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The preferential interaction of L-threonine with transport system ASC in cultured human fibroblasts

Ovidio Bussolati [1], Bianca Maria Rotoli [1], Philip C. Laris [2], Valeria Dall'Asta [1] and Gian C. Gazzola [1]

[1] *Istituto di Patologia Generale, Università di Parma, Parma (Italy) and* [2] *Department of Biological Sciences, University of California, Santa Barbara, CA (U.S.A.)*

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The transport of L-threonine was studied in cultured human fibroblasts. A kinetic analysis of L-threonine transport in a range of extracellular concentrations from 0.01 to 20 mM indicated that this amino acid enters cells through both Na^+-independent and Na^+-dependent routes. These routes are: (1) a non-saturable, Na^+-independent route formally indistinguishable from diffusion; (2) a saturable, Na^+-independent route inhibitable by the analog BCH and identifiable with system L; (3) a low-affinity, Na^+-dependent component (K_m = 3 mM) which can be attributed to the activity of system A since it is adaptively enhanced by amino acid starvation and suppressed by the characterizing analog MeAIB and (4) a high-affinity, Na^+-dependent route (K_m = 0.05 mM). This latter route is identifiable with system ASC since it is insensitive to adaptive regulation, uninhibited by MeAIB, *trans*-stimulated by intracellular substrates of system ASC, markedly stereoselective, and relatively insensitive to changes in external pH. At an external concentration of 0.05 mM more than 90% of L-threonine transport is referrable to the activity of system ASC; in these conditions, the transport of the amino acid exhibits typical ASC-features even in the absence of inhibitors of other transport agencies, and, therefore, it can be employed as a reliable indicator of the activity of transport system ASC in cultured human fibroblasts.

Introduction

System ASC is a Na^+-dependent agency that operates to transport small, zwitterionic amino acids across the plasma membrane of mammalian cells [1]. Other features of the system include the intolerance of N-methylation of substrates [2], the presence of exchange properties that produce *trans*-stimulation of unidirectional substrate fluxes [3], the relative insensitivity to pH [4–5], the marked stereospecificity [4], and, although less consistently, the acceptance of Li^+ as a cosubstrate in the place of Na^+ [6]. Originally detected in Ehrlich ascites tumour cells [2], it has been subsequently described in a number of cell models [7–16]; in some, such as CHO cells [17–18], it constitutes the major entry route for neutral amino acids while in others it is scarcely detected.

While specific substrates exist for two other transport systems for neutral amino acids (systems A and L), no natural or analog amino acid has been consistently defined as a specific substrate or inhibitor for system ASC. This fact has greatly hindered the assessment of mechanistic features of system ASC, which, as a consequence, are not characterized in depth. Moreover, since the system is not constant in its selectivity among the substrates in the various cell models [1], the reactivity of a given amino acid for the system has to be ascertained in each model. In some cell types, L-cysteine [4] and L-homocysteine [19] have been claimed to be specific probes for transport activity of system ASC, but since there is an appreciable entry of these amino acids into cells in the absence of extracellular Na^+, their employment as probes of system ASC requires proper discrimination procedures [4,19]. In rat hepatocytes [8] and leukemia cells [20] L-threonine transport exhibits a high degree of selectivity for system ASC

Abbreviations: DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; EBSS, Earle's balanced salt solution; MeAIB, 2-methylaminoisobutyric acid; BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid.

Correspondence: G.C. Gazzola, Istituto di Patologia Generale, Università di Parma, Via Gramsci, 14, 43100 Parma, Italy.

indicating that it can be employed as a probe for the activity of the system in those models.

In this study we report that L-threonine uptake by cultured human fibroblasts occurs through the amino acid transport systems A, ASC and L. The kinetic features of L-threonine influx through these agencies indicate that in a fairly extended range of concentrations, including the half-maximal concentration for system ASC, the amino acid enters cells mostly through system ASC and can be employed as a probe of the transport activity of this system.

Materials and Methods

Materials

Fetal bovine serum (FBS), growth medium and antibiotics were purchased from Gibco. 2-Methylaminoisobutyric acid (MeAIB) was purchased from Aldrich and 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) was from Calbiochem. L-[3-^{3}H]Threonine (630 GBq/mmol) was obtained from Amersham Laboratories; 2-[1-^{14}C]methylaminoisobutyric acid (1.8 GBq/mmol) and 3-*O*-methyl-D-[U-^{14}C]glucose (11.1 GBq/mmol) were from New England Nuclear. Sigma was the source of all the other chemicals.

Cell culture

Human foreskin fibroblasts were obtained from a 15-y old donor and routinely grown in 10-cm diameter dishes (Falcon) in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. The conditions of culture were: pH 7.4, atmosphere 5% CO_2 in air, temperature 37 °C. All measurements of amino acid transport were made on fibroblast subcultures resulting from $3 \cdot 10^4$ cells seeded into 2-cm^2 wells of disposable 24-well trays (Nunc) and incubated for 3 days in 1 ml of growth medium. Cell density, estimated as protein content, ranged from 30 to 40 μg of cell protein per cm^2.

Experimental design and incubations

The transport of neutral amino acids in mammalian cells is mediated by three major agencies, systems A, ASC and L, each endowed with specific properties of substrate recognition, energization and regulation. The discrimination among these systems is achieved by evaluating: (i) the dependence of amino acid influx upon external sodium (systems A and ASC are sodium dependent; system L is sodium independent); (ii) the *trans*-effects by internal amino acids to which the transport activity is sensitive (system A is *trans*-inhibited; systems ASC and L *trans*-stimulated); (iii) the occurrence of adaptive regulations of transport activity (only the activity of system A is adaptively regulated); (iv) the sensitivity to inhibition by the characterizing substrates of system A (MeAIB), and of system L (BCH).

Before the determination of transport activity, cells were incubated for various times in Earle's balanced salt solution (EBSS), containing 117 mM NaCl, 5.5 mM glucose, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, buffered at pH 7.4 with 20 mM Hepes-NaOH, and supplemented with 10% dialyzed FBS. This treatment causes the depletion of intracellular amino acid pool, thus relieving the *trans*-effects on the activity of the various transport systems by intracellular amino acids [11,21], and at longer times of incubation resulting in the expression of the adaptive increase of the activity of system A [22]. In the remainder of this communication we will define: (i) replete cells, fibroblasts in which no incubation in EBSS has been performed; (ii) unstarved depleted cells, fibroblasts incubated in EBSS for 90 min (this incubation produces a marked depletion of the internal amino acid pool [11,21] but it is too short to achieve a significant adaptive increase of the transport activity of system A [22]); (iii) starved depleted cells, fibroblasts incubated for either 3 or 6 h in EBSS, in which a marked adaptive increase of transport activity of system A is achieved [22].

Uptake assay

Amino acid uptake has been measured under conditions of initial entry rates using the cluster-tray method for the rapid measurement of solute fluxes in adherent cells described by Gazzola et al. [23]. Cell monolayers were incubated for 30 s at 37 °C in EBSS at pH 7.4 in the presence of the labelled substrate and, when required by the experimental design, of unlabelled putative inhibitors. The Na^+-independent uptake was determined in a Na^+-free medium in which choline replaced sodium in EBSS. Transport assay was terminated by three rapid washes of the cell monolayer with ice-cold 0.1 M $MgCl_2$ and the cells were fixed in place by the addition of 0.2 ml of ethanol. Extracts were added to 2.5 ml of scintillation fluid and counted for radioactivity with a Packard 460C liquid scintillation spectrometer. Cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 M NaOH and assayed for protein content directly in the well as described previously [23].

Calculations

Amino acid uptake was expressed as μmol (or nmol) per ml of intracellular water per min. The intracellular fluid volume was estimated from the steady state distribution of 3-*O*-methyl-D-glucose as described by Kletzien et al. [24]. The kinetic parameters were evaluated by the analysis of initial velocity data using a BASIC program applying the Marquardt's algorithm. The equations used were:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad (1)$$

for a single saturable system;

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} + K_d \cdot [S] \quad (2)$$

for a saturable system plus diffusion;

$$v = \frac{V_{max1} \cdot [S]}{K_{m1} + [S]} + \frac{V_{max2} \cdot [S]}{K_{m2} + [S]} \quad (3)$$

for two additive saturable systems;

$$v = v_0 - \frac{I_{max} \cdot [I]}{[I]_{0.5} + [I]} \quad (4)$$

for a system competitively inhibited, where:

$$[I]_{0.5} = \left(1 + \frac{[S]}{K_m}\right) \cdot K_i \quad (5)$$

Results

Discrimination of transport systems contributing to the influx of L-threonine: Na^+-independent components

The discrimination of the transport agencies that contribute to the inward transport of L-threonine has been accomplished by a kinetic analysis of the transport over a wide range of extracellular amino acid concentrations (0.01–20 mM). For these experiments depleted cells have been employed, so as to minimize the influence of *trans*-effects on the kinetic parameters obtained.

The results shown in Fig. 1 and Table I indicate that in the absence of extracellular sodium the transport of

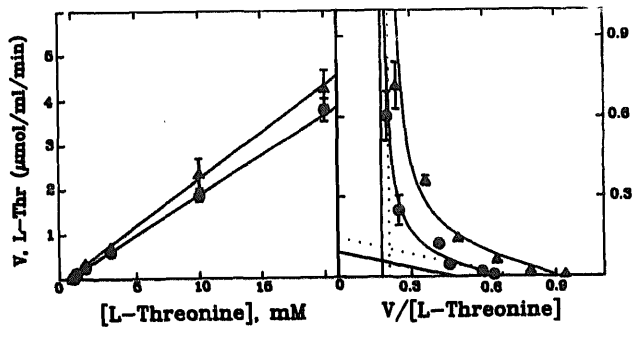

Fig. 1. Kinetic analysis of Na^+-independent L-threonine influx. (Left) Cells grown in 10% FBS-containing DMEM were incubated for 90 min (unstarved cells, ●) or for 6 h (starved cells, ▲) in EBSS containing 10% dialyzed FBS. At the end of this period cell monolayers were washed twice in Na^+-free EBSS with choline substituting Na^+ and L-[^{3}H]-threonine uptake was measured in the same solution. The external concentration of L-threonine ranged from 0.01 to 20 mM. Extracellular osmolality was levelled with proper additions of sucrose. The points are means of three independent determinations with SD indicated. Lines are computer-drawn, best-fit non-linear regressions to Eqn. 2. (Right) Data reported in left panel (up to 3 mM [L-Thr]) are shown in Eadie-Hofstee graphical representation. Curves are computer drawn regressions. Solid and dashed lines are the single components of L-threonine uptake in unstarved and starved cells, respectively.

TABLE I

Kinetic parameters for the Na^+-independent transport of L-threonine

Parameters were obtained by non-linear regression analysis of the data presented in Fig. 1 and are shown with S.E. indicated.

	K_m (mM)	V_{max} (μmol/ml per min)	K_d (min^{-1})
Unstarved	0.20 ± 0.05	0.095 ± 0.022	0.18 ± 0.01
Starved	0.21 ± 0.01	0.15 ± 0.03	0.20 ± 0.01

L-threonine is due to the additive contributions of a single saturable transport agency and of a non-saturable route, formally indistinguishable from diffusion (see Calculations, Eqn. 2). Compared to unstarved cells, only minor increases of V_{max} of the saturable component and of the diffusion constant K_d were detected in starved cells.

The identification of the saturable component was accomplished through an inhibition analysis of Na^+-independent transport of L-threonine. The results, reported in Table II, indicate that a substantial portion of Na^+-independent transport of L-threonine is inhibited by the analog BCH, a specific inhibitor of the Na^+-independent transport system L [11], while both L-glutamate and L-arginine (substrates of the Na^+-independent transport systems x_c^- [25] and y^+ [21], respectively) are without any significant effect. These results suggest that the saturable Na^+-independent route for L-threonine transport is identifiable with transport system L.

Sodium-dependent transport of L-threonine: identification of the low-affinity component with system A

Kinetic analysis of sodium-dependent L-threonine transport was performed in unstarved and in starved

TABLE II

Inhibition of Na^+-independent L-threonine influx by amino acids

Cultured human fibroblasts were washed twice in a sodium-free EBSS. Assay of 0.05 mM L-threonine transport was performed in the same solution in the absence (control) or in the presence of the indicated amino acids, employed as inhibitors after either 90 min (unstarved cells) or 6 h (starved cells) of incubation in EBSS supplemented with 10% dialyzed FBS. Data are means of three independent determinations and are expressed as percentage values of the uninhibited (control) transport. The significance of the inhibition was assessed on transport data (three independent determinations) with an unpaired, two tail *t* test. Values of control were 72.3 ± 6.4 nmol/ml per min and 90.7 ± 7.1 nmol/ml per min in unstarved and starved cells, respectively.

Inhibitor	L-Threonine influx (% of control)	
	unstarved	starved
BCH (2 mM)	34 **	39 **
L-Glu (2 mM)	99	96
L-Arg (2 mM)	96	97

** $P < 0.01$.

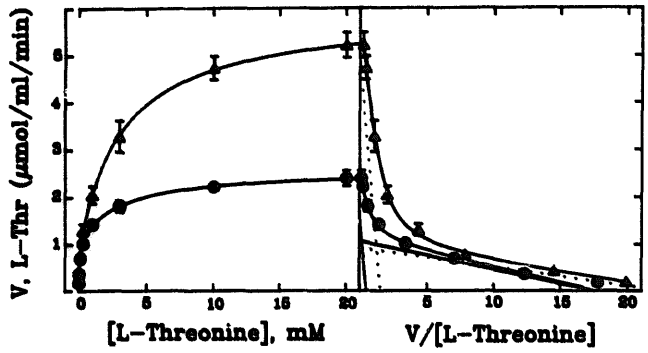

Fig. 2. Kinetic analysis of Na^+-dependent L-threonine influx. (Left) Cells grown in 10% FBS-containing DMEM were incubated for 90 min (unstarved cells, ●) or for 6 h (starved cells, ▲) in EBSS containing 10% dialyzed FBS. At the end of this period cell monolayers were washed twice in EBSS and L-[^{3}H]threonine influx was measured in the same solution (total uptake). Values of Na^+-dependent L-threonine influx were obtained from total influx after subtraction of the influx measured in the absence of extracellular sodium. External concentration of L-threonine ranged from 0.01 to 20 mM with osmolality levelled with proper additions of sucrose. The points are means of three independent determinations with S.D. indicated. Lines are computer-drawn, best-fit non-linear regressions to Eqn. 3. (Right) Data reported in left panel are shown in Eadie-Hofstee graphical representation. Curves are computer drawn regressions. Solid and dotted straight lines are the single components of L-threonine influx in unstarved and starved cells, respectively.

depleted cells both in the absence (Fig. 2) and in the presence (Fig. 3) of the specific inhibitor of system A MeAIB.

In the absence of MeAIB the sodium-dependent transport of L-threonine is satisfactorily accounted for by the additive operations of two distinct transport agencies (see Calculations, Eqn. 3). The calculated kinetic parameters are shown in Table III and indicate that these agencies are endowed with very different

TABLE III

Kinetic parameters for Na^+-dependent influx of L-threonine

Parameters were obtained by non-linear regression analysis of the data presented in Fig. 2 and are shown with S.E.

	K_m (mM)	V_{max} (μmol/ml per min)
High affinity		
Unstarved	0.061 ± 0.008	1.07 ± 0.06
Starved	0.041 ± 0.004	0.93 ± 0.07
Low affinity		
Unstarved	2.86 ± 0.41	1.51 ± 0.05
Starved	3.20 ± 0.40	4.98 ± 0.19

affinities towards L-threonine. A comparison of unstarved and starved cells demonstrated that 6-h starved cells exhibit an enhanced activity of the low-affinity route, kinetically referrable to a 3-fold increase of V_{max}, without significant changes in the apparent K_m. The activity of the high-affinity route is not enhanced by cell starvation.

When the transport assay is performed in the presence of 10 mM MeAIB (Fig. 3), the low-affinity, Na^+-dependent route appears to be completely suppressed. In these conditions the Na^+-dependent transport of L-threonine can be accounted for by the operation of a single agency (see Calculations, Eqn. 1) whose kinetic parameters are similar to those of the high-affinity, Na^+-dependent route and comparable in starved depleted and in unstarved depleted cells.

The inhibition of MeAIB influx by L-threonine is shown in Fig. 4. The pattern of inhibition of MeAIB transport by L-threonine is satisfactorily fitted by an equation (see Calculations, Eqn. 4) that describes a competitive-type inhibition. The maximal inhibition

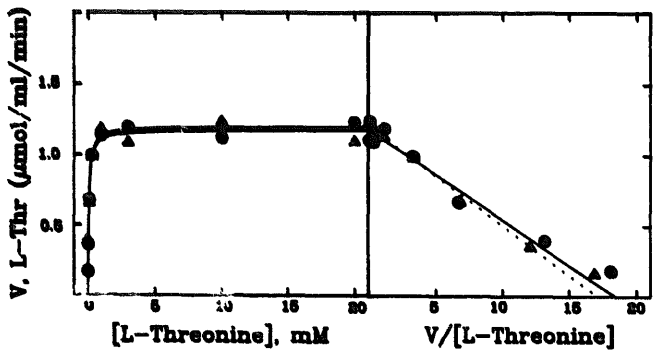

Fig. 3. Kinetic analysis of L-threonine influx in the presence of MeAIB. (Left) The 30-s transport of increasing concentrations of L-threonine (0.01–20 mM) was measured in EBSS in the presence of 5 mM MeAIB both in unstarved (●) and in 6-h-starved cells (▲). Points indicate Na^+-dependent influx obtained for subtraction and are means of three replications. Curves represent the best fit of the data to Eqn. 1. (Right) Data reported in left panel are shown in Eadie-Hofstee graphical representation. Solid and dotted straight lines are L-threonine influx in unstarved and starved cells, respectively.

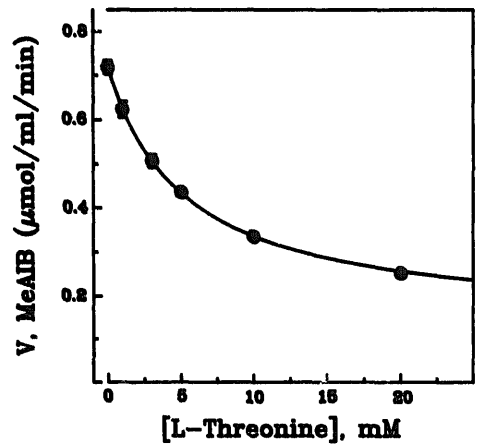

Fig. 4. Inhibition of MeAIB influx by L-threonine. Cultured human fibroblasts were incubated for 6 h in EBSS supplemented with 10% dialyzed FBS. At the end of the incubation, the transport of 0.2 mM MeAIB was determined either in the absence or in the presence of increasing concentrations of L-threonine, as indicated. The curve represents the best fit of the data to Eqn. 4. Points are means of three independent determinations with S.D. shown.

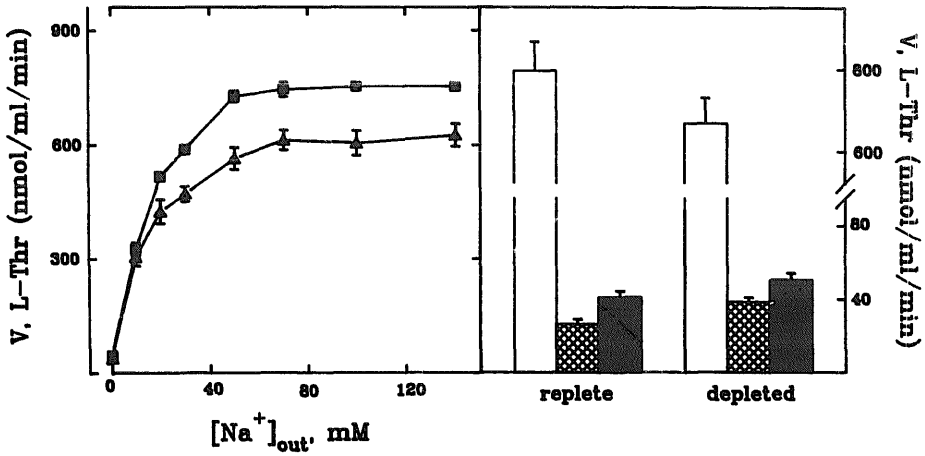

Fig. 5. Sodium dependence of L-threonine transport. (Left) The influx of 0.05 mM L-threonine was measured in replete (■) and in 6-h-starved cells (▲) at increasing $[Na^+]_{out}$. Choline salts substituted for Na^+ salts so as to maintain external osmolality. Data were fitted to an hyperbola; half-maximal transport was obtained at a $[Na^+]_{out}$ of 18.8 ± 2.54 mM for replete and of 15.4 ± 2.00 mM for starved cells. Points are means of three independent determinations with S.D. shown when larger than size of points. (Right) L-Threonine transport was measured both in replete and in 6-h-depleted cells in EBSS (empty bars) or in a modified EBSS in which sodium chloride was substituted by choline chloride (hatched bars) or LiCl (solid bars). Data are means of three independent determinations with S.D. indicated.

calculated accounts for more than 85% of the Na^+-dependent MeAIB uptake measured in the same experiment; the half-maximal inhibition of MeAIB influx is reached at a threonine concentration of 5.2 mM. The kinetic analysis of MeAIB uptake, performed in the same experiment (not shown), yielded a value of 0.224 mM for K_m of MeAIB towards system A; through Eqn. 5 (see Calculations), a K_i of 2.75 mM for inhibition of MeAIB uptake by L-threonine has been calculated. From these results it is possible to conclude that MeAIB and L-threonine interact with the same system with very different affinities, the apparent K_m of the system towards L-threonine being 10-fold higher than that exhibited towards MeAIB.

The above results indicate that the low-affinity component of Na^+-dependent transport of L-threonine is identifiable with system A.

Identification of the sodium-dependent, high-affinity component of L-threonine transport with system ASC

The results described above demonstrate that the sodium-dependent, high-affinity transport component for L-threonine is not adaptively regulated by cell starvation and is not inhibited by MeAIB. These features suggest that this agency can be identified with system ASC. To verify this identification, experiments were performed to compare the properties exhibited by L-threonine transport through the high-affinity, sodium-dependent route with the features generally associated with the operation of system ASC. In these experiments, L-threonine has been employed at an extracellular concentration (0.05 mM) close to the K_m of the amino acid for the component to be identified. From the kinetic parameters enlisted in Tables I and III, it is possible to calculate that the transport of 0.05 mM L-threonine is referrable to this component by more than 80% in starved cells and by more than 90% in unstarved cells. Transport features taken into consideration included: (a) sodium-dependence; (b) *cis*-inhibition by extracellular amino acids; (c) *trans*-stimulation by intracellular amino acids; (d) sensitivity to extracellular pH.

(a) Dependence of influx upon extracellular sodium. The results shown in Fig. 5, left panel, indicate that the influx of L-threonine is dependent on the presence of extracellular sodium by over 90% in both replete and starved cells. The half-maximal activation of the influx of L-threonine is observed at less than 20 mM $[Na^+]_{out}$ under both conditions. Lithium does not substitute effectively for sodium in the activation of the influx of L-threonine (Fig. 5, right panel).

(b)cis-Inhibition of L-threonine influx. Inhibition studies of sodium-dependent L-threonine influx demonstrated (Table IV) that MeAIB has no effect in either replete or depleted unstarved cells. In starved cells, however, MeAIB exerts a small, although significant, inhibition. L-Serine, a substrate of both systems A and ASC [12] markedly inhibits L-threonine transport in all the experimental conditions tested, with inhibitions ranging from 80% (replete cells) to 85% (depleted cells). The inhibition obtained with D-serine is much less, thus indicating that most of L-threonine influx in cultured human fibroblasts occurs through an agency endowed with a marked degree of stereoselectivity. L-Aspartate (a substrate of the Na^+-dependent transport system X_{AG}^- for anionic amino acids [26] and, with low affinity, of system ASC [27]) exerts a small inhibitory effect on L-threonine transport at 2 mM but not at 0.1 mM. D-Aspartate does not exert a significant inhibition of L-threonine influx.

TABLE IV

Inhibition of L-threonine influx by amino acids

Cultured human fibroblasts were washed twice in EBSS. Assay of 0.05 mM L-threonine transport was performed in the same solution in the absence (control) or in the presence of the indicated amino acids immediately (replete cells) or after either 90 min (unstarved cells) or 6 h (starved cells) of incubation in EBSS supplemented with 10% dialyzed FBS. Data are means of three independent determinations and are expressed as percentage values of the uninhibited (control) transport. The significance of the inhibition was assessed on transport data (three independent determinations) with an unpaired, two-tail *t*-test. Values of control were 1.146±0.054 μmol/ml per min for replete cells, 0.712±0.032 μmol/ml per min for unstarved depleted cells, 0.654±0.041 μmol/ml per min for starved depleted cells.

Inhibitor	L-Threonine influx (% of control)		
	replete	unstarved	starved
MeAIB (5 mM)	103	98	89 *
L-Ser (2 mM)	20 **	15 **	16 **
L-Asp (2 mM)	93 *	92 *	91 *
L-Asp (0.1 mM)	98	101	99
D-Ser (2 mM)	56 **	58 **	57 **
D-Asp (2 mM)	101	99	100

* $P < 0.05$.
** $P < 0.01$.

(c)trans-Stimulation of L-threonine influx. L-Threonine influx progressively decreases during the depletion of the intracellular amino acid pool (Fig. 6). Hence, most of L-threonine influx is due to a *trans*-stimulated transport system. In contrast, the transport of L-proline, a preferential substrate of transport system A in cultured human fibroblasts [11], is progressively enhanced by the depletion of amino acid pool. This result is expected from both the decrease of *trans*-inhibition [11] and the triggering of adaptive enhancement of system A activity [22]. Also the transport of L-alanine, a substrate of both transport systems A and ASC in cultured human fibroblasts [12], is enhanced by cell starvation. The results obtained for L-alanine transport demonstrate the predominancy of the contribution of system A to the overall transport of this amino acid.

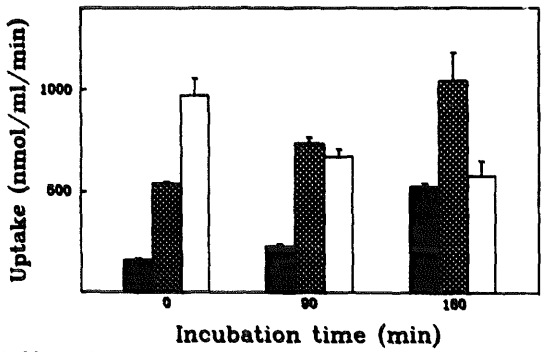

Fig. 6. Neutral amino acid transport in cultured human fibroblasts. Effect of preincubation in an amino acid-free saline solution. Cells were washed twice in EBSS; the 30-s uptake of L-Pro (0.5 mM, solid bars), L-Ala (0.05 mM, hatched bars), or L-Thr (0.05 mM, empty bars) were determined either immediately or after 90 or 180 min of incubation in EBSS supplemented with 10% dialyzed FBS. Data are means of three independent determinations with S.D. indicated.

TABLE V

Trans-stimulation of L-threonine influx

Cultured human fibroblasts were washed twice in EBSS and incubated in the same solution supplemented with 10% dialyzed FBS for 6 h. At the end of this period, cells were incubated for 10 min in the same solution (control) or in EBSS supplemented with the indicated amino acids at a concentration of 1 mM (L-Thr, L-Ala), 5 mM (L-Leu), or 10 mM (MeAIB, L-Glu, L-Arg). The assay of 0.05 mM L-threonine transport was performed after two additional washes in EBSS. The significance of the stimulation was assessed on transport data (means of three independent determinations) with an unpaired, two-tail *t*-test.

Amino acid preaccumulated	L-Threonine influx (nmol/ml per min)	% of control
None (control)	704±31	-
L-Thr	946±27 **	134
MeAIB	680±19	97
L-Ala	1041±13 **	147
L-Glu	671±19	95
L-Leu	788±61	112
L-Arg	688±20	98

** $P < 0.01$.

The experiment recounted in Table V was performed in order to identify the amino acids which *trans*-stimulate the influx of L-threonine. In this experiment cells were first depleted of amino acids for 6 h and L-threonine transport was assessed either immediately or after a 10-min preincubation in the presence of selected amino acids. The results indicate that L-threonine influx is significantly enhanced when cells are preincubated in the presence of L-threonine and L-alanine, but not of L-leucine, L-arginine, L-glutamate or MeAIB.

(d)pH-sensitivity of L-threonine influx. In the experiment shown in Fig. 7, the transport of 0.05 mM L-threonine and of 0.1 mM MeAIB was measured in media at pH ranging from 6.5 to 7.4. In both replete and starved depleted cells the influx of L-threonine exhibits little, if any, sensitivity to pH (Fig. 7, left panel). In contrast, MeAIB influx measured at pH 6.5 is 60–70% lower than that observed at pH 7.4.

Discussion

The results presented here demonstrate that L-threonine enters cultured human fibroblasts through Na^+-dependent and Na^+-independent routes. The relevant kinetic parameters of transport (Tables I and III) indicate that most of the saturable L-threonine uptake is dependent upon the presence of extracellular sodium

and occurs through two distinct agencies endowed with very different affinities towards the amino acid (K_m 0.05 mM vs. 3 mM). The low-affinity system can be identified with system A since its activity is adaptively enhanced by amino acid starvation and is completely inhibited by the analogue MeAIB. Moreover, L-threonine competitively inhibits MeAIB influx with a K_i close to the K_m for the low-affinity system. On the other hand, the activity of the high-affinity system does not change upon cell starvation, it is unaffected by the addition of excess MeAIB, insensitive to changes in external pH in the range 6.5–7.4, endowed with marked stereoselectivity and *trans*-stimulation by intracellular ASC substrates. The above features indicate that this mediation can be identified with transport system ASC.

At variance with results obtained in other models for system ASC [6], is the finding that in cultured human fibroblasts lithium cannot substitute for sodium in activating L-threonine influx. Lithium acceptance, however, is by no means a constant feature of system ASC since it is not observed in many biological models, such as H35 hepatoma cells [28], fetal hepatocytes [28] or CHO cells [18]. Hence, activation by lithium should not be considered a characterizing feature of transport activity mediated by system ASC.

From the kinetic parameters obtained in the present study it is possible to estimate the relative contributions of the three discriminated systems to the saturable inward transport of L-threonine (Fig. 8). The fractional uptake by each system (expressed as the percentage of the total) has been calculated as a function of the external substrate concentration. In the whole range of concentrations considered (from 0.01 to 0.5 mM), system ASC represents most of L-threonine entry in unstarved cells. Even in starved cells, where the capacity of system A is raised towards very high values, over 50% of the saturable L-threonine influx is due to system ASC in the same range of concentrations (not shown). Hence, at low external concentrations of the amino acid system ASC is the major transport component and L-threonine influx can serve as an indicator of transport activity of this agency. L-Threonine appears to be a preferential substrate for system ASC also in other cell models [8,20]; it has already been suggested as a transport probe for system ASC in cultured human fibroblasts [29], although no formal characterization of L-threonine transport was performed in that study.

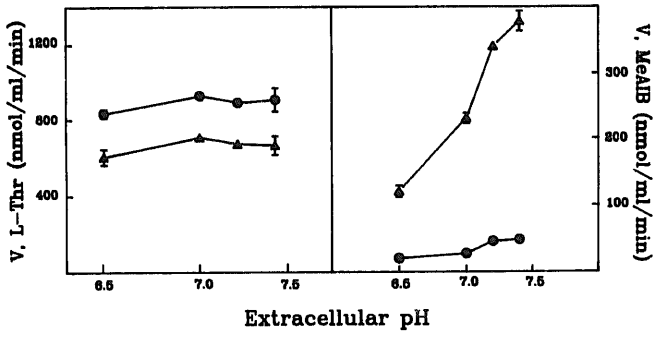

Fig. 7. Sensitivity to pH of L-threonine (left) and MeAIB (right) influx. Cells were washed twice in EBSS and amino acid transport was measured either immediately (●) or after a 6-h incubation in EBSS supplemented with 10% dialyzed FBS (▲). Transport assay was performed in standard EBSS (pH 7.4) or in modified EBSS solutions whose pH had been fixed at 6.5, 7 or 7.2 with additions of 1 M HCl. Results are the mean of three independent determinations with S.D. indicated when larger than size of points.

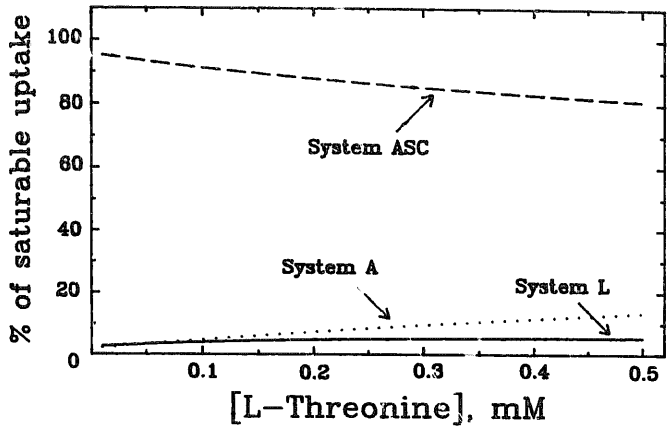

Fig. 8. Percentage contribution of systems A, ASC, and L to the saturable uptake of L-threonine by cultured human fibroblasts as a function of the external concentration of the amino acid. The curves (dashed line, system ASC; dotted line, system A; solid line, system L) have been calculated from the kinetic parameters presented in Tables I and III for depleted unstarved cells.

The greater specificity of L-threonine as a transport probe of system ASC in respect of other substrates of this system is due to both (i) the higher affinity exhibited by L-threonine towards system ASC compared to those exhibited by other substrates of this agency (K_m 0.05–0.06 mM vs. values of 0.08–0.16 mM for L-alanine [12], L-serine [12] and L-glutamine [30]) and (ii) the poor reactivity of the amino acid towards system A (K_m value of 3 mM vs. values of 0.54–0.74 mM found for L-alanine and L-serine [12] and of 1.7–2.5 mM for L-glutamine [30]). It is well known that the presence of an hydroxyl group at a defined distance in the side chain enhances the reactivity of the amino acid towards the receptor site of system ASC [8]. In contrast, the structural features of L-threonine that hinder the interaction of the amino acid with system A are not obvious. It is possible that the position of the hydroxyl group imposes an hindrance on the interaction with system A. Further studies should provide clues for the structure of the binding site of the system as well as for the characterization of analogs for system ASC more specific than L-threonine, that could be used not only as transport probes but also as selective inhibitors of the system. Indeed, it should be noted that the ascertained low-affinity interaction of L-threonine with system A

prevents its employment as a specific inhibitor of system ASC.

At an external concentration close to the K_m of the high-affinity Na^+-dependent system, L-threonine transport exhibits typical features of an ASC-type transport activity even in the absence of inhibitors of other agencies such as system A. The study of *cis*-inhibition and of the *trans*-effects of 0.05 mM L-threonine transport clearly indicates that, at this concentration, the contribution of transport systems other than system ASC is, at best, very limited. Indeed, L-threonine influx exhibits a detectable *cis*-inhibition by high MeAIB concentrations only in starved cells and no significant *trans*-inhibition by internal MeAIB, thus pointing to a very small contribution of system A. Moreover, the small inhibitory effect of L-aspartate cannot be taken as an evidence of the interaction between L-threonine and system X_{AG}^-, since the inhibition of the uptake of L-threonine is only detected at concentrations of L-aspartate at which the anionic amino acid effectively interacts with system ASC [27]. On the contrary, at a concentration of L-aspartate of 0.1 mM, at which system X_{AG}^- is saturated by nearly 90% [26], no inhibition of L-threonine influx is observed.

The elucidation of the operative characteristics of system ASC has been thus far hindered by the absence of a reliable transport probe. Indeed, the experimental procedure usually adopted for the assessment of the activity of the system consists in measuring the Na^+-dependent uptake of alanine or serine in the presence of high concentrations of MeAIB, so as to obstruct the influx of the ASC substrate through system A. This practice may introduce two distinct sources of error. First, the operation of other MeAIB-resistant, Na^+-dependent systems, such as those described in various mammalian models [14,15,31], might be ascribed to system ASC. Second, MeAIB influx through system A can change *per se* important cell parameters such as the membrane potential [32,33] or the intracellular sodium concentration [34]. The employment of L-threonine influx as a probe of system ASC should provide more reliable data on the mechanism of the transport process mediated by this agency in cultured human fibroblasts.

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