low speed (2,000 rpm) centrifugation The supernatant was precipitated with 5% trichloroacetic acid and resuspended in 150 μl of Laemmli sample buffer One-half was separated, and DTT was added to 100 mM Then the two halves (reduced and nonreduced) were boiled for 5 min and stored The pellets were eluted by adding 100 μl of Laemmli sample buffer without DTT and boiling for 5 min Then one-half was separated, and DTT was added to 100 mM Finally, the two halves were boiled once again and stored

SDS-PAGE was performed on total membrane proteins (endo H-treated or not) and the supernatants and pellets from the biotin-labeling experiments according to Laemmli (27) For rBAT Western blotting, reduced samples were transferred to Immobilon (Millipore) Following the transfer the filters were blocked with 5% non-fat dry milk and 0 02% sodium azide in phosphate-buffered saline for 1 h at 37 °C and incubated with the polyclonal antibody anti rBAT MANRX (6) at 1 100 dilution in 1% non-fat dry milk and 0 004% sodium azide in phosphate-buffered saline overnight at room temperature Detection of the immune complex was accomplished using ^{125}I-protein A (ICN) For β1-integrin Western blotting, nonreduced samples were transferred as above, blocked in 10% non-fat dry milk and 0 05% Tween-20 in phosphate-buffered saline for 30 min at 37 °C, and incubated with the monoclonal antibody 8C8 (29) at 1 10 dilution in the blocking solution overnight at room temperature Antibody binding was detected using the ECL Western blot detection system (Amersham Corp) Blots were quantified by scanning densitometry Immunoblots were performed under conditions in which autoradiography detection was in the linear response range All electrophoresis reagents were obtained from Bio-Rad NHS-LC-biotin was from Pierce, and streptavidin-agarose beads were from Sigma

RESULTS

We introduced the naturally occurring mutations M467T and M467K in the rBAT cDNA and, upon transcription *in vitro*, injected them into *Xenopus* oocytes As illustrated in Fig 1, L-Arg-induced transport was clearly reduced in both M467T

[1] The abbreviations used are endo H, endoglycosidase H, DTT, dithiothreitol, PAGE, polyacrylamide gel electrophoresis, ER, endoplasmic reticulum

and M467K compared with the wild type at 1.5 days after the injection. For 2.5 ng the uptake values were 21.4 ± 1.6 pmol/5 min/oocyte for the wild type group, 4.3 ± 1 for the M467T, and 2.3 ± 0.4 for the M467K; for 0.5 ng the values were 12.2 ± 0.9, 2.6 ± 0.8, and 0.7 ± 0.4; and for 0.05 ng the values were 4.4 ± 0.6, 0.1 ± 0.4, and 0 ± 0.4, respectively. Surprisingly, 7 days after the injection of 2.5 ng, the M467T achieved the same level of expression as the wild type (47.7 ± 4 pmol/5 min/wild type oocyte *versus* 42.2 ± 0.6 pmol/5 min/M467T oocyte). This recovery was dose-dependent since it was not observed at the lower doses (72% recovery at 0.5 ng and only 40% at 0.05 ng), suggesting that at physiological levels of rBAT mRNA there is no recovery (Fig. 1). The M467K-induced uptake was also time- and cRNA dose-dependent: at 0.05 ng the uptake was undetectable 1.5 days after the injection and was 13% of the wild type at 7 days; at 2.5 ng the uptake rose from 11 to 25% of wild type values. In other experiments the uptake induced in the M467K-injected oocytes was between 5 and 35% of control values. Similar results were obtained with current measurements (data not shown). Attempts to determine transport over a longer period failed because of the increased oocyte mortality, so we cannot preclude the possibility that the M467K (or the M467T at low cRNA doses) could reach the wild type uptake values. We also performed experiments in which 25 ng was injected and obtained similar results (see Fig. 3).

To assess whether the decrease in function was due at least in part to changes in the K_m for the different substrates we performed kinetic analysis of transport. The induced uptake was studied both by tracer measurements and by electrophysiological techniques: 3–4 days after injection by the former method and 8 days after injection by the latter (Table I). The wild type and the M467T were indistinguishable after 8 days of expression with regard to their similar K_m (in the 100 μM range, as reported in Refs. 1, 3, 22, and 30) and V_{max} for both L-Arg and L-Leu. Only after a shorter time of expression, *i.e.* 3–4 days, was a V_{max} decrease found for the three substrates used (65% for L-cystine, 54% for L-Leu, and 59% for L-Arg). The difference in the K_m of L-Arg and L-Leu observed only in the electric measurements could be due to the voltage clamp of the oocytes at −50 mV; more positive voltages led to a reduction of the L-Leu K_m without changing the L-Arg K_m (22). At day 6 and with 20 ng of cRNA injected, the M467K also showed a V_{max} decrease (65% for L-Arg) without any change in the K_m (27 ± 6 μM for the M467K and 40 ± 7 μM for the wild type). Kinetic analysis of the M467K mutant by electric measurements was also attempted, but the low induced currents precluded accurate estimations of the K_m and V_{max}.

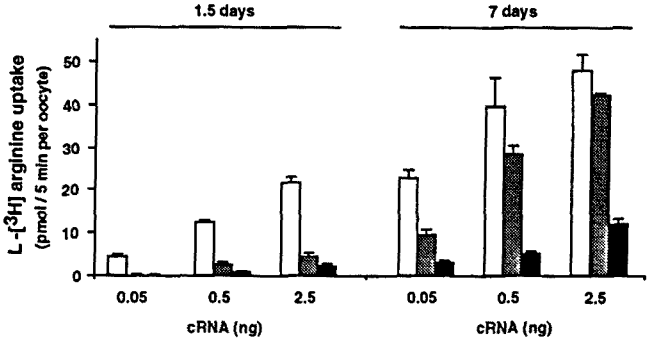

FIG. 1. **Time and cRNA dose dependence of the induced amino acid transport in wild type and mutant-injected oocytes.** Oocytes were injected with the indicated doses of cRNA of the wild type rBAT (*open bars*), M467T (*shaded bars*), or M467K (*closed bars*). The uptake of 50 mM L-Arg was determined for 5 min 1.5 and 7 days after the injection. Each bar is the mean ± S.E. of the induced uptake values measured in 6–8 oocytes. Another independent experiment gave similar results.

rBAT mediates an obligatory exchange of amino acids; no transport of amino acids occurs in the absence of amino acids on any side of the cell. This exchange accounts for accumulation of the substrates in the oocyte and it fully explains the rBAT amino acid-induced currents (4, 5). The L-Arg-induced currents measure the L-Arg inward-L-neutral amino acid outward mode of exchange, and the L-Leu-induced currents measure the reverse mode. Voltage modification leads to changes in these currents, reflecting, at least in part, changes in the modes of exchange; for instance, hyperpolarization reduces L-Leu-induced currents and increases L-Arg-induced currents. Therefore, we examined the possibility of a defect in the exchange by assaying the voltage dependence of L-Arg- and L-Leu-induced currents at day 8 after injection; as shown in Fig. 2 there was no difference between wild type and M467T-injected oocytes. The efflux of amino acids from the oocyte in the absence of amino acids in the extracellular medium was undetectable in either wild type or M467T-injected oocytes, as expected from an obligatory exchange (data not shown). Lastly, we examined the accumulation level of amino acids in both wild type and M467T-injected oocytes, and no differences were found (data not shown). In all, the data indicate no functional difference in the M467T mutant at high cRNA doses but a clear V_{max} decrease in the M467K.

Given that the M467T-injected oocytes displayed no functional defect despite the clear delay in the expression of transport, we next examined rBAT protein expression in the oocytes using the previously characterized polyclonal antibody MANRX in Western blot experiments (6). We injected a large dose of cRNA (25 ng) to ensure high levels of protein expression and measured uptake (Fig. 3*A*) and protein present in total membranes (Fig. 3*B*). The wild type protein appeared as a doublet, with one band of 94 kDa (band I) and one of higher mobility (85 kDa, band II). In contrast, only band II was present in the M467T mutant (Fig. 3*B*). This was also the case for

TABLE I

Kinetic parameters of the wild type (wt)- and M467T (MT)-induced amino acid transport activity

Kinetic parameters for the uninduced activity of transport of L-Arg, L-Leu, and L-cystine are given. Oocytes were prepared and injected with 2.5 ng of cRNA. K_m values are given in μM. V_{max} values are given in pmol/5 min/oocyte and in nA for tracer (3–4 days after injection) and electric (8 days after injection) measurements, respectively. Data (mean ± S.E.) correspond to representative experiments in which induced transport was measured varying the substrate concentration between 10 and 250 μM. 7–8 oocytes per triplicate were used for each data point. Different batches of oocytes from two different laboratories were used for the tracer and the electrophysiological studies. Therefore, the K_m values obtained are not comparable between the tracer and the electrophysiological studies.

J		3–4 days		8 days	
		wt	MT	wt	MT
L-Cystine	V_{max}	9.2 ± 0.6	3.2 ± 0.4	nd	nd
	K_m	43 ± 6	40 ± 10	nd	nd
L-Arginine	V_{max}	196 ± 16	81 ± 6	170 ± 4	181 ± 4
	K_m	93 ± 13	51 ± 8	30 ± 2	27 ± 2
L-Leucine	V_{max}	54 ± 4	24 ± 2	139 ± 3	133 ± 5
	K_m	78 ± 9	88 ± 14	213 ± 20	151 ± 24

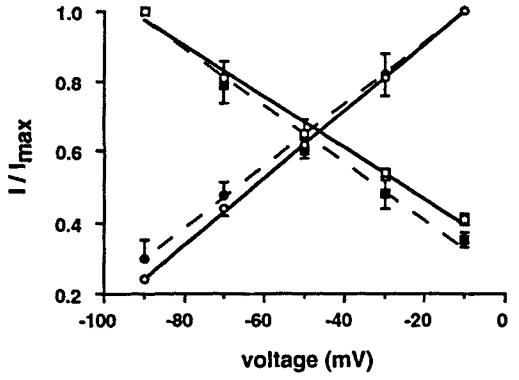

FIG. 2. **Voltage dependence of the wild type- and M467T rBAT-induced currents.** Currents were induced by superfusing wild type (*solid lines, open symbols*)- and M467T (*dashed lines, closed symbols*)-injected oocytes at the given holding potentials between −90 and −10 mV with L-Leu (1 mM, *circles*) or L-Arg (0.1 mM, *squares*) for 30 s. For the graph the currents were normalized against the maximal induced currents in each current-voltage experiment. The L-Leu- and L-Arg-induced currents were always the largest at −10 and −90 mV, respectively. When not visible, error bars are smaller than symbols.

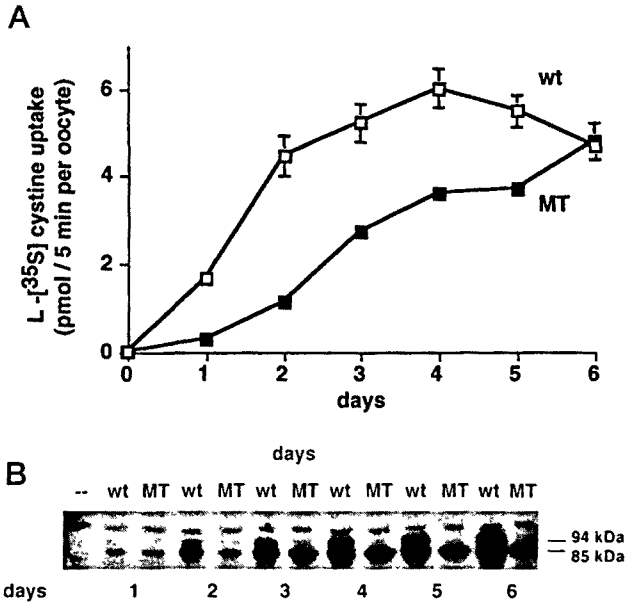

FIG. 3. **Time course of the induced amino acid transport and of the rBAT protein content in total membranes from wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng of cRNA. *A*, the induced uptake of 50 mM L-cystine was measured every day until day 6. Data are the mean ± S.E. of 6–8 oocytes. L-Cystine uptake in uninjected oocytes was 0.83 ± 0.08 pmol/5 min/oocyte at day 1 and 0.81 ± 0.05 pmol/5 min/oocyte at day 6. When not visible, error bars are smaller than symbols. *B*, total membrane proteins of oocytes were collected as described under "Experimental Procedures." 50 μg of reduced samples were run in a 7.5% PAGE system, transferred, incubated with MANRX polyclonal antibody against rBAT, and revealed with ^{125}I-protein A. Notice that the lower mobility band seen in the lanes also appeared in uninjected oocytes. Therefore, it is considered unspecific. --, uninjected oocytes.

the M467K (see Fig. 4). Quantification of the bands obtained in four experiments (data not shown) demonstrated that the total amount of wild type protein compared with the M467T was not significantly different at any day after injection and increased continuously until day 6 without any apparent saturation, despite uptake saturation. The amount of M467K protein was also similar to that of the wild type (data not shown). Electric measurements of the time course gave similar results. Thus, the different profiles of uptake observed for the wild type and M467T cannot be explained by the amount of rBAT protein present in total membranes.

From the Western blot in Fig. 3*B* it appeared that on the day after the injection only band II was detected in wild type-injected oocytes, suggesting that it could be a precursor of band I, most likely a non-fully glycosylated form of the protein. In fact, it is known from other membrane proteins, such as the cystic fibrosis transmembrane conductance regulator (31), low density lipoprotein receptor (32), sucrase-isomaltase (33), α_{IIb} integrin (34), and others, that some of their naturally occurring mutations produce only the endo H-sensitive form of the protein. This indicates intracellular accumulation of the mutant protein (probably in the ER) and failure to reach its normal location. The results of endo H digestions performed with total membranes of wild type-, M467T-, and M467K-injected oocytes are depicted in Fig. 4. Band II (85 kDa), the only one present in M467T and M467K, and the high mobility band of the wild type were endo H-sensitive, producing a new band of 71 kDa. This size agrees with the 72 kDa reported for the rBAT-expressing tunicamycin-treated oocytes (1). Band I shifted to 92 kDa when digested with endo H. rBAT from renal brush borders showed a similar shift, suggesting a similar glycosylation pattern for mature rBAT in oocytes and proximal tubule cells. The failure to detect band I in the M467T mutant suggests that the acquisition of the endo H-resistant condition is not necessary to its activity. This has also been demonstrated for other proteins expressed in oocytes, such as the *Torpedo* acetylcholine receptor γ and δ subunits (35) and glycophorin A (36).

The endo H sensitivity of band II suggested an intracellular location. To examine this hypothesis we performed surface labeling of intact oocytes with the membrane-impermeant reagent NHS-LC-biotin. We also attempted to determine whether the time course of the uptake correlated with the amount of wild type and M467T protein in the plasma membrane. Biotin labeling in oocytes has been successfully carried out in several studies (28, 37, 38). We opted for the method described by Müller and co-workers in Ref. 28 because it provided us with β1-integrin as an internal control for our biotinylation experiments. β1-integrin of *Xenopus* oocytes is expressed as two species of different molecular mass: one of 100 kDa (the pre-β1 form), endo H-sensitive, not biotin-labeled, and presumed to be localized to the cortical ER; and one of 115 kDa (the mature β1), endo H-resistant, biotin-labeled, and localized to the plasma membrane (28). Fig. 5 shows a representative biotin-labeling experiment of oocytes expressing wild type or M467T rBAT 2 or 6 days after the injection of 25 ng of cRNA. Labeled oocytes were lysed, and the biotinylated proteins were isolated by adsorption to streptavidin-agarose beads (see "Experimental Procedures") and probed for the presence of β1-integrin and rBAT. As expected, only the mature β1-integrin was biotinylated. In these conditions, most of the M467T protein remained in an intracellular compartment, whereas most of the wild type rBAT protein reached the oocyte surface. Densitometric measurements from three independent experiments showed that, at day 2 and day 6 in M467T-injected oocytes, 88% of the rBAT protein was in the supernatant. This value was only 35% in wild type-injected oocytes. As for the M467T, the M467K protein remained mostly intracellular, and only very small amounts reached the oocyte surface (data not shown). For the wild type, band II was detected in the intracellular fraction, and its presence in the plasma membrane was scarce; in some experiments it was not detectable, as shown in Fig. 5. In contrast, band I appeared at the plasma membrane, and its presence in the internal membranes was hardly detectable, suggesting an efficient biotin labeling. In agreement with the data obtained with total oocyte membranes (Figs. 3*B* and 4), band I was never observed in M467T-injected oocytes, even in the

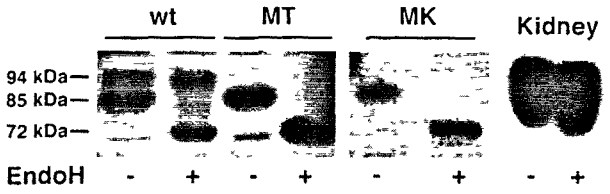

FIG. 4. **Sensitivity of wild type (*wt*), M467T (*MT*), and M467K (*MK*) rBAT proteins to the treatment with endo H.** Oocytes were injected with 25 ng of cRNA. Four days later, total membrane proteins were obtained. 50–100 μg of membrane proteins were digested or not with endo H and immunoblotted with MANRX. An equivalent amount of protein was loaded in each lane, except in kidney lanes, where rat kidney brush border membranes were loaded as a control. Another experiment was performed that gave the same results. The high mobility band seen below 72 kDa in the wild type and mutant lanes is not specific, since it also appeared in uninjected oocytes (not shown).

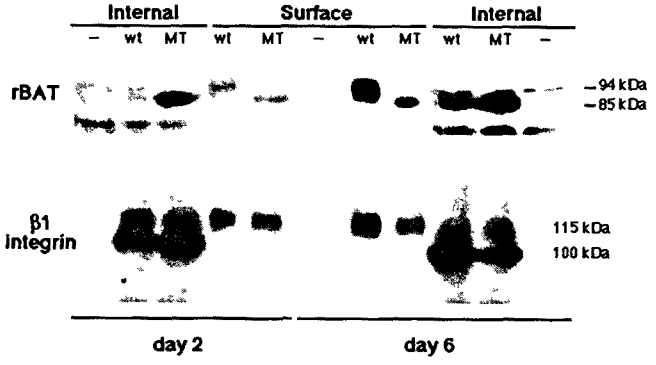

FIG. 5. **Biotin labeling of wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng of cRNA. Biotinylation was performed 2 and 6 days later as described under "Experimental Procedures." A representative experiment is shown. Times of exposure for 2 and 6 days are different (lower for 6 days). In the rBAT panel reduced samples were subjected to SDS-PAGE, probed with MANRX polyclonal antibody against rBAT, and revealed with ^{125}I-protein A. An equivalent of 16.5 oocytes was loaded in the precipitates (*Surface*), and 5 oocytes were loaded in the supernatants (*Internal*). The bands detected above and below the 85-kDa band in the internal fraction of the wild type and mutant lanes at days 2 and 6 are not specific, since they also appeared in uninjected oocytes (—). In the β1-integrin panel nonreduced samples were subjected to SDS-PAGE, probed with 8C8 monoclonal antibody against the oocyte β1-integrin, and revealed with the ECL system (Amersham Corp.) using an anti-mouse IgG coupled to peroxidase. An equivalent of 5 oocytes was loaded both in precipitates and supernatants. (—, uninjected oocytes).

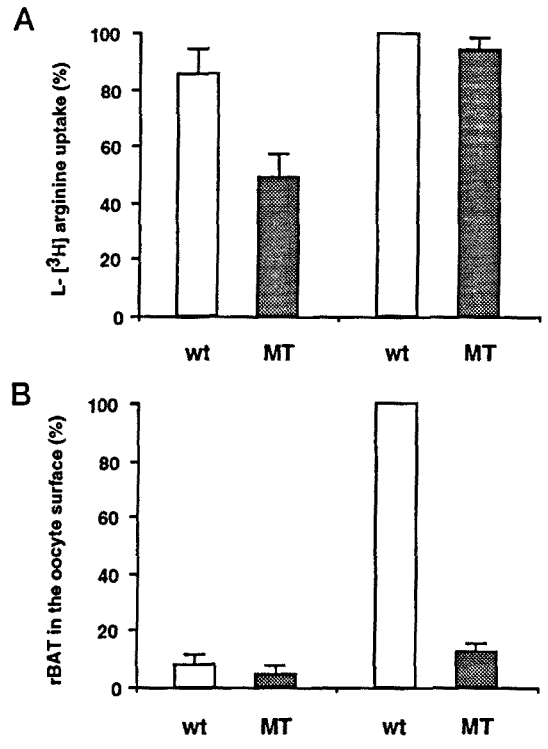

FIG. 6. **Induced amino acid transport and surface rBAT protein in wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng. *A*, 50 μM L-Arg-induced uptake was measured at 2 and 6 days. The wild type-induced uptake at day 6 was adjusted to 100. We present data (mean ± S.E.) of five experiments. *B*, in parallel, oocytes were treated as in Fig. 5. The densitometric quantification of the precipitates corrected for their β1-integrin control precipitates is depicted. The value for wild type at day 6 was adjusted to 100. Data (mean ± S.E.) of three experiments for 2 days and five experiments for 6 days are presented.

highly purified fraction corresponding to the biotin-labeled proteins (surface) (Fig. 5).

Despite the lower levels of M467T protein on the oocyte surface the recovery of the induced transport was total at 6 days. In Fig. 6*B* we present the densitometric quantification of the precipitates for three experiments (day 2) or five experiments (day 6). To obtain a better quantification, the signals in the rBAT precipitates were corrected for their β1-integrin content. The values of transport activity are also shown for comparison (Fig. 6*A*). Although there were no differences in the uptake, M467T surface rBAT protein was only 12% of the amount observed in wild type-injected oocytes at 6 days. Moreover, a 10-fold increase in plasma membrane rBAT protein was measured between 2 and 6 days in wild type-injected oocytes, despite an almost saturated transport at 2 days. Thus, there was a clear dissociation between protein in the plasma membrane and uptake: high increases in surface rBAT protein were not reflected in higher uptake rates. On the other hand, the difference between wild type and M467T surface rBAT protein at 2 days might correlate with the uptake values. The same is true of the increase in M467T rBAT plasma membrane protein from 2 to 6 days. In all, biotin-labeling experiments indicate that, in contrast to the wild type protein, M467T protein is mostly located in an intracellular compartment. A lack of correlation between surface rBAT protein and transport activity was also observed, suggesting the presence of rBAT molecules in the plasma membrane that are not functional. So, as we and others have proposed (1, 2, 20, 21), rBAT may be an essential but not a sufficient component of a complex of two or more proteins that mediates transport function.

DISCUSSION

In this paper we describe the molecular basis for the reduced transport observed in the rBAT point mutations M467T and M467K. Both mutants display an intracellular trafficking defect that impairs their transport to the oocyte surface.

Functional studies showed that transport was impaired in both mutants, the defect being more severe in the M467K. This may be caused by the more drastic amino acid change (from a nonpolar residue to a polar residue in the M467T and to a positively charged residue in the M467K). The magnitude of the defect, however, was dependent on the time after cRNA injection and on the amount of cRNA injected, especially for the M467T mutant. Thus, at high levels of cRNA the M467T mutant achieved the same levels as those induced by the wild type; this was not the case at lower levels. This "cRNA dose and time effect" may be related to the level of saturation of the quality-control machinery in the oocyte: only when the system is saturated (*i.e.* with high cRNA doses or after a longer time) does some M467T rBAT protein reach the surface. The dependence on the cRNA level might be important for the epithelial cell of the tubule *in vivo*; an rBAT mRNA level below the amount injected in the oocytes is conceivable.

The recovery of function is difficult to explain if the mutation affects only the amino acid translocation across the transporter, and it is better supported by an impairment of the intracellular transport of the molecule to its normal localization in the cell. An alteration of the transporter's turnover rate is also difficult to apply in this case because the total amount of wild type protein compared with the M467T is similar. Whole oocyte transport assays indicated no intrinsic functional defect in the M467T mutant: the K_m for the different substrates remained the same and the exchanger activity, measured as the voltage dependence of amino acid-induced currents and the efflux of amino acids in the absence of any amino acid in the extracellular medium, was not different from that of the wild type. Then, as expected from the above data, the amino acid accumulation level in the M467T-injected oocytes was similar to that in wild type-injected oocytes. The K_m of the M467K-induced transport was also the same as that of the wild type, but a V_{max} decrease was observed. This may reflect an additional intrinsic defect in the translocation pathway of amino acids. Nevertheless, the magnitude of this decrease is, as for the M467T but to a lesser extent, dependent on the time and on the cRNA injected, again suggesting a trafficking defect.

The normal rBAT protein accumulated in the oocyte in two forms: an endo H-sensitive form (band II) of 85 kDa and an endo H-resistant form (band I) of 94 kDa. Thus, rBAT followed the general pathway of membrane proteins, passing first through the endoplasmic reticulum and then through the Golgi complex, where it acquired the endo H-resistant condition, on its way to the plasma membrane. Band I was by far the major rBAT protein found in the plasma membrane in wild type-injected oocytes, as revealed by surface labeling with biotin. In contrast, both mutants remained in an endo H-sensitive form. We had no evidence that the two rBAT mutants were ever transported to the Golgi complex. However, we detected the presence of the endo H-sensitive form in the plasma membrane. This also occurred with the M467K mutant (data not shown). The mutants may traverse the Golgi complex without being processed; it has been shown that some naturally occurring mutants of the sucrase-isomaltase gene are endo H-sensitive and are missorted to the basolateral membrane of the epithelial cells of the jejunum. The authors suggested that conformational and/or structural alterations in the protein prevented the acquisition of the endo H-resistant condition (33).

It is worth mentioning the method we used to localize rBAT in the oocyte. Initial experiments with an oocyte membrane subfractionation protocol proposed by Thomas and co-workers (25), based on an earlier method (39), showed, surprisingly, the same localization for both the normal and the M467T mutant, in the "plasma membrane" fraction. Control Western blots with the oocyte β1-integrin showed the endo H-sensitive and the endo H-resistant forms of β1-integrin in the plasma membrane fraction, but neither form was detected in the intracellular fractions (data not shown). β1-integrin has been immunolocalized to the plasma membrane and to the cortical ER (28), which extends around the cortical granules and contacts the plasma membrane of the oocyte (40). The fractionation method may lead to the copurification of the plasma membrane and the cortical ER. Thus, the information so obtained should be interpreted with caution.

Therefore, surface labeling with biotin was used to quantify the amount of rBAT protein in the oocyte surface, using β1-integrin as a control in the biotinylation procedure. We observed a lower amount of M467T and M467K proteins at the plasma membrane and higher amounts located intracellularly. The converse situation was true for the wild type. Moreover, the overall rate of accumulation at the surface was higher for the normal protein. All these data strongly suggest a defect in the delivery of the mutants to the plasma membrane due to their retention in an intracellular compartment, probably the ER. The residence time in the ER is expected to be longer for both mutants. The intracellular retention could be due to an improper folding in the ER, as shown for the ΔF508 CFTR in cystic fibrosis (41) or α_1-antitrypsin deficiency (42). Met-467 lies on the third transmembrane domain according to the model of Tate and co-workers (17). The change to threonine would only slightly impair the folding of the protein, but the introduction of a lysine in the highly hydrophobic environment of the transmembrane domain would have a stronger effect. The complete carrier may be constituted only if rBAT is assembled with other proteins; this assembly might be impaired (see below). Assembly of proteins into native homo- or heterooligomers is essential for their transport out of the ER (43, 44). In eukaryotic cells, proteins that fail to fold or to assemble properly usually follow an ER-associated degradation pathway: ΔF508 CFTR (45) and the subunits of the T-cell receptor (44) are good examples. The slow rate of this pathway at low temperatures (46) makes its involvement in the defect displayed by these mutants unlikely, at least in oocytes, because they are continuously kept at 18 °C. Actually, the ΔF508 CFTR does not appear to be degraded in oocytes and cell lines cultured at low temperature (47–49) but is degraded in cells cultured at 37 °C (45). Whatever the cause of the retention in the ER, we propose that type I cystinuria should be added to the list of putative human protein-folding diseases given by Thomas and co-workers (50).

Here we have shown a clear lack of correlation between the amount of rBAT protein in the oocyte surface and the induced amino acid transport. The simplest explanation is that rBAT alone is unable to sustain its induced amino acid transport activity. So the question arises: what is the functional unit for the rBAT-induced $b^{0,+}$-like activity? Several findings support the hypothesis that it is a complex formed by rBAT and another protein. First, all the cloned metabolite and neurotransmitter transporters contain 8–12 transmembrane domains (19), which is in contrast to the 4-transmembrane domain model proposed for rBAT (17). This suggests that rBAT may not be the carrier but a modulator of it (*i.e.* delivering silent transporters to the cell surface, like the β subunit of the Na^+/K^+ ATPase (51)). Second, rBAT is expressed in kidney and intestine as a 94-kDa band in reducing conditions and as a 120–130-kDa band in nonreducing conditions (6, 20, 21), indicating a disulfide-linked heterodimer with a 30–40-kDa protein. Furthermore, this 120–130-kDa band is the only one detected upon cross-linking of kidney brush border membranes followed by reducing Western blot (20). Third, the rBAT homologous protein 4F2hc induces in oocytes a y^+L-like amino acid exchanger activity (2, 5, 52) and is expressed in different tissues as a disulfide-linked heterodimer of 120 kDa. Its 40-kDa component has been detected by ^{125}I surface labeling followed by immunoprecipitation and reduction of the complex. Unfortunately, only the 85-kDa component (4F2hc) has been cloned (53, 54). We have been unable to immunoprecipitate the rBAT 120–130-kDa band, most probably due to a steric masking of the epitope produced by the tight aposition of the other protein.[2] Fourth, so far the only heterologous system in which rBAT has been successfully expressed is the *Xenopus* oocyte. rBAT transiently expressed in COS cells is unable to reach the plasma membrane (21) or, if it arrives, no amino acid transport induction is detected (55). This correlates with the absence of the 120–130-kDa band in nonreducing Western blots from these cells (21).

[2] R. Estévez and M. Palacín, unpublished results.

Finally, Wang and Tate have recently shown that in nonreducing conditions, several high molecular weight rBAT-specific complexes (*i.e.* not present in uninjected oocytes) are detected in oocyte total membranes (20). In preliminary experiments, we detected such complexes in biotin-labeled oocytes (data not shown). Among them, only the 125-kDa complex was exclusively found at the surface, and its relative amount in wild type and M467T oocytes may correlate with transport function (data not shown). Its identical electrophoretic mobility in wild type and M467T oocytes, however, is surprising; perhaps a very small fraction of M467T protein matures (but so small as to be undetectable, even in overexposed films of the highly purified surface fraction) or, more likely, the endogenous subunit may display an anomalous mobility that renders the same size when it binds to either band I or II. Interestingly, the rBAT homologous protein 4F2hc, when it is bound to its very hydrophobic subunit (4F2 light chain), has an abnormal electrophoretic mobility (54).

In conclusion, the M467T and M467K mutants display a trafficking defect that maintains them in an intracellular location, probably the ER. A slower folding or assembly in the ER could be responsible for this defect. Thus, misfolding or a folding delay of the mutants may decrease the assembly rate with the endogenous subunit. The present data do not allow us to distinguish between these two possibilities. In any case, this phenotype can explain type I cystinuria. Direct confirmation of this requires analysis of the rBAT protein from tissue samples of patients carrying these mutations. A 125-kDa complex composed of rBAT and an endogenous oocyte protein may be the functional unit of transport. However, reconstitution experiments are needed to demonstrate that only a complex of rBAT with another protein is able to mediate transport. These studies await the cloning of this putative protein.

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