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Characteristics of L-glutamine transport during Caco-2 cell differentiation

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Abstract

Glutamine is the main fuel of intestinal epithelial cells, as well as a precursor for the intense nucleotide biosynthesis which arises with the rapid turnover of enterocytes. In order to determine whether glutamine uptake may vary as a function of metabolic demand, glutamine transport across the brush-border membrane of differentiating Caco-2 cells has been investigated. The uptake of L-[^{3}H]glutamine was measured between day 7 and day 21 post-seeding. Kinetic analysis with glutamine concentrations ranging from 6.25 μM to 12.8 mM revealed the involvement of high affinity Na^+-dependent (K_t = 110 μM) and low affinity Na^+-independent (K_t = 900 μM) transport components at day 7. Both components were partially inhibited by L-lysine in a competitive fashion, suggesting that four different systems were responsible for glutamine uptake: B^0, $B^{0,+}$, $b^{0,+}$ and L. All four systems were present during the differentiation process, with systems L and B^0 being responsible for up to 80% of glutamine uptake. Caco-2 cell differentiation was associated with a marked decrease in L-glutamine uptake, which affected both the Na^+-dependent and the Na^+-independent components. In contrast to glucose uptake, the development of L-glutamine uptake across the brush-border membrane of Caco-2 cells may reflect an adjustment to cell metabolic demand rather than the progressive appearance of a vectorial transport process.

Keywords: Glutamine; Amino acid transport; Intestine; Brush-border membrane; Differentiation; Caco-2 cell

1. Introduction

The differentiation of intestinal epithelial cells is characterised by the gradual appearance of vectorial nutrient transport from the gut lumen to the blood. For example, expression of the brush-border Na^+-dependent glucose transporter increases as cells migrate from the crypt to the villus tip and progressively acquire the enterocyte phenotype [1]. The same also applies to the H^+-peptide cotransporter PepT-1, which has been shown to appear during enterocyte differentiation [2]. However, the patterns of expression are probably more complex with respect to amino acid transporters which are involved both in the extraction of dietary amino acids for whole body nitrogen metabolism and in fulfilling of the anabolic requirements of rapidly proliferating epithelial tissue. We recently reported an increase in the glutamic acid transport rate during the differentiation of the intestinal epithelial Caco-2 cell line [3], whereas other studies have shown a decrease in the transport rate

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of alanine and arginine through the B^0 and y^+ systems as cells cease to proliferate and undergo differentiation [4,5].

Among the amino acids, glutamine occupies a central position in intestinal epithelial cells [6]. It constitutes a major metabolic fuel for the rapidly proliferating intestinal epithelium, and also serves as a precursor for the intense nucleotide biosynthesis that arises with the rapid turnover rate of enterocytes [7]. Several studies have shown that glutamine is essential to mucosal growth and to maintain the intestinal function [8]. Its absorption by the small intestine far exceeds that of any other amino acid, and it has been shown to be taken up at a rate equalling that of glucose [9,10]. This study was designed to characterize the transport systems involved in glutamine Na^+-dependent and Na^+-independent uptake at the level of brush-border membrane during the differentiation of Caco-2 cells. This cell line is acknowledged to be a useful model to investigate the processes which occur during the differentiation of intestinal epithelial cells [11]. After confluence, Caco-2 cells undergo spontaneous enterocytic differentiation in culture and mimic the crypt to villus maturation observed in vivo [12,13]. Several epithelial-specific and non-specific transport systems have been shown to be expressed in the brush-border membrane of this cell line [14–17,32]. In 1992, Souba et al. reported that glutamine uptake occurred through a single, high affinity transport pathway resembling system B in 10-day-old Caco-2 cell monolayers [18]. Our results indicate that L-glutamine transport decreases during Caco-2 cell differentiation, and that four different systems are involved in this process. All four systems appeared to be present on both day 7 and day 16 post-seeding.

2. Materials and methods

2.1. Materials

L-[G-^{3}H]glutamine (1.81 TBq mmol^{-1}) was obtained from Amersham (Les Ulis, France). All amino acids and chemicals came from Sigma. Cell culture consumables were obtained from Life Technologies. All amino acids were of the L-isomer, unless otherwise specified.

2.2. Cell culture

The Caco-2 cell line was kindly provided by Dr. G Trugnan (INSERM U450, Paris, France) and was used between passages 47 and 60. Cells were seeded at 4×10^4 cells per cm^{-2} and grown as monolayers in high glucose Dulbecco's modified Eagle's medium (Life Technologies, Cergy-Pontoise, France) supplemented with 15% fetal bovine serum, 1% non-essential amino acids, 6 mM L-glutamine and 200 μg ml^{-1} gentamicin. The culture medium was replaced every 2 days. Confluence was reached on day 7–8 post-seeding. Cells were then sub-cultured using 0.05% trypsin in 0.02% EDTA.

2.3. Transport measurements

In most of the experiments, the uptake of L-glutamine was measured in 24-well plates for 2 min at 37°C using a cluster-tray technique [19]. The culture medium was renewed so as to feed the cells 3 h before the beginning of the transport assay. The culture medium was then discarded and cells were washed for 15 min in HEPES saline buffer (HSB) containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 0.3 mM KH_2PO_4, 10 mM glucose and 10 mM HEPES, adjusted to 7.4 with Tris. Glutamine transport was initiated by adding 1 ml of HSB containing 0.1 mM of L-glutamine trace-labelled with 12.2 GBq L-[G-^{3}H]glutamine (1.81 TBq mmol^{-1}). Uptake was terminated by removing the transport medium and washing the cells with 3×1 ml of ice-cold HSB containing an excess of unlabelled L-glutamine. The cells were harvested in 500 μl of 0.1 N NaOH, and cell-associated radioactivity was determined by liquid scintillation counting (Packard 2200 TriCarb liquid scintillation analyzer). Na^+-independent L-glutamine transport was assessed by omitting NaH_2PO_4 from the HSB and replacing NaCl by choline chloride. The Na^+-dependent transport of L-glutamine was then determined as the difference between the uptakes measured in sodium and choline transport medium. For competition experiments, amino acids were added to the transport medium in 10- or 100-fold excess. All the results presented were corrected for an accumulation of L-glutamine in adherent extracellular fluids, as previously described [20].

The protein contents of wells were determined using the method described by Smith et al. [21], and results were expressed as pmol of amino acid transported per min and per mg of cell proteins.

2.4. Data analysis

Transport measurements were performed in triplicate and all experiments were confirmed using at least two independently seeded generations of cells. Results were expressed as means ± S.E.M. and statistical comparisons were made using Tukey's studentized range test (GLM procedure, SAS 6.03, SAS Institute, Cary, NC, USA). Determination of the kinetic parameters of glutamine transport was achieved by data fitting, using the REG (linear regression) and NLIN (non-linear regression) procedures under the SAS statistical software (SAS 6.03, SAS Institute, Cary, NC, USA).

3. Results

3.1. Kinetics of L-glutamine transport in differentiating Caco-2 cells

The time courses of Na^+-dependent and Na^+-independent glutamine accumulations were first determined on day 7 post-seeding in order to determine the conditions of the subsequent experiments. Glutamine accumulation was shown to proceed at constant rate for up to 4 min for both low (100 μM) and high (12 mM) concentration of glutamine (data not shown). Incubation for 2 min with the radiolabelled substrate was used in the subsequent transport experiments. In the Caco-2 cell line, initiation of the differentiation process began as cells reached confluence, i.e. 6–7 days after plating. uptake of 100 μM glutamine was measured in Caco-2 cells between days 7 and 21 post-seeding, in the presence and absence of extracellular Na^+ (Fig. 1). The gradual increase in the activity of the sucrase-isomaltase marker enzyme and alkaline phosphatase reflected the differentiation process occurring over this period. At any time point, glutamine uptake measured in Na^+-containing transport buffer was about twice that measured in Na^+-free buffer. A dramatic reduction in glutamine uptake was observed between days 7 and 12 (−71%). Thereafter, the glutamine transport rate remained stable until day 21 post-seeding.

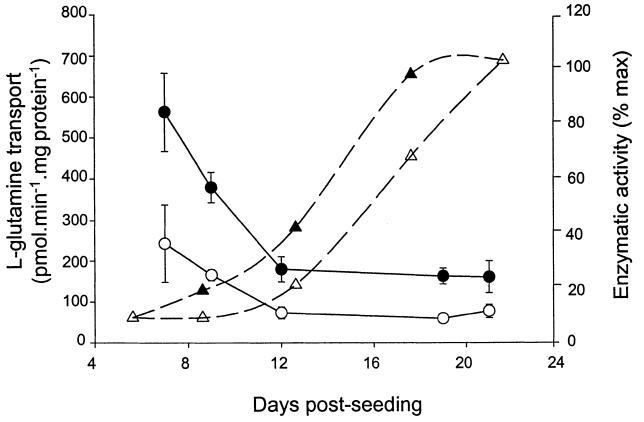

Fig. 1. The effect of cell age on sodium-dependent (●) and sodium-independent (○) glutamine uptake. Initial uptake rates of 100 μM L-[^{3}H]glutamine were measured in NaCl or choline chloride uptake media. Data represent means ± S.E.M. The differentiation of Caco-2 cells was verified by determining sucrase (▲) and alkaline phosphatase (△) activities; the results are expressed as a percentage of maximum activity.

In a second experiment, the kinetics of glutamine uptake across the brush-border membrane of Caco-2 monolayers were measured in the absence and presence of extracellular Na^+, using L-glutamine concentrations ranging from 6.25 μM to 12.8 mM on both day 7 and day 16 post-seeding. As shown in Fig. 2a, the Na^+-independent uptake of L-glutamine was the sum of a saturable process and non-saturable diffusion. Fitting the rate of uptake to Eq. 1 enabled calculation of the permeability coefficient k_{diff} for glutamine across the brush-border membrane.

$$V = V_{max} \times [S]/(k_m + [S]) + (k_{diff} \times [S]) \quad (1)$$

A two-thirds reduction in k_{diff} was observed as the cells differentiated (k_{diff} = 0.75 and 0.25 μl. min^{-1} mg $protein^{-1}$ on day 7 and day 16 post-seeding, respectively). An Eadie–Hofstee plot of the saturable Na^+-independent component of glutamine uptake gave single straight lines on day 7 and day 16 (Fig. 2b). The V_{max} value was significantly higher on day 7 than on day 16 (3213 ± 25 vs. 1548 ± 21 pmol min^{-1} mg $protein^{-1}$, for days 7 and 16, respectively) and the slopes differed.

Na^+-dependent glutamine uptake was determined as being the difference between glutamine uptakes in

the presence and absence of extracellular Na^+. The Na^+-dependent uptake of glutamine was clearly saturable (Fig. 3a). An Eadie–Hofstee plot of Na^+-dependent uptake showed a best fit with a single straight line in both confluent (day 7) and differentiated (day 16 post-seeding) Caco-2 cells (Fig. 3b), with parallel slopes ($K_m = 119$ μM and 106 μM, on day 7 and day 16, respectively). A 75% reduction in the V_{max} value was observed between day 7 and day 16 ($V_{max} = 1371$ and 327 pmol min^{-1} mg^{-1} protein on day 7 and day 16, respectively).

3.2. Amino acid analog inhibition during differentiation

In order to characterize the different transport systems involved in the Na^+-independent and Na^+-dependent uptake of glutamine further, competition experiments were performed using neutral and cationic amino acids and analogs on different days post-seeding. As shown in Table 1, the *N*-methyl amino acid analog methylaminoisobutyric acid failed to inhibit glutamine uptake on both day 7 and day 16. In contrast, there was significant inhibition of both the Na^+-dependent and Na^+-independent uptake of glutamine by 1 mM alanine, leucine, and to a lesser extent, phenylalanine. For both alanine and leucine, inhibition of the Na^+-dependent and Na^+-independent uptake of glutamine was more marked on day 7 than on day 16 post-seeding. In contrast, the inhibition of glutamine uptake by phenylalanine was similar in both confluent and differentiated cells. For

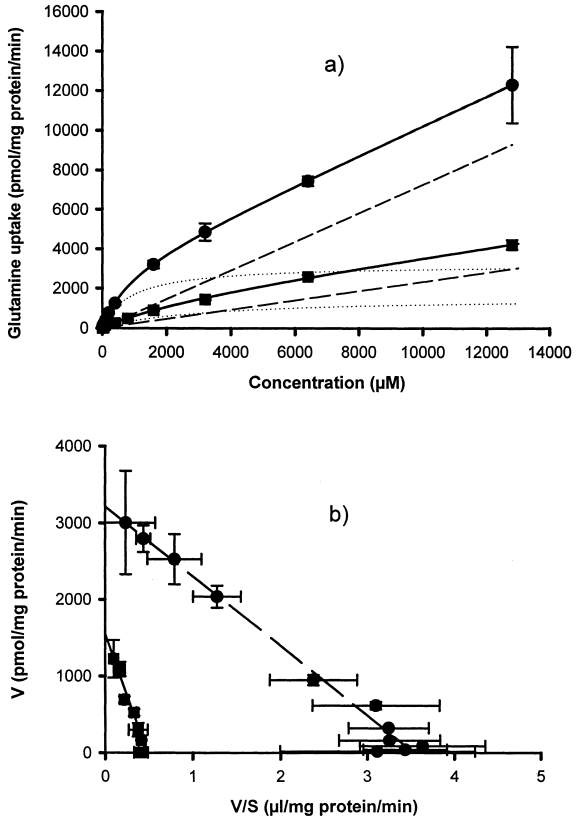

Fig. 2. (a) Na^+-independent component of glutamine transport in Caco-2 cells at day 7 (●) and day 16 (■) with the saturable process (dotted line) and non-saturable diffusion (medium dashed line). (b) Eadie–Hofstee plots of the Na^+-independent saturable component of glutamine transport at day 7 (●) and day 16 (■).

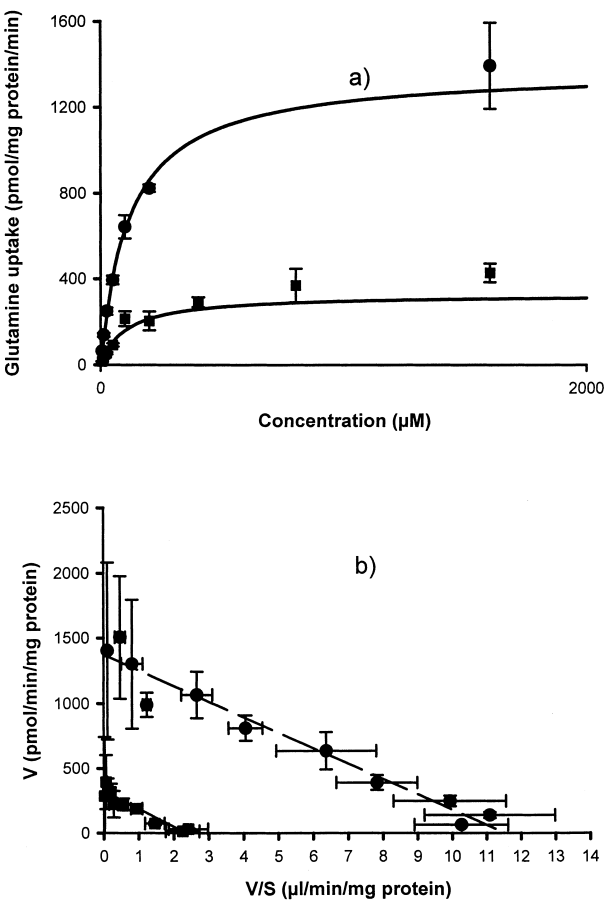

Fig. 3. (a) Na^+-dependent saturable component of glutamine transport in Caco-2 cells at day 7 (●) and day 16 (■). (b) Eadie–Hofstee plots of the Na^+-dependent saturable component of glutamine transport at day 7 (●) and day 16 (■).

this particular competitor, twofold inhibition was achieved for Na^+-independent glutamine uptake as compared to Na^+-dependent uptake. A slight inhibition of Na^+-independent glutamine uptake was also achieved in the presence of 1 mM BCH, a cyclic analog of leucine, whereas this competitor failed to inhibit the Na^+-dependent uptake of glutamine.

L-Glutamine uptake was also partially inhibited by the cationic amino acids lysine and arginine. A significant reduction in Na^+-dependent glutamine uptake was achieved in the presence of a 10-fold excess of arginine or lysine, whereas Na^+-independent uptake was only inhibited in the presence of a 100-fold excess of cationic amino acids. Inhibition of the transport of one amino acid by another does not imply transport via the same carrier unless the inhibition can be shown to be competitive. So as to investigate the nature of the inhibition of glutamine Na^+-dependent and Na^+-independent uptake by lysine further, the Na^+-dependent and Na^+-independent uptake of 100, 200 and 400 μM glutamine was measured in the presence of 2.5, 5 or 10 mM lysine, on both 7-day-old and 16-day-old Caco-2 cells. Dixon plots are presented in Fig. 4. On both day 7 and day 16 post-seeding, inhibition by lysine of Na^+-independent glutamine uptake ($k_i = 6$ and 8 mM, for day 7 and day 16 post-seeding, respectively) and Na^+-dependent glutamine uptake ($k_i = 16$ and 10 mM, for day 7 and day 16 post-seeding, respectively) was clearly competitive.

4. Discussion

The intestinal regulation of neutral (dipolar or zwitterionic) amino acid absorption has been predicted by whole animal and tissue studies [22,23]. These experiments emphasize the multiplicity of transport pathways through which glutamine can be translocated into the cytoplasm of the cell. As a result of such studies, various distinct amino acid transport systems have been defined on the basis of their amino acid selectivities and physicochemical properties, each system presumably relating to a discrete, putative membrane-bound transporter protein.

The present study provides new insight concerning characterization of the transport systems involved in glutamine uptake across the brush-border membrane of Caco-2 cells, and their regulation during the differentiation process occurring after confluence. Our results indicate that: (i) glutamine uptake involves both Na^+-dependent and Na^+-independent saturable transport processes as well as passive diffusion; (ii) the Na^+-dependent uptake of L-glutamine occurs mainly through the B^0 system described by Christensen [24] whereas a small contribution of the cationic and neutral amino acid transporter $B^{0,+}$ can not be excluded; (iii) systems L and $b^{0,+}$ are responsible for the Na^+-independent uptake of glutamine at the brush-border membrane level, and (iv) a reduction in the Na^+-dependent and the Na^+-independent uptake of glutamine occurs as the cell differentiates.

Table 1
Percentage inhibition of specific uptake of L-glutamine (100 μM) uptake by amino acids and their analogs in Caco-2 cells at days 7 and 16 post-seeding

	Na^+-dependent		Na^+-independent	
	Day 7	Day 16	Day 7	Day 16
L-Glutamine				
+L-Alanine 10×	70.17 ± 1.11*	53.95 ± 6.53*	59.75 ± 10.34*	25.77 ± 5.57*
+L-Leucine 10×	42.25 ± 2.55*	27.64 ± 6.24*	52.15 ± 1.93*	34.84 ± 4.60*
+L-Phenylalanine 10×	21.44 ± 3.03*	26.65 ± 6.43	48.09 ± 3.00*	44.77 ± 1.56*
+L-Phenylalanine 100×	56.56 ± 3.36*	53.79 ± 3.65*	76.36 ± 4.80*	38.56 ± 2.20*
+L-Lysine 10×	15.88 ± 3.51*	26.28 ± 3.44*	3.20 ± 2.41	6.93 ± 3.44
+L-Lysine 100×	15.98 ± 5.31*	19.85 ± 7.21*	31.09 ± 2.82*	10.68 ± 2.82
+L-Arginine 10×	31.41 ± 4.47*	9.21 ± 3.07	9.50 ± 3.53	6.29 ± 5.53
+MeAIB 10×	2.57 ± 5.02	26.85 ± 2.88*	−11.35 ± 4.86	−10.48 ± 5.57
+BCH 10×	3.72 ± 4.34	10.28 ± 4.89	36.92 ± 2.29*	21.92 ± 7.56

The uptake of 100 μM L-Glutamine was measured at 37°C for 2 min in the presence and absence of sodium and a 10- or 100-fold excess of the competitor. Points are means ± S.E.M. of $n = 6$–44 determinations. *Denotes a significant inhibition at $P < 0.05$.

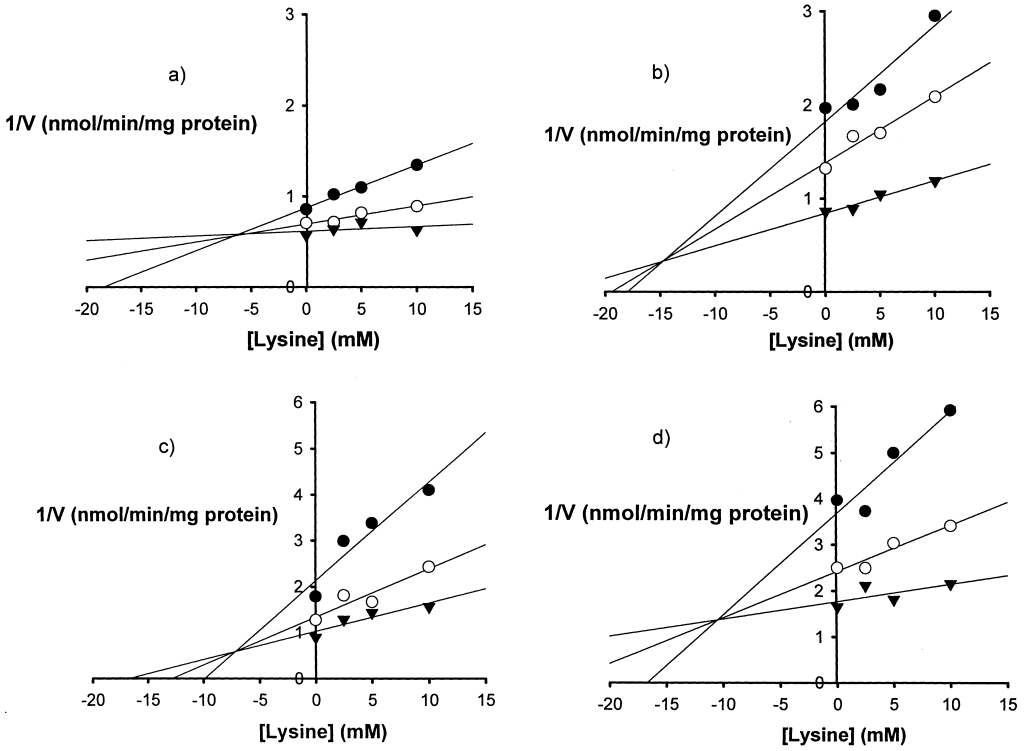

Fig. 4. Dixon plots of the inhibition of Na^+-dependent and Na^+-independent glutamine transport by lysine at days 7 and 16. Initial rates of transport were measured over 2 min in the presence of various inhibitory concentrations in the presence and absence of Na^+. (a) J7 Na^+-dependent (b) J16 Na^+-dependent (c) J7 Na^+-independent (d) J16 Na^+-independent: (●), 0.1 mM glutamine; (○), 0.15 mM glutamine; (▼), 0.25 mM glutamine.

The passive diffusion of glutamine across the brush-border membrane of Caco-2 cells has also been reported by Souba et al. [18] but its precise contribution to glutamine uptake has not been assessed. Our results indicate that, in both confluent and differentiated Caco-2 cells, the passive transmembrane diffusion of glutamine contributes to up to 30% of the Na^+-independent uptake of glutamine for 1 mM glutamine, and exceeds saturable Na^+-independent uptake for glutamine concentrations above 3 mM. It therefore appears that at high substrate concentrations, diffusion plays a major role in glutamine uptake, whereas its contribution is only limited at glutamine concentrations below 1 mM.

Although kinetic analysis of the Na^+-independent uptake of glutamine suggested the involvement of a single, low-affinity transport component, the implication of two different systems, namely systems L and $b^{0,+}$ seems more likely in the light of the results of inhibition experiments. The involvement of system L in the Na^+-independent uptake of glutamine can be inferred from the inhibition of glutamine uptake in the presence of neutral amino acids such as alanine, leucine or phenylalanine, and cyclic analogs such as BCH [25,26], whereas the involvement of system $b^{0,+}$ can be deduced from the competitive inhibition of glutamine uptake by lysine [29]. However, the contribution of the latter system appears to be marginal according to the modest inhibition observed in the presence of lysine and most of the Na^+-independent uptake of glutamine occurs through system L. Two variants of this transport system have been described [24]. The L1 variant corresponds to a high-affinity transport system mediating the uptake of bulky dipolar amino acids such as leucine and phenylalanine whereas the L2 variant (which also transports small neutral amino acids such as alanine and serine) exhibits broader substrate selectivity and K_m in the millimolar range. According to our results, the L2 variant of system L is more likely to be involved in glutamine uptake. Recently, a protein, LAT-2 has been identified as being responsible for the expression of an L2-like transport system when co-expressed with the 4F2-heavy chain [27,28]. This pro-

tein is present in the intestinal epithelium, with a predominant localization at the level of the basolateral membrane [28]. Whether LAT-2 is expressed in the Caco-2 cell line has still to be demonstrated.

According to the results of these kinetic experiments, the Na^+-dependent uptake of glutamine occurred through a single, high-affinity transport system on both days 7 and 16 post-seeding, with a K_m within the same range as that previously reported by Souba et al. [18], whereas a careful examination of the results of our competition experiments showed that they tended towards the involvement of two transport systems with similar kinetic characteristics. According to Christensen [7] and Maillard [30], glutamine uptake at the brush-border membrane level may occur through systems A, ASC, B^0 and $B^{0,+}$. Although results from Nicklin et al. suggest that the system A might be present in the brush-border membrane of Caco-2 cells [31], its involvement in the Na^+-dependent uptake of glutamine can be ruled out, as glutamine uptake was unaffected by a 10-fold excess of the system A-specific substrate MeAIB [32]. Rather, the marked inhibition observed in the presence of a 10-fold excess of alanine, leucine, or phenylalanine together with the slight but significant inhibition observed in the presence of lysine are in favor of a glutamine uptake through system B^0 and $B^{0,+}$. The presence of both transport systems in the brush-border membrane of the Caco-2 cell line have previously been reported [33,34]. Since increasing the lysine concentration in the transport medium to 10 mM did not decrease the Na^+-dependent uptake of glutamine for more than 15% whereas doing the same with phenylalanine resulted in a 50% inhibition of glutamine uptake, it can be inferred that the majority of the Na^+-dependent uptake of glutamine occurs through system B^0 and that the contribution of system $B^{0,+}$ to this process is small. These findings are in line with previous observations of a major involvement of system B^0 in the uptake of glutamine in intestinal brush-border membrane vesicles and in the Caco-2 cell line [18,35]. However, this is the first report of a small but significant contribution of system $B^{0,+}$ to glutamine uptake.

The report of a differentiation-dependent decrease in glutamine uptake represents a major result of our study. This decrease is the consequence of different processes occurring simultaneously during that period. Changes in the lipid and protein composition of the brush-border membrane that are known to occur during the differentiation of intestinal epithelial cells are likely to be responsible for the reduction in the passive diffusion of glutamine across the apical membrane of Caco-2 cells [36] whereas a specific reduction of the expression of transporter proteins might account for the decrease in the carrier-mediated uptake of glutamine. Such a reduction was proposed by Pan and Stevens [4] who have reported a decrease in the uptake of the B^0 substrate alanine during Caco-2 cell differentiation. This reduction may also affect the proteins responsible for the activities of the L and $b^{0,+}$ transport system as the Na^+-independent uptake of glutamine was also found to decrease during the differentiation process. In contrast, the slight increase in lysine inhibition of the Na^+-dependent uptake of glutamine observed during differentiation rather suggests that the expression and/or activity of the $B^{0,+}$ transport system was preserved during this process.

The observation that glutamine uptake across the brush-border membrane decreases as Caco-2 cells differentiate is challenging. A major function of differentiated intestinal epithelial cells is to ensure the vectorial flux of nutrient from the gut lumen to the blood. An increase in the transport capacity of glucose and peptides has long been reported to occur during the differentiation of Caco-2 cells. In terms of glutamine and other neutral amino acids, the decrease in transport capacity observed during the differentiation process is more likely to reflect an adaptation of the transporter expression and/or capacity to meet cell metabolic requirements. Glutamine is known to be essential to intestinal epithelial cell proliferation, and the need for glutamine is likely to decrease as cells cease to proliferate and undergo differentiation. The reduction in glutamine uptake, together with the concomitant increase in glutamic acid transport previously reported, may reflect a gradual shift from glutamine to glutamic acid utilization during Caco-2 cell differentiation.

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