The development of γ -glutamyltransferase in a pig renal-epithelial-cell line *in vitro*

Relationship to amino acid transport

Francisco V. SEPÚLVEDA,* Keith A. BURTON and Jeremy D. PEARSON
A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

(Received 23 July 1982/Accepted 27 August 1982)

The pig kidney-cell line, LLC-PK₁, possesses γ -glutamyltransferase with properties similar to those of the purified renal enzyme. γ -Glutamyltransferase activity increases after cells are seeded at low density to reach values comparable with those found in kidney cortex when the cells are fully confluent. This increase is paralleled by an increase in α -methyl D-glucoside uptake. In contrast, the uptakes of L-leucine and L-alanine decrease during this time. These results suggest that γ -glutamyltransferase is not important as a mediator of the renal transport of neutral amino acids.

γ -Glutamyltransferase (EC 2.3.2.2) is found at highest activity in kidney (Meister & Tate, 1976), where it is located at the luminal surface of the brush-border membrane of the proximal-tubule epithelium (Marathe *et al.*, 1979). The enzyme, which catalyses the first step in the catabolism of GSH, has been proposed to mediate, at least in part, amino acid and dipeptide transport into the epithelial cells (Meister & Tate, 1976).

Continuous cultures of epithelial-cell lines that retain the ability to develop differentiated functions *in vitro* provide useful model systems with which to study the regulation of such functions. LLC-PK₁ cells, a pig kidney-cell line (Hull *et al.*, 1976), develop *in vitro* several characteristics of proximal-tubule epithelial cells, among which the best studied is the Na⁺-dependent phlorrhizin-sensitive hexose-transport system (Mullin *et al.*, 1980; Rabito & Ausiello, 1980).

Perantoni & Berman (1979) first reported histochemical evidence for the presence of γ -glutamyltransferase activity in LLC-PK₁ cells. In the present paper we show that γ -glutamyltransferase is present at high activity in LLC-PK₁ cells and has properties similar to the enzyme from kidney cortex, and we explore how this activity develops during growth *in vitro*. The possible correlation between γ -glutamyltransferase activity and the transport of neutral amino acids is also studied.

Abbreviation used: GSH, reduced glutathione.

* To whom correspondence and reprint requests should be addressed.

Methods

Cell culture

LLC-PK₁ cells were generously given by Dr. R. N. Hull (Lilly Research Laboratories, Indianapolis, IN, U.S.A.). They were obtained at passage 186, and grown at 37°C in Dulbecco's modification of Eagle's medium (Dulbecco & Freeman, 1959) containing 10% (v/v) foetal calf serum with air/CO₂ (19:1) as the gas phase. They were subcultured by brief exposure to 0.1% trypsin + 0.025% EDTA. Cells were cultured in 110 ml (4 fl.oz.) glass bottles containing 10 ml of medium, or in 16 mm-diameter wells of 24-well culture trays (Falcon or Linbor) containing 0.5 ml of medium.

Enzyme assays

Cells were suspended by treatment with a rubber 'policeman' and sonicated (48 kHz) at room temperature for 30 s. γ -Glutamyltransferase activity was assayed at 37°C with 3 mM- γ -L-glutamate *p*-nitroanilide as donor substrate and 40 mM-diglycine as acceptor (Sepúlveda & Burton, 1982) in a buffer containing 5.5 mM-MgCl₂, 75 mM-NaCl and 50 mM-Tris/HCl, pH 8.2. Appearance of *p*-nitroaniline was followed at 405 nm. Proteins were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and DNA by the method of Fiszer-Szafarz *et al.* (1981).

Transport measurements

Initial rates of amino acid transport were measured after 30 s incubations of monolayers in the presence of ¹⁴C-labelled amino acid (L-[U-¹⁴C]-

alanine or L-[1- ^{14} C]leucine) and [^{3} H]inulin as described previously (Sepúlveda & Pearson, 1982). Incubation were terminated by rapid washing with Na^+ -free ice-cold buffer, and uptake was expressed per number of cells, measured with a Coulter counter, after correction for extracellular space measured with inulin. A similar approach was used for the measurement of ^{14}C -labelled α -methyl D-glucoside uptake, but with an incubation time of 30 min (Mullin *et al.*, 1980). All radioactively labelled compounds were obtained from Amersham International.

Results and discussion

Incubation of homogenates of LLC-PK₁ cells in the presence of L- γ -glutamate *p*-nitroanilide and diglycine established that there was substantial γ -glutamyltransferase activity in confluent cell monolayers, and that over 97% of the activity could be sedimented by centrifugation at 100 000 *g* for 60 min. Fig. 1(a) shows that the pH optimum for γ -glutamyltransferase activity was approx. 8.2. In the range of pH used the activity was strongly dependent on the presence of acceptor. The pH-dependence of the enzyme from LLC-PK₁ cells closely resembled that measured in isolated brush-border membranes from kidney cortex and that for the purified enzyme (Glossman & Neville, 1972; Tate & Meister, 1974). Fig. 1(b) shows the effect of GSH on γ -glutamyltransferase activity measured with L- γ -glutamate *p*-nitroanilide as donor; as expected for a true γ -glutamyltransferase activity, the reaction was strongly inhibited by the natural donor, GSH, in a manner similar to that reported for the enzyme isolated from kidney, where the K_i for GSH was 1.1 mM (Tate & Meister, 1974). In Table 1 the ability of a number of amino acids to replace diglycine as acceptor of the γ -glutamyl residue is presented. The order of effectiveness closely matches that observed with the purified enzyme (Tate & Meister, 1974).

The results described above demonstrate that the properties of γ -glutamyltransferase in LLC-PK₁ cells closely resemble those of the enzyme present in proximal tubular epithelium *in vivo*. The specific activities of the enzyme also correspond to those observed in kidney-cortex homogenates (activity in confluent cultures of LLC-PK₁ cells was 186 ± 20 nmol/min per mg of protein; activity in cortex homogenates was 167 ± 27 nmol/min per mg of protein; means $\pm$ S.E.M. for three and five observations respectively), and are substantially higher than those found in other cultured cells. For example, we did not detect γ -glutamyltransferase activity in mouse Balb/C 3T3 cells (less than 0.1 nmol/min per mg of protein), and specific activities of 20 and 4 nmol/min per mg of protein

Fig. 1. Effects of pH (a) and of GSH concentration (b) on the γ -glutamyltransferase activity of LLC-PK₁ cells. For full experimental details see the text. Activity was measured in the presence of 40 mM-diglycine (●) or in the absence of acceptor (▲).

have been reported in MDCK cells (a dog kidney-epithelial-cell line with characteristics of distal tubule) and rat hepatocytes respectively (Richardson *et al.*, 1981; Viña *et al.*, 1982).

Mullin *et al.* (1980) and Rabito & Ausiello (1980) first described an Na^+ -dependent system in LLC-PK₁ cells that transports hexoses actively across the epithelial layer (Misfeldt & Sanders, 1981). This transport function is found *in vivo* only in terminally differentiated epithelia such as those of the proximal nephron or the small intestine. *In vitro*, the

Table 1. Effect of various L-amino acids and diglycine on the γ -glutamyltransferase activity of homogenates of LLC-PK₁ cells

For full experimental details see the text. Acceptors were all used at 40 mM. Activity is expressed as a percentage of that observed in the presence of 40 mM-diglycine (113 nmol/min per mg of protein).

Acceptor	Activity (%)
Diglycine	100
Methionine	58
Alanine	40
Leucine	38
Cysteine	35
Serine	32
α -Aminoisobutyrate	30
α -Methylaminobutyrate	30
α -Amino-n-butyrate	30
Phenylalanine	29
None	11

transport-system activity is very low in cells seeded at low density and increases with cell density to a maximum in fully confluent monolayers (Mullin *et al.*, 1980; Amsler & Cook, 1982). We have measured γ -glutamyltransferase activity in similar experiments. LLC-PK₁ cells were plated at low density and allowed to grow until they became fully confluent, with fresh medium being fed to them on alternate days. The growth curve is shown in Fig. 2(a); by day 7 after plating numerous domes were visible; these are indicative of water transport across the confluent monolayer (Hull *et al.*, 1976). Fig. 2(b) confirms the previous observations of an increase in the ability of the cells to take up α -methyl D-glucoside as cell density increases, and demonstrates that there was a parallel increase (at least 30-fold) in the γ -glutamyltransferase activity. This increase was also obvious when the activity was expressed per cell DNA or protein content.

In an experiment in which cells were seeded at a range of densities similar to that in Fig. 2(c) and used 24 h later, γ -glutamyltransferase activity correlated positively with cell density, but the maximal values reached were lower than those shown in Fig. 2(b).

It has been suggested that the development of the hexose-transport system *in vitro*, as the cells progress from the subconfluent state, represents a process of differentiation analogous to that occurring in proximal-tubule epithelium *in vivo* (Mullin *et al.*, 1980). In the same way, this increase in γ -glutamyltransferase activity mimics the ontogeny of the enzyme in the kidney *in vivo* (Tate & Meister, 1975).

Fig. 2. Activities of γ -glutamyltransferase and transport of hexose and amino acids during growth of LLC-PK₁ cells

For full experimental details see the text. Cells were seeded at low density and cell numbers were measured every 2 days (a). (b) shows γ -glutamyltransferase (GGT) activity (●) and uptake of 0.1 mM- α -methyl D-glucoside (α MG) (▲). (c) shows the uptake of 0.05 mM-L-leucine (▲) and 0.25 mM-L-alanine (●). All values are means for three determinations performed with different monolayers; s.e.m. is shown when bigger than symbol.

The presence of high activities of γ -glutamyltransferase in epithelial membranes involved in

amino acid transport led to the hypothesis that one of the functions of this enzyme is to translocate amino acids and dipeptides across the plasma membrane; part of the support for the hypothesis comes from positive correlations between γ -glutamyltransferase activity and amino acid transport (Meister & Tate, 1976). Fig. 2(c) shows the transport of alanine and of leucine into LLC-PK₁ cells during cell growth. We have shown that alanine is taken up predominantly by a fully Na⁺-dependent transport system and that leucine is transported mainly by an Na⁺-independent pathway (Sepúlveda & Pearson, 1982). The uptake of both amino acids decreased as cells became confluent, in a way that might reflect the down-regulation of amino acid-transport systems that occurs in non-growing cells (Guidotti *et al.*, 1978). Thus γ -glutamyltransferase activity shows no correlation with amino acid transport, suggesting that if the enzyme mediates amino acid transport in LLC-PK₁ cells this is a minor component of alanine or leucine uptake.

In conclusion, the parallelism observed between the development of high activities of γ -glutamyltransferase and of Na⁺-dependent hexose transport suggests that the γ -glutamyltransferase activity is another expression of a differentiated phenotype corresponding to proximal-tubule epithelial cells that is found in LLC-PK₁ cells. The biosynthesis of γ -glutamyltransferase in cortical slices has been examined by Nash & Tate (1982). The finding that the enzyme arises in LLC-PK₁ cells during differentiation *in vitro* suggests that the cell line can be used to study the biogenesis of this membrane protein under controlled conditions. It also provides a cellular model in which to study the importance of γ -glutamyltransferase in the catabolism of intra- or extra-cellular glutathione.

References

- Amsler, K. & Cook, J. S. (1982) *Am. J. Physiol.* **242**, C94–C101
- Dulbecco, R. & Freeman, G. (1959) *Virology* **8**, 396–397
- Fiszer-Szafarz, B., Szafarz, D. & Guevara de Murillo, A. (1981) *Anal. Biochem.* **110**, 165–170
- Glossman, H. & Neville, D. M. (1972) *FEBS Lett.* **19**, 340–344
- Guidotti, G. G., Borghetti, A. F. & Gazzola, G. C. (1978) *Biochim. Biophys. Acta* **515**, 329–366
- Hull, R. N., Cherry, W. R. & Weaver, G. W. (1976) *In Vitro* **12**, 670–677
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, N. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marathe, G. V., Nash, B., Haschmeyer, R. H. & Tate, S. S. (1979) *FEBS Lett.* **107**, 436–440
- Meister, A. & Tate, S. S. (1976) *Annu. Rev. Biochem.* **45**, 559–604
- Misfeldt, D. S. & Sanders, M. J. (1981) *J. Membr. Biol.* **59**, 13–18
- Mullin, J. M., Weibel, J., Diamond, L. & Kleinzeller, A. (1980) *J. Cell. Physiol.* **104**, 375–389
- Nash, B. & Tate, S. S. (1982) *J. Biol. Chem.* **257**, 585–588
- Perantoni, A. & Berman, J. J. (1979) *In Vitro* **15**, 446–454
- Rabito, C. A. & Ausiello, D. A. (1980) *J. Membr. Biol.* **54**, 31–38
- Richardson, J. W., Scalera, V. & Simmons, N. L. (1981) *Biochim. Biophys. Acta* **673**, 26–36
- Sepúlveda, F. V. & Burton, K. A. (1982) *FEBS Lett.* **139**, 171–173
- Sepúlveda, F. V. & Pearson, J. D. (1982) *J. Cell. Physiol.* **112**, 182–188
- Tate, S. S. & Meister, A. (1974) *J. Biol. Chem.* **249**, 7593–7602
- Tate, S. S. & Meister, A. (1975) *J. Biol. Chem.* **250**, 4619–4627
- Viña, J., Boix, J. J., Izquierdo, M. & Iradi, A. (1982) *Biochem. Soc. Trans.* **10**, 112–113