

# Activation of system L heterodimeric amino acid exchangers by intracellular substrates

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**System L-type transport of large neutral amino acids is mediated by ubiquitous LAT1-4F2hc and epithelial LAT2-4F2hc. These heterodimers are thought to function as obligatory exchangers, but only influx properties have been studied in some detail up until now. Here we measured their intracellular substrate selectivity, affinity and exchange stoichiometry using the *Xenopus* oocyte expression system. Quantification of amino acid influx and efflux by HPLC demonstrated an obligatory amino acid exchange with 1:1 stoichiometry. Strong, differential trans-stimulations of amino acid influx by injected amino acids showed that the intracellular substrate availability limits the transport rate and that the efflux selectivity range resembles that of influx. Compared with high extracellular apparent affinities, LAT1- and LAT2-4F2hc displayed much lower intracellular apparent affinities (apparent  $K_m$  in the millimolar range). Thus, the two system L amino acid transporters that are implicated in cell growth (LAT1-4F2hc) and transcellular transport (LAT2-4F2hc) are obligatory exchangers with relatively symmetrical substrate selectivities but strongly asymmetrical substrate affinities such that the intracellular amino acid concentration controls their activity.**

**Keywords:** epithelial cell polarity/glycoprotein-associated amino acid transporter/LAT1-4F2hc/LAT2-4F2hc

## Introduction

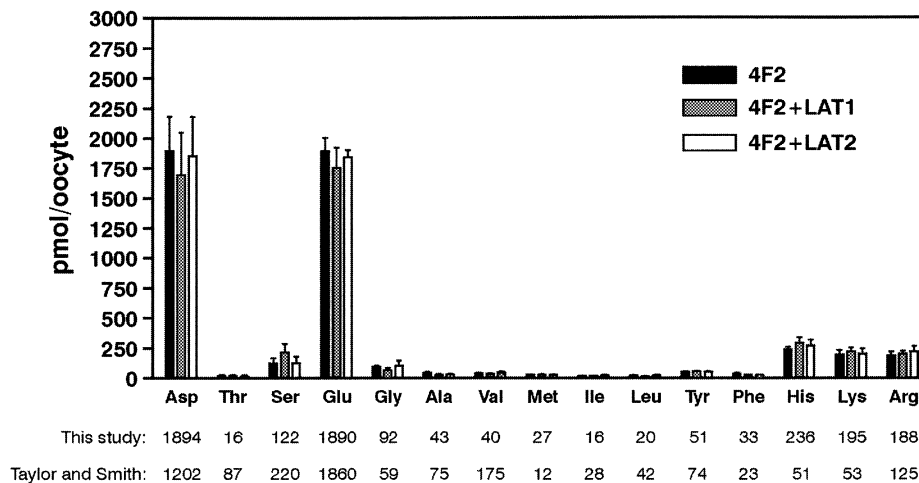
Two heterodimeric transporters for large neutral amino acids that correspond to the Na<sup>+</sup>-independent system L have recently been identified (Kanai *et al.*, 1998; Mastroberardino *et al.*, 1998; Pineda *et al.*, 1999; Prasad *et al.*, 1999; Rossier *et al.*, 1999; Segawa *et al.*, 1999; Rajan *et al.*, 2000). These heterodimers contain catalytic subunits named LAT1 and LAT2, which belong to the family of glycoprotein-associated amino acid transporters (gpaATs) and are also called light chains (Verrey *et al.*, 1999, 2000). A disulfide bond covalently links these gpaATs to their associated glycoprotein 4F2hc/CD98.

Functional experiments performed in expression systems suggest that the two L-type transporters function as exchangers (Mastroberardino *et al.*, 1998; Pineda *et al.*, 1999; Rossier *et al.*, 1999). In the case of LAT2-4F2hc,

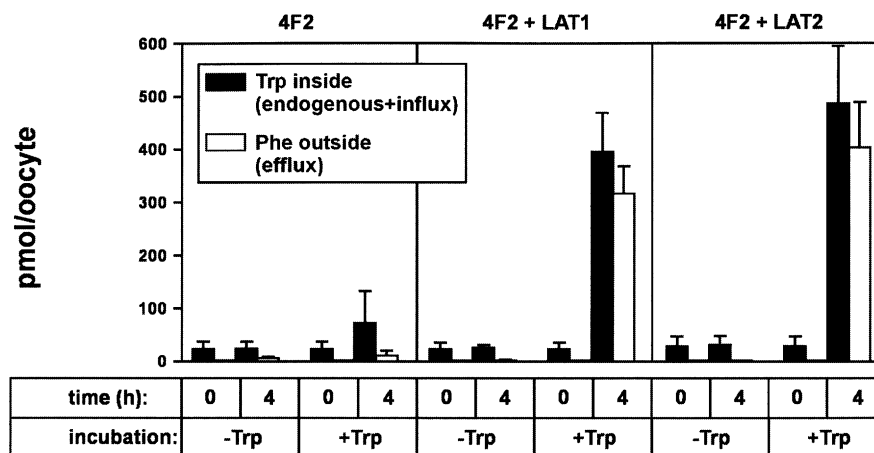
some contradictory data have been published. Our laboratory and that of Palacin have shown that the efflux of L-Phe and of L-Ile depend on the presence of an extracellular uptake substrate, as expected for an obligatory exchange (Pineda *et al.*, 1999; Rossier *et al.*, 1999). In contrast, an efflux of L-Leu observed by Kanai and co-workers was interpreted as a facilitated diffusion (Segawa *et al.*, 1999). Some functional differences between the two L-type transporters were reported, in particular the fact that LAT2-4F2hc has a broader selectivity range than LAT1-4F2hc in that it also mediates the uptake of smaller neutral amino acids.

The tissue distribution and subcellular localization of the two L-type transporters suggest that they must play different roles. LAT1-4F2hc is found quite ubiquitously and is highly expressed in proliferating tissues, in particular also in tumors, suggesting a role in amino acid uptake (Wolf *et al.*, 1996; Nakamura *et al.*, 1999; Campbell *et al.*, 2000) (also see the schematics in Figure 6). In contrast, LAT2-4F2hc is found only in tissues containing epithelial barriers (Bassi *et al.*, 1999; Pineda *et al.*, 1999; Rossier *et al.*, 1999; Segawa *et al.*, 1999; Rajan *et al.*, 2000). In (re)absorptive epithelia of the small intestine and proximal kidney tubule, it is highly expressed at the basolateral side of the epithelial cells (Rossier *et al.*, 1999). We have suggested that its role could be to extrude a number of neutral amino acids into the extracellular space in exchange for other extracellular neutral amino acids. These latter amino acids would, in turn, be recycled into the extracellular space by a parallel functioning, unidirectional export system that has not yet been characterized and probably displays a limited selectivity range (Verrey *et al.*, 1999, 2000). A cDNA encoding a possible candidate for this efflux pathway has recently been cloned (Kim *et al.*, 2001). However, this new transporter, which resembles H<sup>+</sup>/monocarboxylate transporters, cannot be the basolateral efflux pathway for the kidney proximal tubule since it is not expressed in that organ (Kim *et al.*, 2001). The role of LAT2-4F2hc in transepithelial transport thus appears to be complementary to that of y<sup>+</sup>LAT1-4F2hc, which extrudes cationic amino acids basolaterally in exchange for extracellular neutral amino acids plus Na<sup>+</sup> (Torrents *et al.*, 1998; Pfeiffer *et al.*, 1999b; Kanai *et al.*, 2000).

To understand the functional role of the two L-type transporters in proliferating cells (LAT1-4F2hc) and in the context of transepithelial transport (LAT2-4F2hc), their exchanger function needed to be verified and their exchange stoichiometry measured. Furthermore, their intracellular selectivity range and apparent affinity for amino acids needed to be determined. This present study addresses these issues in the *Xenopus* oocyte expression system by using amino acid injection to modify the intracellular amino acid pool, and tracer



**Fig. 1.** The endogenous amino acid pool of *Xenopus* oocytes is not modified by the expression of LAT1- or LAT2-4F2hc in the absence of extracellular amino acids. Amino acids contained in oocytes were separated by HPLC and quantified as described in Materials and methods. The means of 16 oocytes pooled from four independent experiments are shown. The mean values for 4F2hc-expressing oocytes are given below the bar graph in pmol/oocyte. For comparison, the values from Taylor and Smith (1987) are given.



**Fig. 2.** Amino acid analysis reveals a 1:1 stoichiometry of amino acid exchange by LAT1-4F2hc and LAT2-4F2hc. Oocytes expressing 4F2hc alone or together with LAT1 or LAT2 were injected with 1 nmol of L-phenylalanine. Amino acid analysis was then performed immediately after injection (0 h) or after a 4 h incubation in ND96 with or without 500  $\mu$ M L-Trp. The means of eight oocytes from two independent experiments  $\pm$  SD are shown.

measurements and amino acid analysis to quantify the amino acid fluxes.

## Results

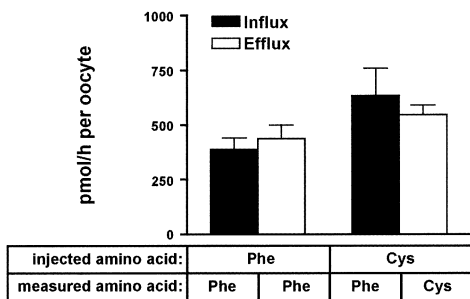
### Endogenous pool of amino acids in *Xenopus* oocytes

Measurements of the endogenous free amino acids in oocytes expressing 4F2hc alone or together with LAT1 or LAT2 showed that the expression of the transporters had no visible effect on the amino acid pool within the oocytes (Figure 1). The lack of amino acid depletion in oocytes expressing transporters that were maintained in amino acid-free buffer confirmed indirectly our earlier conclusion that these transporters do not mediate facilitated diffusion. The absolute amounts of amino acids measured in the oocytes were mostly similar to the values published by Taylor and Smith (1987), with the exception of some amino acids (L-Thr, L-Val, L-His, L-Lys and L-Arg), the

amount of which was substantially different (Figure 1). The reason for this discrepancy is not clear.

### Stoichiometry of LAT1- and LAT2-4F2hc-mediated amino acid exchange

To measure the stoichiometry of exchange, oocytes were injected with 1 nmol of L-Phe and incubated for 4 h with or without extracellular L-Trp (500  $\mu$ M). These amino acids were chosen because they are well transported substrates for both L-type transporters and because preliminary experiments had shown them not to be significantly metabolized in the oocytes during the 4 h incubation period. During this incubation, there was no significant change in intra- or extracellular amino acids in oocytes expressing 4F2hc alone (negative control), even in the presence of extracellular L-Trp (Figure 2). When LAT1 or LAT2 was co-expressed with 4F2hc, the mean amount of amino acid (L-Phe) that leaked from oocytes during the 4 h incubation in the nominal absence of extracellular substrate was not significantly different from the negative



**Fig. 3.** Amino acid flux rate measurements reveal a 1:1 stoichiometry of amino acid exchange mediated by LAT2-4F2hc. Tracer influx and efflux measurements were performed with oocytes expressing 4F2hc alone or together with LAT2; 1 nmol of L-Phe or 1 nmol of L-Cys was injected per oocyte (for efflux measurements together with 1 pmol of L-[<sup>3</sup>H]Phe or L-[<sup>35</sup>S]Cys, respectively). Influx and efflux were measured in the presence of 1 mM L-Phe outside. The bars represent means  $\pm$  SE of 12 oocytes or 12 aliquots (for influx or efflux, respectively) pooled from two independent experiments.

control [ $12.5 \pm 7.6$  and  $1.1 \pm 0.4$  pmol/oocyte versus  $4.2 \pm 2.2$  pmol/oocyte ( $n = 6$ )]. Thus, there is probably no relevant trans-zero efflux of L-Phe via the system L transporters. Alternatively, if such a facilitated diffusion efflux exists, it amounts to <9% of the corresponding exchange rate in the case of LAT1-4F2hc and 4% in the case of LAT2-4F2hc (upper limit of 95% confidence interval).

In contrast, when oocytes were incubated in buffer containing L-Trp, L-Phe appeared at the outside and L-Trp on the inside of the oocytes. The amounts of endogenous amino acids did not change measurably, independently of co-expression of the light chains or the presence of L-Trp outside (data not shown).

The amounts of L-Phe appearing outside and of L-Trp appearing inside (L-Trp after 4 h incubation minus L-Trp before the incubation) measured on the same pool of oocytes were nearly identical. The small, non-significant difference (<15% less L-Phe efflux than L-Trp influx) might be explained by the contribution to the efflux of some endogenous amino acids other than L-Phe (i.e. L-His, L-Tyr, etc.). The result, however, fully supports the hypothesis that the amino acid exchange via the two L-system transporters is coupled with a stoichiometry of 1:1.

These experiments showed that in the presence of a high amount of injected efflux substrate, only small amounts of endogenous amino acids participate in the exchange. Thus, tracer flux experiments should also yield a stoichiometry in the range of 1:1, even without correction for endogenous amino acids. Indeed, tracer experiments performed in LAT2-4F2hc-expressing oocytes showed that influx and efflux rates were similar for the homo-exchange of L-Phe and for the hetero-exchange of extracellular L-Phe against intracellular L-Cys (Figure 3). These experiments thus confirmed with other influx (L-Phe) and efflux (L-Cys) substrates the 1:1 exchange stoichiometry of LAT2-4F2hc.

#### **Trans-stimulation of amino acid influx by injected amino acids**

Measurements of transport rates (influx and efflux) using amino acid analysis (Figure 2), as well as efflux measure-

ments performed on oocytes injected with radiolabeled amino acids (Figure 3 and data not shown), confirmed that the efflux via LAT1- and LAT2-4F2hc depends on the presence of an extracellular influx substrate. In view of the 1:1 exchange stoichiometry (Figures 2 and 3), the reverse should also be true, namely that the influx rate depends on the availability of an intracellular efflux substrate (Verrey *et al.*, 2000). Thus, changing the intracellular amino acid availability should correspondingly affect (trans-stimulate) the substrate uptake rate, provided that the range of intracellular concentration is clearly below that allowing maximal efflux velocity.

To test the hypothesis that the concentration of endogenous amino acids in oocytes limits the rate of amino acid uptake, we first injected 1 nmol of L-Phe (or only H<sub>2</sub>O) into the oocytes and measured the uptake rate of L-[<sup>3</sup>H]Phe 4 h later. The amount of injected L-Phe (1 nmol) chosen was much higher than that of endogenous amino acids (see Figure 1) and was estimated to lead to an additional intra-oocyte L-Phe concentration of ~2.5 mM. This latter estimate is based on the assumption that the intra-oocyte distribution volume for L-Phe is ~0.4  $\mu$ l, corresponding to the water volume determined for stage 5 and 6 *Xenopus* oocytes by Taylor and Smith (1987).

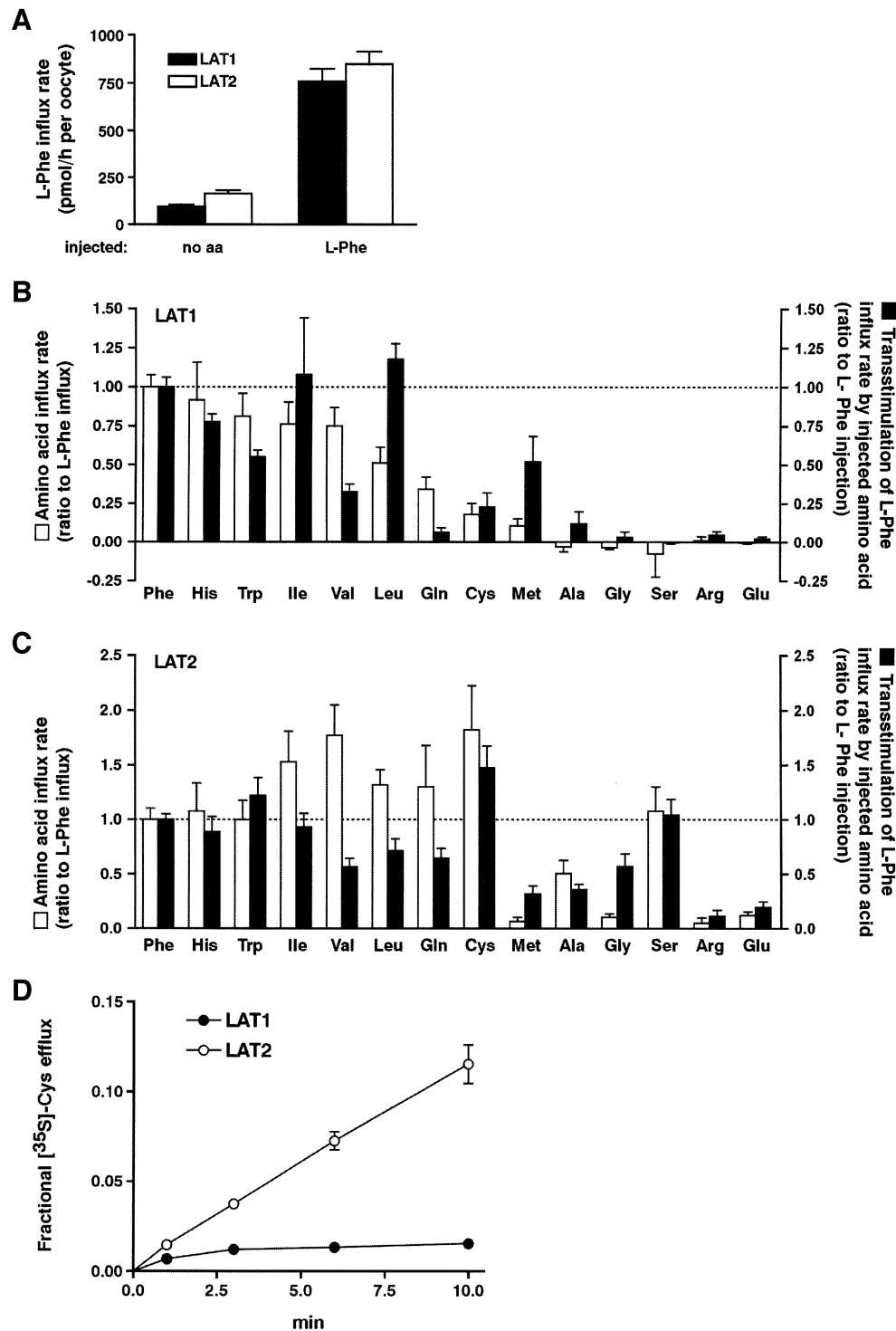
As shown in Figure 4A, pre-injection of 1 nmol of L-Phe massively trans-stimulated the influx rate of L-[<sup>3</sup>H]Phe, demonstrating that the concentration of endogenous substrate amino acids is much lower than that required for maximal transporter activation. In this series of experiments, the mean increase in the L-[<sup>3</sup>H]Phe uptake rate was 9-fold in the case of LAT1-4F2hc-expressing oocytes and 6.5-fold for LAT2-4F2hc-expressing oocytes.

#### **Determination of the intracellular amino acid selectivity of LAT1-4F2hc and LAT2-4F2hc**

We addressed the question of the intracellular amino acid selectivity of both L-system transporters by comparing the trans-stimulation induced by various pre-injected amino acids (1 nmol, corresponding to ~2.5 mM) with that induced by 1 nmol of L-Phe. Figure 4B and C shows a summary in which the trans-stimulation exerted by each injected amino acid is expressed as a ratio to that exerted by L-Phe. For comparison, the relative influx rates for the same amino acids (100  $\mu$ M) are shown in the same panels [data compiled from Mastroberardino *et al.* (1998), Rossier *et al.* (1999) and the current study (L-Cys and L-Ser)].

Some pre-injected amino acids displayed differential trans-stimulation efficiencies on LAT1-4F2hc- and LAT2-4F2hc-mediated L-[<sup>3</sup>H]Phe uptake. In other words, the two L-type transporters displayed differential intracellular substrate selectivities. In the case of LAT1-4F2hc, none of the tested amino acids induced a higher trans-stimulation than L-Phe and, in the case of LAT2-4F2hc, only L-Cys and possibly L-Trp supported higher transport rates than L-Phe.

Importantly, the epithelial LAT2-4F2hc had a broader efflux substrate selectivity range than LAT1-4F2hc. Indeed, approximately half of the injected amino acids trans-stimulated uptake only in the case of LAT2-4F2hc expression (Gly, L-Ser, L-Gln) or at least better in the case of LAT2-4F2hc than LAT1-4F2hc (L-Trp, L-Val, L-Ala,



**Fig. 4.** LAT1- and LAT2-4F2hc-mediated L-[<sup>3</sup>H]Phe uptake is strongly trans-stimulated by injected substrate amino acids. L-Phe uptake (3 min) was measured 4 h or 30 min (for L-Leu, L-Arg and L-Glu) after injection of 1 nmol of the indicated amino acid. (A) Mean L-[<sup>3</sup>H]Phe uptake rate with or without pre-injection of 1 nmol L-Phe.  $n = 78-80$  oocytes, pooled from 13 independent experiments. (B and C) Trans-stimulation of L-[<sup>3</sup>H]Phe influx by various injected L-amino acids (black bars). The results were normalized for each experiment to set to 1 and 0 the relative trans-stimulation levels exerted by the injection of 1 nmol of L-Phe and vehicle, respectively. For each amino acid, the mean of 14–56 oocytes pooled from 2–4 independent experiments is shown. Influx rates (relative to L-[<sup>3</sup>H]Phe) of the same amino acids (100  $\mu$ M) are shown for comparison (white bars). Influx data are compiled from Mastroberardino *et al.* (1998), Rossier *et al.* (1999) and new experiments. (D) Efflux measurements were performed with oocytes injected with 1 nmol of L-Cys (with 1 pmol of L-[<sup>35</sup>S]Cys as a tracer). The oocytes were incubated in (+Na)-solution containing 1 mM L-Phe. The means of six aliquots pooled from two independent experiments are shown.

L-Cys). An exception was L-Leu, which exerted a stronger trans-stimulation on LAT1-4F2hc- than on LAT2-4F2hc-mediated uptake.

To confirm that the observed trans-stimulation of L-Phe influx corresponded to the efflux of the injected amino acid, we compared the efflux time courses of injected

L-[<sup>35</sup>S]Cys via the two transporters (Figure 4D). As expected from the differential trans-stimulation results (Figure 4B and C), a substantial rapid and constant efflux in the presence of extracellular L-Phe was only observed when LAT2-4F2hc was expressed.

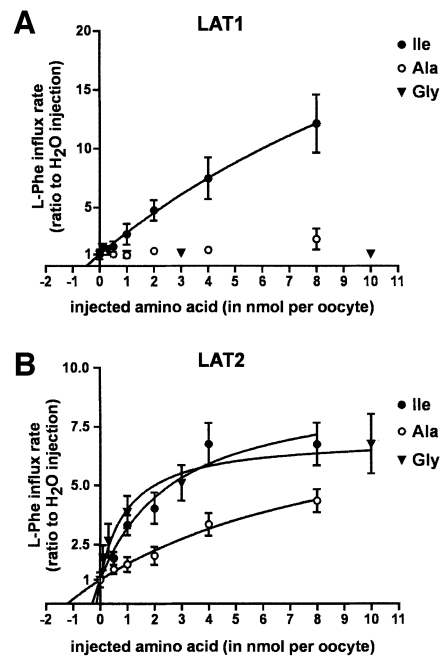
Some of the trans-stimulation measurements used for Figure 4 were performed at 30 min instead of 4 h after amino acid injection. This was the case, for instance, for L-Leu, for which we had noticed that an injected amount of 1 nmol decreased by ~50% over 4 h, whereas injected L-Phe and L-Trp remained stable. This decrease in L-Leu was not accompanied either by changes in other intracellular amino acids or by the appearance of L-Leu outside and was thus probably due to metabolic breakdown. Trans-stimulation by L-Glu and by L-Arg was also performed at 30 min after their injection because first experiments had shown that a high intracellular level of these amino acids leads to a time-dependent increase in trans-stimulation that is suggestive of the appearance of a metabolite(s) acting as efflux substrate(s). The fact that the trans-stimulation by injected L-Glu or L-Arg was higher for LAT2- than LAT1-4F2hc is compatible with the hypothesis that this metabolite is L-Gln since this is a better efflux substrate for LAT2- than for LAT1-4F2hc.

Taken together, the trans-stimulation experiments presented in Figure 4B and C show that the selectivity range and rank order for efflux via LAT1- and LAT2-4F2hc to a large extent correspond to the influx.

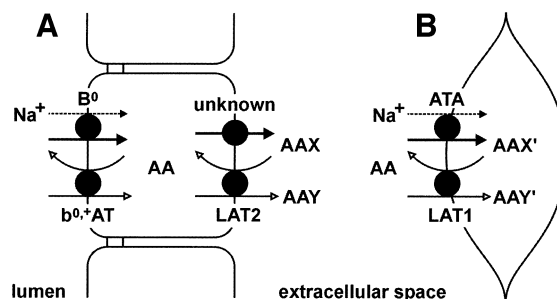
#### Low apparent affinity of intracellular amino acids for LAT1- and LAT2-4F2hc

To obtain an estimate of the intracellular amino acid concentration that is required for half-maximal activation of LAT1- and LAT2-4F2hc, we performed trans-stimulation experiments with increasing amounts of injected amino acids. We chose to inject L-Ile, L-Ala and Gly because of their differential trans-stimulation abilities (see Figure 4B and C), their stability in oocytes and their solubility in water. The results are shown in Figure 5 and expressed as a fractional increase in the rate of L-[<sup>3</sup>H]Phe uptake over that observed in control oocytes injected with water (endogenous amino acid pool only). In all cases in which substantial trans-stimulation was measured, fitting the modified Hill equation to the experimental points yielded Hill coefficients of 1 (between 1.00 and 1.07). Thus, curves corresponding to Michaelis–Menten kinetics were fitted, and  $V_{max}$  and apparent  $K_m$  values could be derived (Table I). In the case of LAT1-4F2hc, the maximal trans-stimulation by injected L-Ile was ~40-fold, meaning that the endogenous intracellular amino acid pool of oocytes supports only ~2.5% of  $V_{max}$ . The small amino acids L-Ala and Gly did not substantially trans-stimulate uptake via LAT1-4F2hc. Half-maximal trans-stimulation of uptake was estimated to be achieved by the injection of ~19 nmol of L-Ile per oocyte. Assuming a distribution volume of 0.4  $\mu$ l, this corresponds to an apparent  $K_m$  for L-Ile of ~50 mM (see Materials and methods for calculations). The absolute value of the  $x$ -axis intercept produced by the curve fit corresponds to the amount of L-Ile that would trans-stimulate the influx rate to the same extent as the mixture of endogenous amino acids.

In the case of LAT2-4F2hc, the maximal trans-stimulation was ~9-fold for L-Ile and L-Ala, and somewhat



**Fig. 5.** Dose dependence of trans-stimulation by injected amino acids reveals low intracellular apparent affinities. Four hours after injection of various amounts of amino acids, L-Phe uptake was performed. The uptake rates were normalized to the mean value obtained for H<sub>2</sub>O-injected oocytes. (A)  $n = 12$  oocytes per data point, pooled from two independent experiments (L-Ile). L-Ala and Gly:  $n = 6$  per data point from one experiment. (B)  $n = 12$  oocytes per data point, pooled from two independent experiments for all three amino acids.



**Fig. 6.** Schematic illustration of the role played by LAT2- and LAT1-4F2hc in broadening the amino acid transport selectivity range in an epithelial and a non-epithelial cell, respectively. (A) Neutral amino acids (AA) enter the epithelial cell from the luminal side via apical transporters (B<sup>0</sup> and b<sup>0</sup>+AT-rBAT and/or others). We postulate that some neutral amino acids (AAX) cross the basolateral membrane via an efflux pathway that has not yet been characterized and has a limited substrate selectivity. Some of these amino acids that are uptake substrates for the obligatory exchanger LAT2-4F2hc are recycled back into the cell, thereby allowing the efflux via LAT2-4F2hc of other neutral amino acids (AAY) which have accumulated in the cell. (B) In this example, a mixture of preferentially small neutral amino acids (AAX') is accumulated into a (growing) cell via a secondary active transport system (system A). Some of these amino acids that are efflux substrates of LAT1-4F2hc are recycled by this exchanger into the extracellular space, and thus drive the uptake of large neutral amino acids (AAY').

lower for Gly. This indicates that the intracellular amino acid pool of oocytes supports ~11% of  $V_{max}$  and that bound Gly might be less efficient than the other two amino acids in allowing the transition of the transporter from the conformation with an intracellular binding site to that with

**Table I.** Comparison of intra- and extracellular kinetics of LAT1- and LAT2-4F2hc

	LAT1-4F2hc			LAT2-4F2hc		
	External apparent $K_m$ (M) <sup>a</sup>	Maximal influx trans-stimulation (fractional change) <sup>b</sup>	Internal apparent $K_m$ (M) <sup>b</sup>	External apparent $K_m$ (M) <sup>a</sup>	Maximal influx trans-stimulation (fractional change) <sup>b</sup>	Internal apparent $K_m$ (M) <sup>b</sup>
L-Ile	$38.7 \pm 5.3 \times 10^{-6}$	$39.7 \pm 5.7$	$49.5 \pm 9.5 \times 10^{-3}$	$36.5 \pm 9.5 \times 10^{-6}$	$9.3 \pm 1.4$	$7.0 \pm 2.5 \times 10^{-3}$
L-Ala	$>10 \times 10^{-3}$	no significant effect	–	$167 \times 10^{-6}$	$9.1 \pm 1.9$	$27.5 \pm 9.5 \times 10^{-3}$
Gly	$9.0 \pm 2.7 \times 10^{-3}$	no significant effect	–	$4.2 \pm 0.7 \times 10^{-3}$	$7.0 \pm 0.5$	$2.6 \pm 0.6 \times 10^{-3}$

<sup>a</sup>External substrate concentrations supporting half-maximal influx rates (apparent  $K_m$ ) were determined as in Rossier *et al.* (1999). In the case of L-Ala, the indicated values are from Rossier *et al.* (1999).

<sup>b</sup>Data are from the influx trans-stimulation experiments shown in Figure 5.

an extracellular binding site. The derived intracellular apparent affinities were higher than for LAT1-4F2hc with the following rank order: Gly > L-Ile > L-Ala (Table I).

In summary, the intracellular apparent affinities of the substrate amino acids for the L-system transporters are much lower than the apparent affinities at the extracellular side.

## Discussion

### **Absence of facilitated diffusion in either direction**

The lack of measurable amino acid efflux from oocytes expressing LAT1-4F2hc or LAT2-4F2hc in the absence of extracellular amino acids, even when a high amount of amino acid was injected into oocytes, confirms that the L-type transporters do not provide a facilitated diffusion pathway for amino acid efflux (Figure 2). In terms of an alternating access model, this means that the transporter does not undergo (at a measurable rate) the transition from a conformation with an extracellular-facing binding site to one with an intracellular-facing binding site in the absence of ligand. From the present results, one can extrapolate that the reverse applies as well, namely that in the absence of intracellular ligand the transporter does not undergo (at a measurable rate) the transition from the intracellular- to the extracellular-facing binding site conformation. This can indeed be deduced from the fact that injected efflux substrates exert a high level of uptake trans-stimulation that follows simple Michaelis–Menten kinetics (Figures 4 and 5) and that the efflux matches the influx (Figures 2 and 3). However, the possibility that a small trans-zero influx (facilitated diffusion) could take place has not been formally excluded. Because such an influx would only amount to a very small fraction of the transports observed in the presence of physiological amino acid concentrations, it would not play any functional role. We can thus conclude that there is a tight bidirectional coupling of influx and efflux (obligatory exchange) that does not allow any physiologically relevant facilitated diffusion of amino acids, either for influx or for efflux. This implies that system L transporters cannot mediate net vectorial amino acid transfer and that their function is rather that of complementing/extending the action of unidirectional transporters located in the same membrane (Figure 6).

### **Similarity of influx and efflux selectivities but asymmetry of apparent affinities**

The comparison of the selectivity range of LAT1- and LAT2-4F2hc for the uptake of extracellular amino acids (100  $\mu$ M) with their selectivity range for trans-stimulation by intracellular amino acids (1 nmol per oocyte, corresponding to  $\sim 2.5$  mM) (Figure 4B and C) shows a high level of similarity, indicating that the influx substrates are generally also efflux substrates. However, the apparent affinities at the intracellular side of the transporters (apparent  $K_m = 10^{-3}$ – $10^{-1}$  M) appear to be generally lower by two to three orders of magnitude than those at the extracellular side (apparent  $K_m = 10^{-5}$ – $10^{-3}$ ) (see L-Ile and L-Ala in Table I). A particular case is that of Gly, which for the epithelial LAT2-4F2hc displays similar apparent  $K_m$  values at the intra- and extracellular sides (4.2 and 2.6 mM, respectively; Table I).

Knowing the influx and efflux properties of LAT1- and LAT2-4F2hc now allows us to make some prediction about the function of these transporters in the context of cell growth and transcellular transport. In that respect, it will be interesting to test whether the low apparent affinity of system L-transporters for intracellular substrates is independent of the cell type and/or regulation or whether it is subject to the regulatory/modulatory action of other gene products not expressed in *Xenopus* oocytes.

### **Saturating concentration of extracellular amino acids**

Under normal conditions, the extracellular amino acid concentration is sufficient to nearly saturate the activity of the L-system transporters. Indeed, we have estimated the ‘substrate activity’ exerted by a physiological extracellular amino acid mixture on LAT1- and LAT2-4F2hc based on published apparent  $K_m$  values of various amino acids for these transporters and on their normal plasma concentrations (see Materials and methods for calculation) (Divino Filho *et al.*, 1997; Fauci *et al.*, 1998; Mastroberardino *et al.*, 1998; Rossier *et al.*, 1999; Segawa *et al.*, 1999). For LAT2-4F2hc, the ‘substrate activity’ calculated with the available data (Rossier *et al.*, 1999; Segawa *et al.*, 1999) is equivalent to 20 times the  $K_m$  value. According to Michaelis–Menten kinetics, this ‘substrate activity’ would lead to a transport amounting to  $\sim 95\%$  of  $V_{max}$ . In the case of LAT1-4F2hc, the available apparent affinity data are less complete (Mastroberardino

*et al.*, 1998), but suggest that the 'substrate activity' is at least 10 times higher than the  $K_m$ , and thus would lead to a transport rate amounting to >90% of the  $V_{max}$ . Thus, in both cases, the activity of the transporter is essentially not controlled by the extracellular amino acid concentration and, therefore, the control of a stable extracellular amino acid concentration cannot be the physiological role of these transporters.

#### **Implications for the physiological role of LAT1**

Because they are obligatory exchangers, the L-type transporters do not modify the overall amino acid concentration ratio between the two sides of the membrane, but act on the proportional distribution of amino acids. As discussed above, they display similar selectivity ranges for intracellular and extracellular substrate amino acids. For instance, the ubiquitous transporter LAT1-4F2hc accepts, from the outside, mainly large neutral amino acids, in particular L-Phe, L-Tyr, L-Leu, L-Ile, L-His, L-Trp and also L-Val and L-Met. The relative rate of transport of these different amino acids by LAT1-4F2hc depends on their 'substrate activity', i.e. the ratio of their local concentration to their apparent  $K_m$ . Because the apparent  $K_m$  values for uptake of these various amino acids as well as their normal extracellular concentrations lie within similar concentration ranges, we expect that these amino acids will be transported into cells at similar rates. The rate of L-Val transport is expected to be similar as well, since the slightly higher extracellular  $K_m$  of this amino acid is compensated by its higher extracellular concentration.

The amount of amino acid that was injected into oocytes for the trans-stimulation experiments shown in Figure 4B and C was well below the apparent  $K_m$  and thus within the linear range of the activation curves (see Figure 5). Therefore, we can consider that the relative trans-stimulation effects of various injected amino acids shown in Figure 4B are roughly proportional to their apparent intracellular affinities ( $1/K_m$ ), assuming that the various amino acids display similar  $V_{max}$ . Because all typical system L substrates mentioned above produce a similar trans-stimulation, we can conclude that their efflux rates via LAT1-4F2hc are mostly determined by their respective intracellular concentration.

In the case of *Xenopus* oocytes, the low intracellular concentration of endogenous amino acids limits the transport rate via LAT1-4F2hc to as little as ~2.5% of  $V_{max}$  (see the 40-fold trans-stimulation in Figure 5). Published intracellular amino acid concentrations (Divino Filho *et al.*, 1997) suggest that in other cell types the intracellular amino acid 'substrate activity' can be quite higher than in oocytes but remains below that required for half-maximal activation of efflux via LAT1-4F2hc. Thus, changes in intracellular amino acid concentration due to influx, metabolism or protein synthesis/degradation modify the exchange activity of system L accordingly. For instance, if the secondary active amino acid transporter system A is induced and imports amino acids, the intracellular concentration of small amino acids and also that of L-His and L-Met increase (Reimer *et al.*, 2000; Sugawara *et al.*, 2000a,b; Varoqui *et al.*, 2000; Albers *et al.*, 2001). Those of the accumulated amino acids that are efflux substrates for

LAT1-4F2hc (e.g. L-His and L-Met) can then be exchanged via LAT1-4F2hc against other substrates, leading to an equilibration of the amino acid concentration inside the cell (Figure 6B). In other words, LAT1-4F2hc can extend the selectivity range of amino acid uptake to large neutral amino acids when it is expressed in the same membrane as active system A. Therefore, it is conceivable that the expression of system L might play, in some circumstances, a rate-limiting role for cell growth and proliferation by controlling the availability of essential amino acids such as L-Leu, as suggested by recent transfection experiments (Campbell and Thompson, 2001). Another indication that the expression of this transporter might play an important role in cell proliferation is the fact that it is expressed in many tumors and proliferating tissues (Campbell *et al.*, 2000).

Similar reasoning applies in the case of the blood-brain barrier where LAT1 has been shown to be expressed on both luminal and contraluminal membranes of endothelial cells (Boado *et al.*, 1999; Duelli *et al.*, 2000; Kageyama *et al.*, 2000; Matsuo *et al.*, 2000). Thus, the role of LAT1-4F2hc is most likely equilibration of the amino acid distribution across the two membranes, whereas other transporters determine the actual net amino acid flux.

In the case of the placenta, the situation is more complex because of the apparent asymmetrical distribution of LAT1, which has been suggested to be enriched in the brush border membrane (maternal side), whereas LAT2 and y<sup>+</sup>LAT1 would be expressed at the basal membrane (fetal side), analogous to their basolateral localization in the small intestine and the proximal kidney tubule (Kudo and Boyd, 2000, 2001; Jansson, 2001; Ritchie and Taylor, 2001). The role of LAT1 in the brush border membrane lining the maternal side of the placenta syncytiotrophoblast is probably analogous to its role in growing cells, namely to extend to large neutral amino acids the selectivity range of the amino acids that are taken up from the maternal blood via unidirectional transporters.

#### **Physiological implications for LAT2-4F2hc function in epithelial barriers and placenta**

From its localization in basolateral membranes of amino acid-transporting epithelia, such as in the small intestine and the kidney proximal tubule, and its probable localization to the basal membrane of placenta syncytiotrophoblast, it is clear that LAT2-4F2hc exerts a function in transcellular amino acid transport (Figure 6A). Compared with LAT1-4F2hc, this transporter has a selectivity range for influx and efflux that is slightly extended towards smaller neutral amino acids (Pineda *et al.*, 1999; Rossier *et al.*, 1999; Segawa *et al.*, 1999) (Figure 4B and C). The fact that the same endogenous amino acid pool of *Xenopus* oocytes supports a higher fractional activity of LAT2-4F2hc (11%) than of LAT1-4F2hc (2.5%) is certainly due in part to the broader selectivity range, but also appears to be due to somewhat higher intracellular apparent affinities (Table I). We suggest that these differential intracellular apparent affinities of LAT1-4F2hc and LAT2-4F2hc might favor vectorial, transcellular flux of large neutral amino acids.

An interesting aspect is the relatively high apparent affinity of Gly for the intracellular binding site of LAT2-4F2hc, which suggests an important role for

LAT2-4F2hc in the vectoriality of transcellular transport and of secretion of this small amino acid. In contrast, L-Leu appears to be a poor efflux substrate for LAT2-4F2hc relative to its high apparent affinity for influx. Thus, if there were a parallel efflux pathway for L-Leu, this amino acid could serve as a recycled species to drive the basolateral efflux of other neutral amino acids by LAT2-4F2hc. One of these efflux substrates is certainly L-Cys, which was most efficient in our trans-stimulation assay (Figure 4B). This is interesting in view of the fact that L-Cys enters the kidney proximal tubule cells to a substantial extent as L-cystine via the apical heterodimeric dibasic amino acid transporter  $b^{0,+}AT-rBAT$ , which is defective in cystinuria (Chairoungdua *et al.*, 1999; Feliubadalo *et al.*, 1999; Pfeiffer *et al.*, 1999a). Thus, it will be interesting to investigate whether the dysfunction of LAT2-4F2hc also leads to an increase in urinary L-Cys.

In summary, both LAT1- and LAT2-4F2hc enhance the selectivity range of amino acids transported across membranes to large neutral amino acids. Both are obligatory exchangers that display relatively low intracellular apparent substrate affinities, which contrast with their high extracellular affinities; therefore, they respond to changes in intracellular amino acid concentration by adapting their exchange activity accordingly. We suggest that the differential kinetic and selectivity properties of LAT1- and LAT2-4F2hc favor their role as 'selectivity range enhancers' in the context of amino acid uptake and efflux, respectively.

## Materials and methods

### cRNA synthesis

Plasmids containing the cDNAs of *h4F2hc* (vector pSport) (Teixeira *et al.*, 1987), *hLAT1* (pcDNA1/Amp-pSP64T) (Mastroberardino *et al.*, 1998) and *mLAT2* (pSD5easy) (Rossier *et al.*, 1999) were linearized using the restriction sites *HindIII*, *EcoRV* or *BglIII*, respectively. cRNA was synthesized with T7 and SP6 RNA polymerases (Promega), respectively, according to standard protocols.

### *Xenopus laevis* oocytes

Oocytes were treated with collagenase A for 2–3 h at room temperature in  $Ca^{2+}$ -free buffer containing 82.5 mM NaCl, 2 mM KCl, 1 mM  $MgCl_2$  and 10 mM HEPES pH 7.4, and then kept at 16°C in ND96 buffer containing 96 mM NaCl, 2 mM KCl, 1 mM  $MgCl_2$ , 1.8 mM  $CaCl_2$  and 5 mM HEPES pH 7.4.

### Amino acid analysis

Oocytes were injected with 5 ng of the corresponding cRNAs dissolved in 33 nl of water and then incubated for 18 h at 16°C in ND96 buffer. Where indicated, 1 nmol of L-Phe dissolved in 33 nl of water was then injected into the oocytes. Subsequently, four oocytes per condition were washed six times in ND96 and incubated for 4 h in 100  $\mu$ l ND96  $\pm$  supplementation with 500  $\mu$ M L-Trp. To quantify the change in amino acid levels inside and outside of the oocytes, amino acid analysis was performed on oocytes and buffer before and after the incubation and on the buffer of the last wash before the incubation (zero value). Samples were prepared as follows: oocytes were washed six times in ND96 and lysed in 120  $\mu$ l of oocyte lysis buffer containing 50 mM Tris-HCl pH 8.0, 120 mM NaCl and 0.5% IGPAL CA-630 (Sigma). Lysates were vortexed for 20 s, incubated briefly on ice, and centrifuged for 30 min at 12 000 r.p.m. in an Eppendorf centrifuge at 4°C. Proteins were precipitated with 5-sulfosalicylic acid (2.5 g/100 ml final concentration). The samples were vortexed for 20 s, incubated for 1 h on ice and centrifuged for 5 min at 15 000 r.p.m. at 4°C. The supernatants were extracted with phenol/chloroform/isoamyl alcohol. To analyze the amino acid content of the extracellular buffer, samples of 90  $\mu$ l were taken and their pH adjusted using citric acid (0.1 M final concentration). Amino acid analysis was performed with a Biochrom 20 amino acid analyzer

(Pharmacia, Biotech) by the post-column derivatization method using *o*-phthalaldehyde as detection reagent.

### Trans-stimulation of L-[ $^3H$ ]phenylalanine influx

The *Xenopus* oocyte presents characteristics that are very useful for the investigation of the intracellular kinetics of exogenously expressed amino acid transporters. First, the endogenous transport (background) is very low. Secondly, the concentration in endogenous neutral and cationic amino acids is relatively low and, thirdly, oocytes are easy to inject with known amounts of amino acid substrates, the level of which remains mostly stable during the course of the experiments. For trans-stimulation experiments, oocytes were injected with the corresponding cRNAs and, 18 h later, with the given amounts of the indicated amino acid (dissolved in 33 nl of  $H_2O$ ). After 30 min or 4 h at 16°C in ND96, oocytes were washed six times with ND96 and 5–12 oocytes per condition were pre-incubated for 2 min at 22°C. The buffer was replaced with ND96 supplemented with 500  $\mu$ M L-Phe and L-[ $^3H$ ]Phe as a tracer. Uptake was measured for a 3 min period, for which pilot experiments had shown a constant transport rate. The uptake was stopped by washing the oocytes five times with 3 ml of ND96. Oocytes were then distributed to individual vials, lysed in 2% SDS and the radioactivity determined by liquid scintillation.

### Calculation of substrate activity and of intra- and extracellular apparent $K_m$ values

The 'substrate activity' of an amino acid mix is defined here as the capacity of this mix to activate a transporter, on a given side of the membrane. The 'substrate activity' can be calculated as follows, assuming that each amino acid substrate of the mix is transported according to Michaelis–Menten kinetics and supports the same  $V_{max}$ . This latter assumption is based on data reported by Segawa *et al.* (1999) and obtained in our laboratory (L.Mastroberardino and C.Meier, unpublished data), which show that the  $V_{max}$  values for the influx of different amino acid substrates are mostly quite similar to each other. Then, substrate activity is given by:

$$\text{Substrate activity} = [aa_1]/K_{m,aa_1} + [aa_2]/K_{m,aa_2} + \dots + [aa_n]/K_{m,aa_n} \quad (1)$$

where  $[aa_n]$  stands for the concentration and  $K_{m,aa_n}$  for the apparent affinity of each substrate amino acid present in the mix. Thus, if an amino acid mixture displays a substrate activity of 1 (dimensionless) for a given transporter, it supports a half-maximal transport rate.

To estimate the apparent  $K_m$  for any single extra- or intracellular amino acid from dose-dependence experiments, curves were fit to data according to Michaelis–Menten kinetics using Prism (Version 3.02; GraphPad, San Diego, CA). For the activation of amino acid uptake by extracellular substrates, the Michaelis–Menten equation used was:

$$v = V_{max} \times [aa]/([aa] + K_m) \quad (2)$$

For the trans-stimulation of influx elicited by any single injected amino acid, equation (2) was modified to account for the fact that oocytes injected with no amino acid display a baseline influx rate that is due to the endogenous pool of cellular amino acids. In each experiment, this baseline rate was used to normalize the influx rates measured at different amounts of injected amino acid, yielding relative transport rates ( $v_r$ ,  $V_{max,r}$ ) that correspond to relative trans-stimulation levels ( $T_r$ ). The endogenous amino acid pool was expressed as amount  $B$  of the injected amino acid (nmol/oocyte) that would trans-stimulate influx to the same extent as does the endogenous pool (and thus would produce a  $T_r$  of 1). Introducing relative transport rates and  $B$  (in nmol/oocyte) in equation (2) yields:

$$T_r = V_{max,r} \times ([aa] + B)/([aa] + B) + K'_m \quad (3)$$

where  $K'_m$  stands for  $(K_m - B)$  and is expressed in nmol/oocyte, and where the amount of injected amino acid  $[aa]$  is also given in nmol/oocyte. Equation (3) can now be further simplified for the situation where no amino acid is injected ( $[aa] = 0$ ) and thus  $T_r = 1$  (see above), such that  $B$  (nmol/oocyte) can be expressed as follows:

$$B = K'_m/(V_{max,r} - 1) \quad (4)$$

Substituting equation (4) in equation (3), the relative trans-stimulation becomes:

$$T_r = V_{max} \times \frac{([aa] + (K'_m/(V_{max} - 1)))}{(K'_m/(V_{max} - 1)) + K'_m} \quad (5)$$

Using this equation, curves were fit to mean values (12 oocytes from two experiments) using the method of least squares. The intracellular

apparent  $K_m$  (M) was then calculated from the derived  $K'_m$  and  $B$ , taking  $0.4 \mu\text{l}$  as intra-oocyte amino acid distribution volume (see Results):

$$K_m = (K'_m + B)/0.4 \mu\text{l} \quad (6)$$

### Efflux of injected amino acids from *Xenopus* oocytes

*Xenopus* oocytes were injected with 5 ng of cRNA dissolved in 33 nl water and incubated for 18 h at 16°C in ND96 buffer. Then, oocytes were injected with the indicated radioactively labeled amino acid dissolved in 33 nl of water. Between the pre-injection and the efflux measurements, oocytes were kept for 4 h at 16°C in ND96 to test for a possible leak and to bypass possible volume regulatory effects. No reproducible leak of labeled amino acid was observed during this period. Oocytes were then washed six times with (+Na)-buffer containing 100 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and 10 mM HEPES pH 7.5, and again three times at the start of the efflux measurement. For each group of four oocytes, the buffer was replaced with 300  $\mu\text{l}$  of (+Na)-buffer supplemented with the indicated concentration of amino acid. Three aliquots of 4  $\mu\text{l}$  of buffer were taken at different time points after the start of the incubation (22°C), and the radioactivity was determined by liquid scintillation. Cell L-[<sup>3</sup>H]Phe or L-[<sup>35</sup>S]Cys was determined by liquid scintillation after oocyte lysis in 2% SDS. Absolute efflux values for calculating the stoichiometry were calculated taking into account the measured loss of radioactivity that occurred during the injection and the subsequent pre-incubation, and amounted to 0.2–25%.

### Statistics

Data are expressed as means  $\pm$  SE (SD in the amino acid analysis). The difference between control values (oocytes expressing 4F2hc alone) and test values (4F2hc + LAT1 or LAT2) was evaluated using Student's *t*-test (two tailed, unpaired). The upper limit for a possible facilitated diffusion (efflux) was evaluated using ANOVA followed by Dunnett's multiple comparison test.

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