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Involvement of system A in proline transport in the intestinal crypt-like cell line IEC-17

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Although the morphologic and enzymatic acquisitions associated with enterocytic differentiation have been extensively investigated, little is known about the changes in amino acid transport during this process. The transport of proline was examined in the rat epithelial cell line IEC-17, which presents the characteristics of an undifferentiated crypt cell. Proline uptake involves both Na^+-independent non-saturable diffusion and Na^+-dependent components. The Na^+-dependent transport of proline is sensitive to the pH and is inhibited by hydroxyproline, glycine, alanine, and MeAIB. Non-α amino acids, such as β-alanine and γ-aminobutyric acid, do not compete with proline, whereas leucine is a weak inhibitor. Therefore, proline transport appears to occur through the system A, whereas the intestinal specific systems (B and IMINO) are not involved in this process. These observations suggest that these two systems are either absent or expressed at a very low level in this undifferentiated epithelial cell line. (J. Nutr. Biochem. 7:431–436, 1996.)

Keywords: transport; differentiation; proline; epithelial cell line; system A

Introduction

The absorption of amino acids across the intestinal epithelium occurs mainly in the upper-third of the intestinal villi and involves epithelium-specific as well as ubiquitous transport systems.[1,2] The villus absorptive cells originate from the intestinal crypts and undergo differentiation during their migration to the villus tip. The differentiation of epithelial cells includes the acquisition of the brush-border and of the enterocyte-specific hydrolases and transporters, such as sucrase, lactase, and the Na^+-glucose cotransporter SGLT-1. The time course of these acquisitions is now well documented.[3,4,5] However, the appearance of the amino acid transport systems during the differentiation of the intestinal epithelial cells has received little attention. It is thus necessary to identify the transport systems present in the crypt cells. The rat intestinal epithelial cell line IEC-17 represents a convenient model to investigate the transport of amino acid in small intestine crypt cells. This rapidly proliferating cell line has been shown to express intestinal-specific antigens and to exhibit scarce microvilli but lacks the villus markers of differentiated enterocytes.[6] Proline has been our focus because it has been found in rats to be absorbed across the intestinal brush-border membrane through both the IMINO and B transport systems,[1] whereas in non-epithelial cells (such as fibroblasts) proline is absorbed mainly through the transport system A.[7] The Na^+-dependent transport system designated as B accepts almost all of the bipolar amino acids, including branched-chain amino acids and proline and is specific of the intestinal epithelium.[1,8,9] Another epithelium-specific Na^+-dependent transport system designated as IMINO has also been described for imino as well as non-α amino acids such as β-alanine.[10–12] The absorption of bipolar amino acids also occurs through the ubiquitous Na^+-dependent systems A and ASC as well as through the Na^+-independent branched-chain preferring system L.[1,7] In

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the present study, IEC-17 was used to investigate the mechanisms involved in the transport of proline and to investigate the present of epithelial-specific transport systems in this undifferentiated crypt cell model.

Methods and materials

Materials

L-2,3-^{3}H-proline (2.22 TBq.mmol^{-1}) was obtained from The Radiochemical Centre (Amersham, Les Ulis, France). Cell culture reagents were purchased from Gibco (Cergy-Pontoise, France) and plastic dishes from Falcon (Strasbourg, France). All other reagents were obtained from Sigma (La Verpillère, France).

Cell culture

The rat IEC-17 cell line was kindly provided by Dr. Kedinger (INSERM, Strasbourg, France). The cells were used between passage 21 and 40. They were cultured at 37°C in humidified atmospher with 5% CO_2 and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g.L^{-1} glucose, 200 μg.mL^{-1} Gentamycin, 4 μg.mL^{-1} Insulin, and supplemented with 5% heat decomplemented fetal calf serum (FCS). They were seeded at 2 × 10^4 cells.cm^{-2} and grown in 75 cm^2 flasks. They reached confluence in 4 to 5 days and were detached with 0.5% trypsin 0.2% EDTA (subculture).

Transport assays

Proline uptake was measured in confluent IEC-17 cells cultured in 24-well plates (2.3 cm^2/well). The culture medium was renewed 3 hr before the beginning of the experiment. The cells were preincubated at 37°C for 15 min with 1 mL of Hepes-buffered Saline (10 mM Hepes-Tris, 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mM NaH_2PO_4, and 0.3 mM KH_2PO_4) containing 10 mM glucose and adjusted to pH 7.4 (buffer HSG). This buffer was then removed and the cells incubated at 25°C with 1 mL of HSG containing unlabeled proline (at concentrations ranging from 0.1 to 20 mM) and L-2.3-^{3}H-proline (3.7 KBq). The experiment was stopped by aspiration of the medium and the wells were rapidly rinsed twice with 1 mL of ice-cold buffer containing 100 μM unlabeled proline. The cells were harvested in 500 μL of 0.1 N NaOH and the cell-associated radioactivity determined by liquid scintillation counting. In most experiments, proline uptake into IEC-17 monolayers was measured after 5 min. To investigate the sodium dependence, sodium chloride was replaced by choline chloride and NaH_2PO_4 was omitted. In competition experiments, an inhibitor was added to the transport medium in a 4 to 100 fold excess.

No attempt was made to balance the osmolarity of the transport buffer when using high concentration of proline or competitor because increasing the osmolarity of the transport medium from 300 to 320 mosmol did not affect the accumulation of 100 μM proline (data not shown). The extracellular fluid that adhered to the cells during the washing procedure was determined using the non-absorbable marker ^{14}C-mannitol,[13] and was used to estimate the zero time value for the accumulation of proline. The amount of extracellular proline that remained associated with the cells after washing was calculated from the amount of ^{14}C-mannitol recovered after a 5-min incubation, and assessed to represent the zero time value for the determination of uptake rate.[14] The extracellular space was found to be 2.35 μL.mg protein^{-1}.

The protein contents of the well were determined by the Bradford method[15] and results are expressed as pmol of proline transported per mg of cell protein.

Calculations

Results were expressed as means ± standard deviations and statistical comparisons were done using the Tukey's studentized range test (GLM procedure, SAS 6.03, SAS Institute, Cary, NC, USA). Transport kinetic parameters were obtained by fitting data to Michaelis-Menten equation or to a linear model (NLIN and REG procedures, SAS 6.03, SAS Institute, Cary, NC, USA).

Results

Time course of proline transport at different temperatures

The time course of L-proline uptake in IEC-17 cells was studied at 25°C and at 4°C (*Figure 1*). For both temperatures, the amount of proline accumulated in the cells increased linearly for the first 10 min and reached a plateau afterwards. The slope of the accumulation curve was four times greater at 25°C than that at 4°C.

Effects of sodium gradient and extracellular pH

The uptake of proline in IEC-17 monolayers was significantly reduced when choline chloride was substituted for sodium chloride in the transport buffer (61 ± 9 vs 278 ± 57 pmol.mg protein^{-1}.min^{-1} for Na^+-free and Na^+ buffer, respectively) (*Table 1*). Lowering the pH of the transport buffer from 7.4 to 6.0 resulted in a 62% reduction of proline accumulation when transport was measured with Na^+-containing buffer. However, there was no effect of the extracellular pH on proline uptake when the transport was carried out in a Na^+-free buffer.

Concentration dependence of proline transport

The accumulation of proline in IEC-17 monolayers was also measured in Na^+-containing and Na^+-free buffer for concentrations of proline ranging from 0.15 to 20 mM (*Figure 2*). The uptake rate of this amino acid increased linearly with its extracellular concentration when the transport medium was devoid of Na^+. The apparent permeability coefficient for proline was calculated from the slope of the curve according to the following equation[16]: $P_{app} = dQ/(C.dt).1/A$, where dQ/(C.dt) is the transport rate of proline (expressed as pmol. min^{-1} per culture well) divided by the concentration of proline in the transport buffer and A is the surface of the well. The apparent permeability coefficient for proline in the IEC-17 cell line was found to be 0.44 × 10^{-6} m.min^{-1}. In contrast to Na^+-free conditions, a saturation was observed when the transport was carried out in a Na^+-containing buffer. This saturation was more evident by considering the Na^+-dependent transport, calculated as the difference between the transport measured in Na^+-containing and Na^+-free buffers. The Eadie-Hofstee plot of the Na^+-dependent proline transport suggests the presence of one component with a K_t of 0.77 mM and a V_{max} of 8 nmol.mg protein^{-1}.min^{-1}.

Competition experiments

The specificity of proline uptake was first investigated by adding another amino acid in a 100 fold excess to the Na^+-

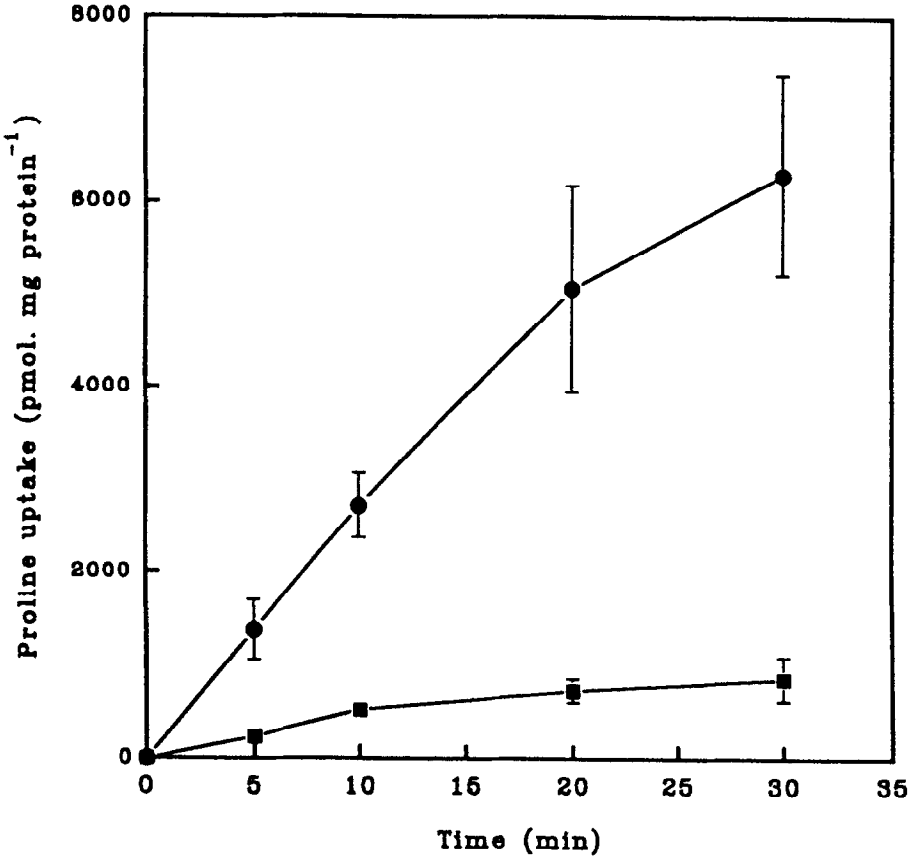

Figure 1 Time course of proline uptake (0.1 mM) into confluent IEC-17 cell monolayers. The experiment was carried out in a Na^+-containing buffer at T = 25°C (●) or T = 4°C (■). Points are means ± SD of n = 12 determinations.

containing transport buffer (*Table 2*). Under these conditions, the accumulation of proline in IEC-17 cells was highly reduced in the presence of excess alanine, methylamino-isobutyric acid (MeAIB), glycine and hydroxyproline. It was also reduced although to a lesser extent, by leucine, whereas lysine, β-alanine, and GABA had almost no effect on proline transport. The competition between proline and alanine, MeAIB and leucine was further investigated using Na^+-free and Na^+-containing transport buffers and a concentration of a competitor ranging from 400 μM to 10 mM (4 to 100 fold excess) (*Figure 3*). In no case was the Na^+-independent accumulation of proline reduced by the competitor. With Na^+-containing buffer, similar patterns of inhibition were obtained with alanine and MeAIB with a 60% reduction of the total transport, i.e. 71% reduction of the Na^+-dependent transport with a 4 fold excess of the competitor. Conversely, the inhibition pattern observed with leucine was different. No reduction in proline transport was observed for 400 μM and 2 mM leucine, whereas a 43% reduction in total transport, i.e. a 52% reduction in Na^+-dependent transport, was achieved with a 100 fold excess of the competitor.

Table 1 Effect of extracellular pH and extracellular sodium on proline uptake in IEC-17 cells

Transport (pmol.mg protein^{-1}.min^{-1})	pH 7.4	pH 6
Total	278 ± 57	107 ± 21
Na^+-independent	61 ± 9	58 ± 12
Na^+-dependent	217 ± 57	49 ± 21

Proline uptake (0.1 mM) was measured after 10 min at pH 7.4 and pH 6. The total transport was measured in the presence of sodium and the Na^+-independent transport was measured in the absence of sodium. The Na^+-dependent transport is the total uptake minus the Na^+-independent transport. Data represent means ± SD of n = 12 determinations.

Discussion

Although the absorption mechanism of amino acids in intestinal villus cells has been extensively investigated, the nature of the transport systems present in the undifferentiated crypt cells has received little attention. The present results strongly suggest that the saturable Na^+-dependent component exhibits most of the characteristics of system A and do not support the involvement of the epithelial-specific, Na^+-dependent systems B and IMINO.

Proline uptake in IEC-17 epithelial cells appears to involve both Na^+-dependent and Na^+-independent components. The Na^+-independent component of proline transport is not saturable in the range 0.15 mM to 20 mM. This observation, together with the absence of stimulation of the Na^+-independent transport when the extracellular pH is de-

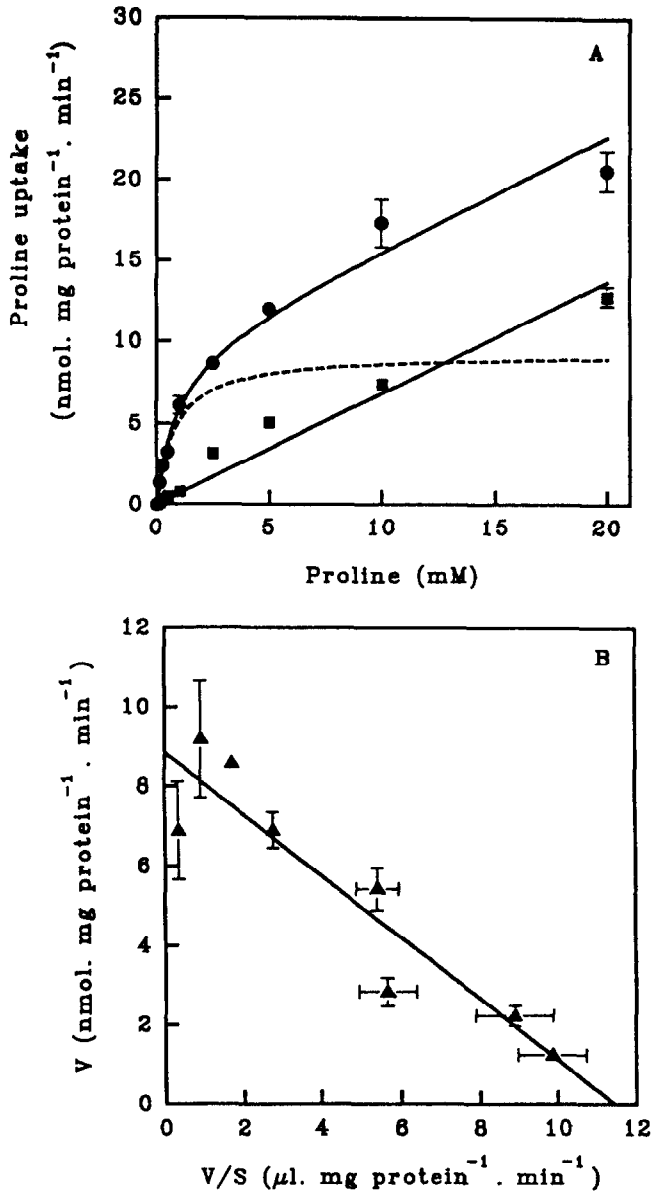

Figure 2 The concentration dependence of proline transport. The uptake was measured after 10 min in the presence and absence of sodium. (A) *V* versus *S* plots of total uptake of proline in the presence of Na^+ (●): the absence of Na^+ (■): and, the Na^+-dependent component (i.e. transport in the presence of Na^+ minus that in the absence of Na^+) (---). (B) Eadie-Hofstee representation of Na^+-dependent transport (▲). Points are means ± SD of $n = 6$ determinations.

creased from 7.0 to 6.0, rules out the possibility of a Na^+-independent H^+-proline cotransport similar to that reported by Thwaites et al. in the differentiated epithelial cell line Caco-2.[17] Moreover, there was no inhibition of the Na^+-independent transport of proline by MeAIB and alanine, whereas Thwaites et al. have suggested that the H^+-cotransport system present in the brush-border of Caco-2 cells was shared by these three amino acids as well as by glycine and β-alanine.[18] Taken together, our results indicate that the Na^+-independent transport of proline occurs through passive transmembrane diffusion.

Table 2 The effect of amino acid competitors on total transport of proline in IEC-17 monolayers

Addition	Total proline uptake (% of control)
None	100[a]
Lysine	93 ± 3[a]
GABA	88 ± 6[b]
β-Alanine	78 ± 4[b]
Leucine	58 ± 10[c]
Alanine	30 ± 6[d]
Glycine	26 ± 5[d]
Hydroxyproline	25 ± 4[d]
MeAIB	24 ± 6[d]

The accumulation of 0.1 mM proline was measured after 10 min of incubation in the presence of a 100 fold excess of competitor. Results are means ± SD of $n = 6$ determinations and are expressed as the percentage of the transport measured in the absence of competitor, the value of the control was 258 ± 41 pmol.mg protein^{-1}.min^{-1}. Means with different letters are significantly different ($P < 0.05$) from each other.

Contrary to the Na^+-independent component, the Na^+-dependent transport of proline in IEC-17 cells exhibits a clear saturation pattern in the range 0.15 mM to 20 mM. Kinetic analysis of this component indicates the presence of a single transport system with a K_t of 0.77 mM and a V_{max} of 8 nmol.mg protein^{-1}.min^{-1} in confluent IEC-17 cells. This K_t is slightly higher than that reported by Stevens and Wright[19] for proline with rabbit brush-border membrane vesicles (0.25 mM) and contrasts with that reported by Nicklin et al. in Caco-2 cells for Na^+-dependent transport of proline (5.28 mM).[20] In this epithelial cell line, proline transport was reported to occur through the Na^+-dependent system A,[20] whereas the system IMINO was responsible for the transport measured with rabbit brush-border membrane vesicles.[19] An extensive characterization of the system IMINO has been done by Munck et al.[10–12] In the rat, this system is Cl^--dependent and is shared by proline, N-methylated amino acids and non-α amino acids such as β-alanine and γ-aminobutyric acid. In IEC-17 cells, proline uptake was significantly reduced by a 4 fold excess of MeAIB but was unaffected by a 100 fold excess of γ-aminobutyric acid. Therefore, it appears that the transport of proline in the undifferentiated epithelial cell-line IEC-17 does not occur through the system IMINO. In the same way, the involvement of the system B is also not supported by our results because proline transport was efficiently inhibited by MeAIB, which is not a substrate for this transport system.[21] However, our results indicate that in the IEC-17 cell line, proline is transported through the system A. The widespread amino acid transport system is Na^+-dependent and has been shown to be sensitive to the extracellular pH.[22] It is involved in the transport of most of the small bipolar amino acids including alanine, glycine, and proline and of N-methylated amino acid analogues such as MeAIB,[1,7] whereas its role in the transport of branched-chain amino acids is only marginal. The former amino acids were found to be effective competitors for proline transport in our study, whereas the inhibition of proline uptake by leucine was limited and observed only when a large excess of leucine was added to the medium (×100).

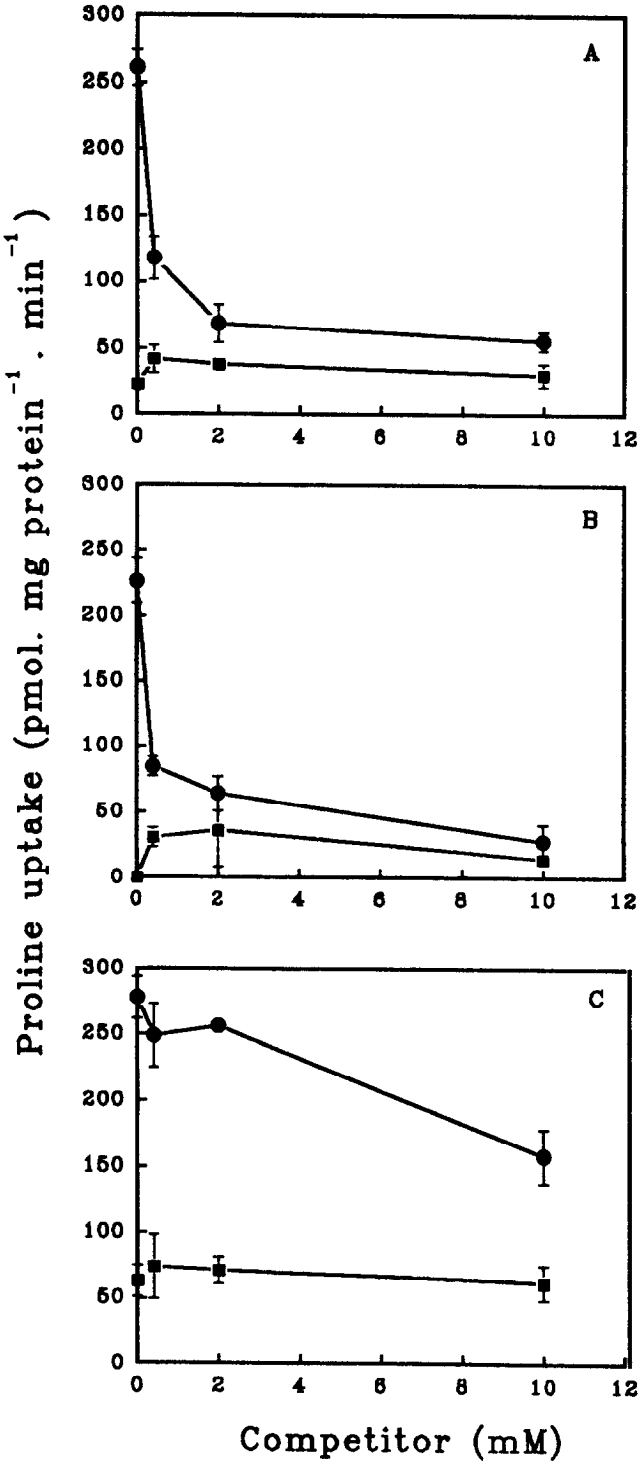

Figure 3 The inhibition of proline uptake by alanine (A), MeAIB (B), and leucine (C). The transport of proline (0.1 mM) was measured after 5 min in the presence (●) and absence of sodium (■). Competitor was added in a concentration of 0.4, 2 or 10 mM. Points are means ± SD of $n = 6$ determinations.

The presence of Na^+-dependent system A in the IEC-17 cell line is not surprising since other studies indicate that this system is also present in the differentiated intestinal cell line Caco-2 and isolated guinea-pig enterocytes.[20,23] However, the fact that Na^+-dependent transport of proline occurs only through system A suggests that system B and IMINO are absent or expressed at a very low level in the IEC-17 cell line. At the moment, there have been no cloned transporters responsible for neutral amino acid transport, and it is not possible to give a clear-cut response concerning the expression of the different transport systems in this cell line. Our results are, however, consistent with the idea that ubiquitous amino acid transport systems are present in the undifferentiated crypt cells, whereas intestinal-specific systems appear during the differentiation of epithelial cells.

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