EAAT1 is involved in transport of L-glutamate during differentiation of the Caco-2 cell line

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Received 9 September 1999; accepted in final form 24 March 2000

ENTEROCYTE DIFFERENTIATION is associated with increased expression of the enzymes and transporters involved in the digestive and absorptive functions of the intestinal epithelium. Epithelial cell lines undergoing differentiation and mimicking enterocyte differentiation represent unique tools for the analysis of these functions during enterocyte differentiation (25, 30). The Caco-2 cell line, which undergoes spontaneous enterocytic differentiation, is well recognized as being the most relevant in vitro model to investigate the impact of differentiation on the absorptive functions of epithelial cells (30). In this regard, the uptake of a variety of ions and organic nutrients including hexoses, bile acids, dipeptides, inorganic phosphate, and vitamin B12 by Caco-2 cells resembled that which occurs in the small intestinal epithelium (3, 7, 11, 18, 34). This model and others have helped us to gain an understanding of the acquisition of hydrolase activities during epithelial cell differentiation (25). In contrast, few data are currently available regarding the expression of amino acid transporters, primarily because of the overlapping specificity of these transporters and a lack of molecular characterization (19).

The dicarboxylic amino acid L-glutamate is mainly involved in energy metabolism and thus plays a key role in epithelial cell physiology. Five L-glutamate transporters mediating L-glutamate uptake through an X_A,G transport system have recently been identified in the central nervous system and cloned from a human cDNA library (1, 2, 9). These proteins exhibit similar functional characteristics and constitute the five subtypes of the excitatory amino acid transporter (EAAT) family. The expression of EAAT2, EAAT4, and EAAT5 is mainly restricted to the brain and the retina, whereas sites in which the expression of EAAT1 and EAAT3 transcripts has been reported include heart, lung, liver, kidney, placenta, and small intestine (1, 12). Whether the effects associated with the enterocyte differentiation extend to the transport of L-glutamate has not so far been investigated.

In this study, we addressed the transport of L-glutamate in Caco-2 cells and its regulation during the enterocytic differentiation process. Our results show an increased transport of L-glutamate in differentiated Caco-2 cells. Furthermore, the cell line expresses the messenger for EAAT1, whereas EAAT3 mRNA are barely detectable. Interestingly, we have observed an increased expression of EAAT1 in differentiated Caco-2 cells, suggesting that the increased transport of L-glutamate in differentiated Caco-2 cells is related to increased expression of the EAAT1 transporter.
EXPERIMENTAL PROCEDURES

Cell Culture

The Caco-2 cell line was kindly provided by Dr. G. Trugnan (INSERM U410, Paris, France) and was used between passages 50 and 79. Cells were seeded at 4 × 10^5 cells/cm^2 and grown as monolayer cultures 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-glutamate, and 200 μg/ml gentamicin. The medium was replaced every 2 days. Confluen ce was reached on days 7–8 after seeding. Cells were then subcultured using 0.05% trypsin in 0.02% EDTA.

Transport Measurements

The uptake of L-glutamate was measured in 24-well plates for 2 min at 37°C using a cluster-tray technique (5). The culture medium was renewed to feed cells 3 h before the transport assays started. Cells were then prepared for transport experiments by incubation of the monolayer for 15 min in HEPES-saline buffer (HSB) containing (in mM) 137 NaCl, 5.4 KCl, 2.8 CaCl_2, 1 MgSO_4, 0.3 NaH_2PO_4, 0.3 KH_2PO_4, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with Tris. This buffer was then discarded, and L-glutamate transport was initiated by adding 1 ml of HSB containing 0.01–2 mM L-glutamate trace-labeled with 9.25 kBq of L-[3H]glutamate (1.8 TBq/mmol; Amersham, Les Ulis, France). Uptake was terminated by removing the transport medium and washing the cells with 3 ml of ice-cold HSB containing an excess of unlabeled L-glutamate. The cells were harvested in 500 μl of 0.1 N NaOH, and the cell-associated radioactivity was determined by liquid scintillation counting. In selected experiments, Na^+ -independent L-glutamate transport was evaluated. For this purpose, NaH_2PO_4 was omitted from the HSB, and NaCl was replaced by choline chloride. Na^+ -dependent L-glutamate transport was calculated as the difference between the transport measured in Na^+ -containing and Na^-free buffers. For competition experiments, amino acids were added to the transport medium at a final concentration of 100 μM. In one experiment, L-glutamate accumulation was compared in (aminooxy)acetate-treated and nontreated Caco-2 cells to evaluate the contribution of transport and metabolism to L-glutamate accumulation. The transaminase inhibitor (aminooxy)acetate (final concentration 2.5 mM) was added in the culture medium 15 min before the beginning of the transport experiment. The uptake of L-glutamate was then measured after 5 min, as described above. All the results presented were corrected for the accumulation of L-glutamate in adherent extracellular fluids, as previously described (16). The protein contents of wells were determined using the method developed by Smith et al. (32), and results were expressed as picomoles of L-glutamate transported per milligram of cell proteins.

RNA Extraction

Total RNA from Caco-2 cells seeded in six-well plates was extracted using TRI InstaPure reagent (Eurigenter, Seraing, Belgium). One milliliter of TRI InstaPure was added per ten square centimeters, and the cell lysates were homogenized by repeated pipetting. RNA extraction was performed according to the manufacturer’s specifications, and the RNA was resuspended in RNase-free water. The control and normalization of RNA samples was achieved using densitometry analysis of the ribosomal 28S and 18S on an ethidium bromide-stained agarose gel and optical density quantification.

RT-PCR

For RT-PCR assays, the EZ rtth RNA PCR kit (Perkin Elmer, Branchburg, NJ) was used with primers chosen from the cDNA sequence of the human EAAT1 and EAAT3 transporters identified by Arriza et al. (Ref. 1; Table 1). Before amplification, total RNA samples were treated with deoxycy-

Table 1. EAAT1 and EAAT3 primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence</th>
<th>cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAT1-5’</td>
<td>5’-ACC ACC ACC AAC GTA CTG GG-3’</td>
<td>1468–1487</td>
</tr>
<tr>
<td>EAAT1-3’</td>
<td>5’-TTG GTT TCA CTG TCG ATG GG-3’</td>
<td>1633–1652</td>
</tr>
<tr>
<td>EAAT3-5’</td>
<td>5’-GCT CTC CAA GAA GGA GCT GG-3’</td>
<td>1410–1429</td>
</tr>
<tr>
<td>EAAT3-3’</td>
<td>5’-TCC AGT CAT CTG CAG CCA GG-3’</td>
<td>1598–1617</td>
</tr>
<tr>
<td>T7 EAAT1-3’</td>
<td>5’-TAA TAC GAC TCA CTA TAG GTT TCA CTG TCG ATG GG-3’</td>
<td>1633–1649</td>
</tr>
</tbody>
</table>

The primers used for RT-PCR and RPA riboprobe construction were chosen from the cDNA sequence of the transporters cloned by Arriza et al. (1). EAAT, excitatory amino acid transporter; RPA, ribonuclease protection assay. Bold letters refer to T7 promoter.
the transcription reaction. After digestion with the restriction enzyme, the template was purified using the Magic DNA clean-up system (Promega, Madison, WI). The antisense EAAT3 riboprobe was synthesized using the same method as that used for the EAAT1 probe (BrightStar BIOTINscript kit, Ambion).

**Ribonuclease Protection Assay**

RPA was performed as described in the RPA II kit (Ambion). Total Caco-2 RNA (25–100 μg) was hybridized with 1,250 pg of the biotinylated riboprobe (EAAT1 or EAAT3) at 45°C overnight, and digestion was ensured with the 1/1,000 diluted RNase A-RNase T1 mixture of the kit for 30 min at 37°C. The protected fragments were separated on a 5% denaturing polyacrylamide gel. After electrophoresis on a nylon membrane (Ambion) the detection procedure was carried out using the BrightStar Biodetect kit (Ambion), and the membrane was washed with both Kodak X-OMAT AR radiography film (1 h) and Ilford photographic paper (overnight). The scanning of areas was achieved using NIH Image 1.60.

**Enzymatic Activities**

All enzyme activities were measured spectrophotometrically by recording the appearance of the reaction product as a function of time. Sucrase activity was determined according to Dahlqvist (4). Alkaline phosphatase activity was assessed according to the Eichholz technique (8) using p-nitrophosphosphate as substrate.

**Calculations and Statistics**

All experiments were conducted in triplicate, and all experiments were confirmed using at least two independently seeded generations of cells. Results are expressed as means ± SE; statistical comparisons were made using the Tukey’s studentized range test (GLM procedure, SAS 6.03, SAS Institute, Cary, NC). Transport kinetic parameters were obtained by fitting data to the Michaelis-Menten equation or to a linear model (NLIN and REG procedures, SAS 6.03).

**RESULTS**

**Characterization Of L-Glutamate Transport in Confluent Caco-2 Cells**

The uptake of L-glutamate was measured on Caco-2 cells cultured in plastic dishes on day 8 after seeding.

Fig. 1. Initial time course of L-glutamate uptake into day 8 Caco-2 cells. The uptake of 10 μM L-glutamate was measured in the presence of 137 mM extracellular sodium. Values are means ± SE of n = 6 determinations.

In the absence of extracellular Na⁺, there was no detectable uptake of L-glutamate after correction of the data for accumulation in extracellular adherent fluids (data not shown). In the presence of 137 mM NaCl, the rate of L-glutamate accumulation was almost constant over a 10-min period and slightly decreased thereafter (Fig. 1). The rate of glutamate accumulation for the first 5 min was not affected by cell pretreatment with the transamination inhibitor (aminooxy)acetate [uptake rate = 92 ± 6 and 87 ± 10 pmol mg protein⁻¹ min⁻¹ in (aminooxy)acetate-treated and nontreated cells, respectively]. Incubation for 2 min with the radiolabeled substrate was used in the subsequent transport experiments. The stoichiometry of Na⁺-L-glutamate cotransport was investigated by measuring the uptake of 10 μM L-glutamate with an extracellular Na⁺ concentration ([Na⁺]) ranging from 0 to 140 mM (Fig. 2). These data were fitted to the Hill equation, \( V/V_{\text{max}} = [\text{Na}^+]^{n_H}/K_m([\text{Na}^+]^{n_H} + [\text{Na}^+]^{n_H}) \) (where \( V_{\text{max}} \) is maximum membrane uptake capacity, \( K_m \) is the Michaelis-Menten constant, and \( n_H \) is the Hill coefficient), using the NLIN procedure of the SAS software. The solid curve representing the best fit of the point was clearly sigmoidal. \( K_m(\text{Na}^+) \) was 65 ± 2 mM and \( n_H \) was 2.9 ± 0.3, suggesting that three sodium ions are cotransported with one glutamate. The measurement of Na⁺-dependent L-glutamate accumulation with L-glutamate concentrations ranging from 0.020 to 2 mM showed that transport of this amino acid was concentration dependent (Fig. 3A). Analysis of the kinetic data for Na⁺-dependent L-glutamate transport by the Eadie-Hofstee method suggested the existence of a single high-affinity transport system in Caco-2 cells, with a \( K_m \) of 0.107 ± 0.021 mM and \( V_{\text{max}} = 1,143 ± 118 \frac{\text{pmol}}{\text{mg protein} \cdot \text{min}}\).
pmol · mg protein⁻¹ · min⁻¹ (Fig. 3B). The possible inhibition of L-glutamate accumulation by different amino acids was also investigated to further characterize the transport system involved in L-glutamate transport in the Caco-2 cell line (Table 2). The rate of L-glutamate uptake into day 8 Caco-2 cells was affected neither by neutral amino acids such as L-alanine, L-proline, and L-leucine nor by the cationic amino acid L-lysine. The addition of 100 μM D- or L-aspartate reduced L-glutamate accumulation by 77% and 84%, respectively, whereas no competition was observed between L- and D-glutamate.

Transport of L-Glutamate During Caco-2 Cell Differentiation

Dramatic changes have been shown to occur during Caco-2 cell proliferation and differentiation. The rate of glutamate uptake was measured in Caco-2 cells between day 2 and day 22 after seeding (Fig. 4). On reaching confluence, Caco-2 cells underwent differentiation as assessed by the sharp increase in alkaline phosphatase and sucrase observed between day 8 and day 17 after seeding. During that period, a dramatic increase (+160%) was also seen in the rate of L-glutamate uptake. At any time after seeding, the inhibition of 10 μM L-glutamate uptake by 100 μM D-aspartate was similar to that measured on confluent cells (−71%, −91%, and −89% on days 5, 10, and 17, respectively). As previously described in confluent Caco-2 monolayers (21), L-glutamate accumulation in fully differentiated Caco-2 cells was insignificant when choline was substituted for Na⁺ (data not shown). The kinetic characterization of Na⁺-dependent L-glutamate uptake on day 17 after seeding was achieved using an L-glutamate concentration in the 0.02–2 mM range (Fig. 5). The Eadie-Hofstee representation clearly demonstrated that L-glutamate transport involved a single component with a $K_m$ of 0.118 ± 0.070 mM, i.e., similar to that determined on day 8 after seeding, and a $V_{max}$ of 2,666 ± 310 pmol · mg protein⁻¹ · min⁻¹, i.e., a 2.5-fold increase compared with confluent cells.

Table 2. Effect of amino acid competitors on L-glutamate uptake in Caco-2 cells

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>L-Aspartate</th>
<th>D-Aspartate</th>
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<tbody>
<tr>
<td>L-Alanine</td>
<td>66 ± 13</td>
<td>17 ± 4*</td>
</tr>
<tr>
<td>L-Proline</td>
<td>65 ± 6</td>
<td>12 ± 3*</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>63 ± 3</td>
<td>17 ± 4*</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>63 ± 3</td>
<td>12 ± 3*</td>
</tr>
<tr>
<td>D-Glutamate</td>
<td>77 ± 5</td>
<td>17 ± 4*</td>
</tr>
<tr>
<td>D-Leucine</td>
<td>63 ± 3</td>
<td>12 ± 3*</td>
</tr>
</tbody>
</table>

The transport of 10 μM L-glutamate was measured for 2 min in the presence of extracellular sodium and a competitor (100 μM). Values are means ± SE of $n = 6$ determinations. *Significant inhibition at $P < 0.05$. 

Fig. 3. Concentration dependence of Na⁺-dependent L-glutamate transport 8 days after seeding of Caco-2 cells. Uptake was measured for 2 min in the presence of extracellular sodium over a range of 0.02–2 mM L-glutamate into day 8 Caco-2 cells. Na⁺-dependent L-glutamate transport was calculated as the difference between the transport measured in Na⁺ and choline uptake media. A: V vs. substrate concentration plot. B: Eadie-Hofstee representation. Values are means ± SE of $n = 6$ determinations.

Fig. 4. Effect of cell age on L-glutamate transport in Caco-2 cells. The uptake of 10 μM L-glutamate (●) was measured for 2 min in the presence of extracellular sodium in Caco-2 cells of various ages ranging from 2 days to 22 days after seeding. A confluent monolayer was attained on day 8. Values are means ± SE of $n = 6$ determinations. The differentiation of Caco-2 cells was controlled by the determination of sucrase (●) and alkaline phosphatase (▲) activities; the results are expressed as the percentage of maximum activity.
EAAT1 and EAAT3 Expression During Caco-2 Cell Culture

With RT-PCR, both EAAT1 and EAAT3 mRNA were seen to be present in the total RNA of day 17 Caco-2 cells (Fig. 6). The expression levels of EAAT1 and EAAT3 mRNA were then measured in Caco-2 cells at different ages (2, 6, 10, 14, 17, and 21 days after seeding) by RPA using 25 µg of total RNA. On day 2 and day 6 after seeding, the level of the EAAT1 transcript was below the detection limit. However, EAAT1 mRNA was clearly detected on day 10, and its level steadily increased between day 10 and day 17 (Fig. 7). Densitometric analysis indicated a fivefold increase in EAAT1 transcript abundance between day 10 and day 21. Under the same conditions, we failed to detect any EAAT3 transcript on any day after seeding (data not shown). With a larger amount of total RNA (100 µg) in the RPA, a faint signal from the protected fragment corresponding to the EAAT3 transcript was observed on day 17 after seeding (Fig. 8).

DISCUSSION

Although several studies have focused on the uptake of dipolar and cationic amino acids in the Caco-2 cell
line and suggested a decrease in the transport capacity through systems B0 and γ+ during enterocytic differentiation (22, 23), the transport of anionic amino acids and its evolution during epithelial cell maturation have received little attention. Our results demonstrate that 1) L-glutamate uptake occurs through a single, high-affinity Na+−dependent component that exhibits the functional characteristics of the XAG system; 2) expression of the XAG-like system is modulated during epithelial cell differentiation, with a steep increase in Vmax occurring when cells cease to proliferate and undergo enterocytic differentiation; 3) L-glutamate uptake in the Caco-2 cell line is associated with expression of the EAAT1 transporter subtype; and 4) enhanced L-glutamate transport in differentiated Caco-2 cells is correlated with increased levels of EAAT1 transcripts.

Our results indicate that the uptake of L-glutamate in the Caco-2 cell line involved a single, high-affinity Na+−dependent transport system. Assuming a cell volume of 3.7 μL/mg protein (3, 6), a 12-fold ratio between the intracellular and extracellular glutamate concentrations was achieved after 5 min. This high ratio did not reflect glutamate metabolism but was the consequence of a true accumulative transport process, as reflected by comparison of the rate of uptake measured in (aminoxy)acetate-treated and nontreated Caco-2 cells. In contrast to our previous observation in the rat IEC-17 intestinal cell line (17), we have found no evidence for the existence of Na+−independent L-glutamate transport or for a low-affinity Na+−dependent transport component. Striking differences have already been reported between these two intestinal epithelial cell lines concerning amino acid transport: the Caco-2 cell line was shown to exhibit a high transport capacity for dipolar amino acids through the B0 transport system soon after passaging, whereas we found no evidence for the presence of this epithelium-specific transport system in the IEC-17 cell line (16). The IEC-17 intestinal cell line exhibits many of the characteristics of undifferentiated intestinal crypt cells (26). It may represent a much earlier stage in the differentiation process than preconfluent Caco-2 cells and thus may express transport proteins that are lost during the first steps of enterocytic transformation. Alternatively, interspecies differences may account for these discrepancies, because the presence of low-affinity Na+−dependent glutamate transport has also been reported in rat small intestine (24), whereas the high-affinity, Na+−dependent component accounts for all glutamate uptake in the human jejunal brush-border membrane (27).

We characterized the specificity, kinetic properties, and stoichiometry of L-glutamate uptake in the Caco-2 cell line. In agreement with the results reported by Nicklin et al. (21), we observed that L-glutamate uptake in the Caco-2 cell line was inhibited by L− as well as D-aspartate and was unaffected by D-glutamate and neutral and cationic amino acids. The Km of the transport reaction in our study was similar to that determined in human brush-border membrane vesicles (90 μM) (27), being of the same magnitude as that reported by Nicklin et al. (21) for transepithelial fluxes across Caco-2 cell monolayers (65.0 μM). The Hill coefficient of 2.9 calculated from the measurement of glutamate uptake in the presence of Na+− concentrations ranging from 0 to 140 mM suggests a Na+−glutamate stoichiometry of 3:1, this being consistent with the stoichiometry reported for EAAT1 and EAAT3 transporters responsible for the expression of the XAG transport system (14, 15). Together, these characteristics indicate that glutamate transport occurs through the widespread XAG system.

The term “transport system” is used to designate a physiological membrane functional process that may be catalyzed by different polypeptides or “transporters.” Five different genes encoding five polypeptides that could mediate glutamate uptake through an XAG transport system have been cloned, and two of them, EAAT1 and EAAT3, are expressed in peripheral organs. With the use of transient expression in COS cells, it has been shown that EAAT1- and EAAT3-mediated L-glutamate uptake exhibit similar inhibition profiles and kinetic characteristics (1). The Km calculated for the uptake of L-glutamate by either of these two proteins was similar to that determined in our experiments (48 and 62 μM for EAAT1 and EAAT3, respectively), and the Km calculated in the d-aspartate transport was similar for the two proteins (Km = 60 and 47 μM for EAAT1 and EAAT3, respectively). It is therefore difficult to determine which of these two proteins is responsible for glutamate transport solely on the basis of functional experiments. Using RT-PCR, we detected the presence of both EAAT1 and EAAT3 transcripts in the Caco-2 cell line. However, the EAAT3 transcript was barely detectable when we used the highly specific and sensitive ribonuclease protection assay, whereas EAAT1 was still clearly expressed in this cell line. The presence of the EAAT1 transcript and the weak expression of EAAT3 in Caco-2 cells were quite unexpected. The latter protein has been cloned in humans by Arriza et al. (1) and Kanai and Hediger (12) (hEAAC1 protein), and the expression of hEAAC1 mRNA has been demonstrated in human intestine (13). In contrast, our results constitute the first report of the presence of the EAAT1 glutamate transporter in an intestinal epithelial cell line. The reasons for these unexpected features may arise from the karyotypic specificity of the Caco-2 cell line. This cell line was established from a colon carcinoma and has been shown to be hypertetraploid, with the loss of normal chromosomes 9 and 21 (10). The chromosomal loci for the EAAT1 and EAAT3 genes have recently been established as 5p11–p12 and 9p24, respectively (31, 33). Reassignment of the EAAT3 gene to a poorly transcribed region during chromosomal alteration may result in a drastic reduction of EAAT3 expression. On the other hand, it should be borne in mind that Caco-2 cells have a colonic origin and may exhibit certain differences with enterocytes, including the expression of different transporter subtypes.
The third important result of our study was the demonstration of a 2.5-fold increase in the glutamate transport rate during Caco-2 cell differentiation. The increase in L-glutamate transport capacity (V_max) probably results from an increase in the amount of active transporter in the brush-border membrane rather than from the expression of a different transporter subtype with a different affinity, because K_m did not vary during differentiation. The magnitude of L-glutamate uptake was correlated to the amount of EAAT1 transcripts in postconfluent cells, suggesting that glutamate transport upregulation may result from an increase in gene transcription or the stabilization of EAAT1 mRNA during cell differentiation. Contrasting with our results concerning glutamate, the uptake of cationic and neutral amino acids through systems y^+ and B^0, respectively, was previously shown to be downregulated when Caco-2 cells cease to proliferate and undergo differentiation (22, 23). Both systems serve the uptake of essential amino acids, the requirement for which depends on protein synthesis rate and may thus decrease as cells cease to proliferate. In contrast, the physiological meaning of up-regulation of system X_A,G after confluence is more speculative. L-Glutamine and L-glutamate are major metabolic fuels of the enterocyte (20, 28), and the increase in L-glutamate transport may compensate for decreased L-glutamine uptake through system B^0. The rise in glutamate uptake may also aid postmitotic Caco-2 cells in maintaining adequate glutathione concentrations and thus resist oxidative aggressions, because it has recently been demonstrated that dietary glutamate (but not de novo synthesized glutamate) is used for glutathione synthesis in the intestinal epithelium (29).

In conclusion, this study provides the first demonstration of the involvement of EAAT1 in L-glutamate transport in the Caco-2 intestinal epithelial cell line and its upregulation during the enterocytic differentiation process. This change may reflect the gradual settling of transporters involved in the vectorial transport of amino acids across the intestinal mucosa as well as modifications to epithelial cell amino acid requirements in relation to their differentiation. Further investigations are needed to explore these hypotheses.

Critical reading of the manuscript by P. N. Boyaka is gratefully acknowledged.

A. Mordrelle was supported by Grant no. 94231 from the French Department of Higher Education and Research.

REFERENCES


