Alkaline Phosphatase and γ-Glutamyl Transpeptidase As Polarization Markers during the Organization of LLC-PK₁ Cells into an Epithelial Membrane

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Confluent monolayers of LLC-PK₁ cells, an epithelial cell derived from a normal pig kidney, contain high levels of alkaline phosphatase and γ-glutamyl transpeptidase activities. Inhibition studies show a close similarity between alkaline phosphatase and the so-called liver-bone-kidney isoenzyme. Nearly complete recovery of both activities in the microsomal fraction demonstrates the membrane-bound characteristics of these enzymes. Histochemical localization of the enzymes activities on the apical membrane of LLC-PK₁ cells confirms this observation. The activity of both enzymes decreases to very low levels when the cells are in exponential growth. Confluent monolayers of LLC-PK₁ cells plated at saturation density with cells that were in active growth show a progressive increase in the activity of alkaline phosphatase and γ-glutamyl transpeptidase. This increase is dependent on the synthesis de novo of RNA and protein and supports the conclusion that the activity of both enzymes is regulated at the transcriptional level. The development of these enzymes is delayed with respect to the development of the occluding junctions. This delay and the direct appearance of alkaline phosphatase activity in the apical membrane indicate that the different components of this membrane are inserted after the limits of the membrane have been established by the synthesis and assembly of the occluding junctions. Alkaline phosphatase activity of confluent monolayers can be further induced by reducing the concentration of phosphate in the medium. When the junctions are dissociated by incubating the monolayers in Ca²⁺-free medium, the alkaline phosphatase activity migrates freely beyond the limits of the apical membrane until it covers the entire cell surface. Both enzymes are synthesized even in the absence of contact between the cells, suggesting that the occluding junctions in LLC-PK₁ monolayers are involved in the polarized distribution, but not in the modulation, of the synthesis of these enzymes. In addition, the progressive decrease in enzyme synthesis that is obtained by reducing the cell-substratum adhesion supports the idea that it is the cell-to-substrate and not the cell-to-cell interaction which is involved in the modulation of these enzymatic markers of the apical membrane.

The active vectorial transport of solutes and water across epithelial cells results from the different biochemical and functional properties of the apical and basolateral aspects of the plasma cell membrane. The specific location of different transport systems (i.e. alkaline phosphatase, (Na⁺-K⁺)-ATPase) and hormonal receptors (i.e. antidiuretic hormone, parathyroid hormone) in each of these domains accounts for that polarization. Although the importance of this event has been recognized, witnessed by the incorporation of epithelial cell polarization as the main premise in nearly all of the current models used to explain transepithelial active transport, the mechanisms involved in its subcellular and molecular development have not been elucidated. The present study concerns the functional and biochemical changes that occur within the cellular membrane during the development of epithelia from free individual cells in tissue culture (1). The cell selected for this study is the LLC-PK₁, a cell line derived from a normal Hampshire pig kidney (2). These cells, grown on a permeable support, provide a cell culture model that exhibits several characteristics of in vivo epithelial membranes. For instance, we have demonstrated that monolayers of LLC-PK₁ cells have Na⁺-dependent hexose (3), phosphate (4), and amino acid (5) transport systems with characteristics similar to those observed in the proximal tubule. Like their in vivo counterpart, the uphill, saturable, and Na⁺-dependent step is localized in the apical membrane of the cultured cells (6).

The apical membrane (brush border) represents a highly specialized section of the plasma cell membrane in epithelial cells. In addition to containing a large number of transport systems in which sodium is co-transported with hexoses, amino acids, and phosphate, this membrane also possesses a series of characteristic enzymes. For instance, alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) and γ-glutamyl transpeptidase (γ-glutamyl)-peptide-amino acid 5-glutamyl transferase, EC 2.3.2.2) are present in large amounts in epithelial membranes, such as the intestinal mucosa and renal proximal tubule. Histochemical studies have located the enzymes in the apical membrane of the epithelial cells (7, 8).

The present study analyzes the presence and changes in activity and localization of the enzymes that may occur during the reorganization of the monolayers. By monitoring the enzymes during the passage of a single cell type from nonpolarized isolated cells to an organized polarized transporting epithelium, we can obtain some information on the mechanisms involved in the development of the epithelial cell polarization.

MATERIALS AND METHODS
Cell Culture and Monolayer Preparation—LLC-PK₁ cells obtained from the American Type Culture Collection (CRL 1992) were main-
tained by serial passages in 10-cm diameter plastic tissue culture dishes. The cells were fed with Dulbecco’s modified Eagle’s medium with l-glutamine. In addition, the medium contained 10% fetal bovine serum. All cultures were maintained in an atmosphere of 5% CO2 in air at 37 °C. When cell growth reached saturation density, subcultures were prepared by using a 0.02% EDTA, 0.05% trypsin solution. The cells which were at the 182nd and 186th passages with passage numbers 5 were used. Monolayers on a permeable support were prepared using a polycarbonate filter membrane with 0.2 µm pore size and 25-mm diameter (Nucleapore Corp., Pleasanton, CA). The filters were covered with a very thin film of 0.5% collagen dispersion (Ethicon, Sommerville, NJ) and applied to a standard microscope slide. The methods for collagen aggregation and sterilization of the collagen-coated membrane have been described elsewhere (9).

Alkaline Phosphatase Assay—Alkaline phosphatase was determined by a modification of the colorimetric procedure designed by DeChatelet et al. (10). The assay was performed as follows: 0.5 ml of p-nitrophenyl phosphate stock solution (4 mg/ml) was added to 0.5 ml of 2-amino-2-methyl-1-propanol buffer solution (1.5 M, pH 10.3), and the resulting mixture was allowed to equilibrate at a 37 °C waterbath. The reaction was initiated by the addition of sonicated cells. After 15 min, the reaction was terminated by the addition of 10 ml of 0.1 M glycylglycine in 0.1 M Tris-HCl buffer, pH 9.0. The absorbance of the samples at 405 nm was determined by a modification of the colorimetric procedure designed by Lowry et al. (13). The fixed cells, attached to the culture dish, were incubated at pH 9 in the following standard medium: 0.04 M Tris-maleate buffer, pH 9.0, 10 mM β-glycerophosphate, 4.0 mM lead nitrate, and 200 mM sucrose. The supernatant of this centrifugation (S3) was saved and stored at -25 °C. The pellet (P3) representing the microsomal fraction was resuspended in a magnetic stirring bar. Transepithelial potential differences were measured with a Keithley Model 606 digital electrometer. The electrolytes used were pairs of calomel electrodes connected to the bathing solutions by bridges of 3.0 M KCl immobilized by 4% agar at 1 mm from the tissue. Current conducted by Ag/AgCl electrodes on the opposite sides of the membranes and at the rear of the chamber was measured with a Weston D.C. microammeter, model 633 (Weston Electrical Instrument Corp., Newark, NJ).

Variation of Substratum Adhesiveness—The adhesiveness of the plastic tissue culture was reduced as described in Ref. 16 by applying increasing concentrations of poly(2-hydroxyethyl methacrylate) in an alcohol solution. The plates were dried under a sterile hood for 72 h in a desiccator.

Materials—Culture media, fetal bovine serum, and trypsin-EDTA solution were obtained from Grand Island Biological Co. Levamisole and 3,5-diaminobenzoic acid hydrochloride were obtained from Aldrich Chemical Co. Poly(2-hydroxyethyl methacrylate) was obtained from Hydron Laboratories Inc.

RESULTS

Effect of Different Inhibitors on Alkaline Phosphatase Activity in LLC-PK1, Cel-LLC-PK1, and LLC-PK2 Cells—Cells were confluent monolayers 7 days after plating were exposed for 30 min to different inhibitors. The results from 6 to 16 monolayers indicate that alkaline phosphatase is extremely sensitive to heat inactivation. Incubation at 60 °C for 30 min completely abolished all enzymatic activity that in control condition was 0.62 ± 0.02 unit µg−1 DNA. Other organ-specific inhibitors also affected this enzyme. For instance, homogarginine at a concentration of 20 mM and Levamisole at a concentration of 1 mM inhibited the enzymatic activity by 82% (0.09 ± 0.002 unit µg−1 DNA) and 70% (0.19 ± 0.01 unit µg−1 DNA), respectively. L-Phenylalanine, however, also at a concentration of 20 mM, inhibited only 35% (0.40 ± 0.01 unit µg−1 DNA) of the enzymatic activity. This small effect seems to be restricted to the L-form since the D-form of this amino acid produced only a 17% inhibition (0.50 ± 0.01 unit µg−1 DNA).

Histochemical Localization of γ-Glutamyl Transpeptidase Activity—Ultrastructural localization of γ-glutamyl transpeptidase activity in LLC-PK1 monolayers was carried out essentially as described by Seligman et al. (15). Monolayers of LLC-PK1 cells grown to confluence in 10-cm diameter plastic Petri dishes were fixed for 30 min at 4 °C with 1% formaldehyde prepared by the depolymerization of paraformaldehyde. The fixed monolayers were washed overnight in Dulbecco’s phosphate-buffered solution. The fixed monolayers were gently scraped from the culture dish and floated on the incubation medium containing γ-glutamyl-(4-methoxy)-2-naphthylamide, glycglycine, sucrose, and freshly diazotized 4-aminophthalhydrazide in phosphate buffer, pH 7.4, for 60 min at 25 °C. Controls were performed by incubation in medium without substrate or by addition to the incubation medium of 1 mM Levamisole. The cells were then rinsed briefly in 0.1 M cacodylate buffer, pH 7.4, for 60 min.

Histochemical Localization of Alkaline Phosphatase Activity in LLC-PK1, Cel-LLC-PK1, and LLC-PK2 Cells—Enzymatic markers during the organization of a epithelial membrane

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Subcellular Distribution of Alkaline Phosphatase and γ-
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Glutamyl Transpeptidase—Table I shows the distribution of alkaline phosphatase and γ-glutamyl transpeptidase in different fractions of a homogenate of LLC-PK₁ cells. Differential centrifugation allows the separation from the total homogenate of several fractions containing different enzymatic activities. Alkaline phosphatase and γ-glutamyl transpeptidase activities were enriched 14- and 9-fold, respectively, in the microsomal fractions in comparison to the total homogenate.

**TABLE I**

*Distribution of alkaline phosphatase and γ-glutamyl transpeptidase in various subcellular fractions obtained by differential centrifugation*

The results are the mean of six determinations ± S.E. from three different total homogenates.

| Fraction | Alkaline phosphatase | γ-Glutamyl transpeptidase |
|----------|----------------------|---------------------------|
|          | unit µg⁻¹ protein    |                           |
| Total homogenate | 0.041 ± 0.001      | 0.057 ± 0.003            |
| P₁       | 0.005 ± 0.0003      | 0.092 ± 0.002            |
| P₂       | 0.011 ± 0.001      | 0.153 ± 0.007            |
| P₃       | 0.103 ± 0.004      | 0.142 ± 0.003            |
| P₄       | 0.574 ± 0.005      | 0.519 ± 0.006            |
| S₁       | 0.002 ± 0.0001     | 0.007 ± 0.0003           |

Both enzymes, however, are practically absent from the supernatant of the 48,000 × g centrifugation designated S₁.

Fig. 1 shows the distribution of alkaline phosphatase in LLC-PK₁ monolayers as determined by histochemical methods. Although the monolayers were floated in the reaction mixture to provide access through both sides, the lead precipitate appeared only on the apical membrane of the epithelial cells. The polarized distribution of the enzyme persisted independent of the cell passage number. Occasionally, however, in high passage number cultures, cells without reaction were observed in close contact with cells having a clear lead precipitate in the apical pole.

The distribution pattern of γ-glutamyl transpeptidase is identical with the distribution of alkaline phosphatase. The enzyme is exclusively localized in the apical membrane of LLC-PK₁ cells (Fig. 2).

Alkaline Phosphatase and γ-Glutamyl Transpeptidase Activities in LLC-PK₁ Monolayers under Different Culture Conditions—Table II indicates that the activity of alkaline phosphatase and γ-glutamyl transpeptidase depends extensively on the growing condition of the monolayer. LLC-PK₁ cells in exponential growth show a decrease of 78 and 81% in the activity of alkaline phosphatase and γ-glutamyl transpeptidase, respectively, as compared with the confluent monolaye-

**FIG. 1. Histochemical localization of alkaline phosphatase in LLC-PK₁ monolayers.** LLC-PK₁ monolayers were fixed in glutaraldehyde and stained for alkaline phosphatase activity as described under “Materials and Methods.” Note the intense lead precipitate in the apical membrane. The microphotograph was selected to show a rare event: the presence of a cell (cell in lower left corner) without a reaction for alkaline phosphatase in close contact with a cell with an intense lead precipitate in the apical side. × 7500.
the γ-glutamyl transpeptidase activity (0.90 ± 0.03 unit μg⁻¹ DNA). The specific removal of Ca²⁺ from the incubation medium as occurs during the incubation of the monolayers in minimal essential medium for suspension cultures (Spinner medium) does not inhibit the activity of the alkaline phosphatase (0.69 ± 0.02 unit μg⁻¹ DNA) and γ-glutamyl transpeptidase (0.72 ± 0.08 unit μg⁻¹ DNA). The alkaline phosphatase activity in the presence of EDTA, however, was completely restored by the incorporation of Zn²⁺ at 2 mM concentration to the assay mixture (0.69 ± 0.03 unit μg⁻¹ DNA).

**Phosphate Medium Concentration and Alkaline Phosphatase Activity in Confluent LLC-PK₁ Monolayers—Partial removal of phosphate from the culture medium increases the activity of alkaline phosphatase without any noticeable change in the activity of the γ-glutamyl transpeptidase. For instance, Table III shows that the reduction in the phosphate concentration in the culture medium from 1.0 to 0.01 mM produces a gradual increase in the activity of the alkaline phosphatase that reaches, after 48 h of incubation, a value 67% higher than the control value at time 0.

**Alkaline Phosphatase Activity and Transepithelial Electrical Resistance during the Reorganization of LLC-PK₁ Monolayers—**LLC-PK₁ cells removed by treating the confluent monolayers with trypsin-EDTA were plated at saturation density on collagen-coated Nuclepore filters. To avoid further cell division, the cells were incubated in Dulbecco’s culture medium containing 10 mM thymidine. The nonadherent cells were removed by changing the culture medium 1 h after plating. Fig. 3A shows the changes in the alkaline phosphatase activity and transepithelial electrical resistance that occurred immediately after plating. The electrical resistance increased from values below 10 ohm.cm⁻² 2 h after plating to a plateau value of about 200 ohm.cm⁻². Alkaline phosphatase also increased with time after plating from a value 0.10 ± 0.02 unit μg⁻¹ DNA at zero time to a plateau value of 0.75 ± 0.025 unit μg⁻¹ DNA. Both events, however, required different times to reach steady state. The transepithelial electrical resistance stabilized after only 18 h from the time of plating compared with the 48 h required by the alkaline phosphatase activity. Changes in the enzyme activity of confluent monolayers prepared with cells in exponential growth followed essentially the same time course as described before for monolayers prepared with cells in the stationary state (cells harvested from confluent cultures without cell growth). The electrical resistance, however, took 48 h rather than 18 h to reach steady state (Fig. 3B).

**γ-Glutamyl Transpeptidase Activity during the Reorganization of LLC-PK₁ Monolayers—**Fig. 4 shows the changes in the γ-glutamyl transpeptidase activity in the same experiment described previously for alkaline phosphatase. The broken lines correspond to changes in the transepithelial electrical resistance.

### Table II

Alkaline phosphatase and γ-glutamyl transpeptidase activities in LLC-PK₁ monolayers grown under different culture conditions

| Culture condition | Harvest method | Alkaline phosphatase (unit μg⁻¹ DNA) | γ-Glutamyl transpeptidase (unit μg⁻¹ DNA) |
|-------------------|----------------|-------------------------------------|------------------------------------------|
| Confluent         | Scraped        | 0.58 ± 0.01                         | 0.86 ± 0.05                              |
| Exponential       | Scraped        | 0.08 ± 0.004                        | 0.63 ± 0.06                              |
| Growth            | Trypsin-EDTA   | 0.13 ± 0.006                        | 0.16 ± 0.01                              |
|                   | Trypsin-EDTA   | 0.06 ± 0.004                        | 0.19 ± 0.06                              |

### Table III

Effect of the incubation in low phosphate culture medium on the alkaline phosphatase and γ-glutamyl transpeptidase activities of LLC-PK₁ monolayers

| Time of incubation (h) | Alkaline phosphatase (unit μg⁻¹ DNA) | γ-Glutamyl transpeptidase (unit μg⁻¹ DNA) |
|------------------------|-------------------------------------|------------------------------------------|
| 0                      | 0.61 ± 0.02                         | 0.86 ± 0.05                              |
| 12                     | 0.63 ± 0.04                         | 0.81 ± 0.02                              |
| 24                     | 0.65 ± 0.05                         | 0.76 ± 0.02                              |
| 36                     | 0.78 ± 0.06                         | 0.75 ± 0.03                              |
| 48                     | 1.03 ± 0.11                         | 0.81 ± 0.04                              |
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Fig. 3. Time course of alkaline phosphatase and transepithelial electrical resistance in monolayers of LLC-PK$_1$ cells. A, at time 0, cells from stationary cultures were plated at saturation density on collagen-coated Nuclepore filters. The alkaline phosphatase activity (O—O) and transepithelial electrical resistance (•—•) were recorded periodically after plating. B, alkaline phosphatase activity (O—O) and transepithelial electrical resistance (•—•) in monolayers plated at saturation density with cells from actively dividing cultures. Each value is the mean of six monolayers ± S.E.

Resistant replotted from Fig. 3. The enzyme activity in monolayers prepared with cells in the stationary state (cells harvested from confluent monolayers) shows a small but significant decrease from 1.02 ± 0.084 unit μg$^{-1}$ DNA at time 0 to 0.76 ± 0.031 unit μg$^{-1}$ DNA 6 h after plating (Fig. 4A). The activity, however, increases again and 48 h after plating reaches a new steady state value of 0.89 ± 0.029 unit μg$^{-1}$ DNA, very close to the value reported at time 0. The enzymatic activity of confluent monolayers prepared with cells harvested from cultures in exponential growth, starts with a value of 0.35 ± 0.032 unit μg$^{-1}$ DNA at time 0 and increases with the culture time to reach a steady state of 0.94 ± 0.062 unit μg$^{-1}$ DNA 72 h after plating (Fig. 4B). There is no difference in the steady state value of the electrical resistance and enzyme activity in monolayers prepared with cells in stationary state or in exponential growth.

Subcellular Distribution of Alkaline Phosphatase during the Reorganization of LLC-PK$_1$ Monolayers—Fig. 5 shows the distribution of alkaline phosphatase during the reorganization of LLC-PK$_1$ monolayers as determined by histochemical methods. In order to obtain a semiquantitative estimation of the enzyme, the monolayers were plated 48, 24, and 12 h before the histochemical reaction was initiated. This procedure allows the simultaneous performance of the histochemical reactions in all the preparations. The figure in the upper left shows the electron microscopy of a monolayer 12 h after plating. The absence of lead precipitate in the apical side is evident. The figure in the upper right shows the electron microscopy of a monolayer 24 h after plating. Although the monolayer was floated in the incubation solution to provide access to the reagents through both sides, the lead precipitate occurs only in the apical side. Similar distribution was observed in monolayers incubated for 48 h; however, the reaction was more intense than in the monolayers incubated for 24 h (figure in the lower left).

Effects of Different Protein and Glycoprotein Synthesis Inhibitors on the Activity of Alkaline Phosphatase and γ-Glutamyl Transpeptidase in LLC-PK$_1$ Cells—Cells from stationary (confluent) or actively dividing cultures were harvested by trypsin-EDTA treatment and plated at saturation density on collagen-coated Nuclepore filters. Ninety minutes after plating, the medium was replaced by a new culture medium containing the indicated concentration of the inhibitor and 10 mM thymidine. The enzymatic activities determined 48 h after plating are shown in Table IV. In monolayers prepared with cells from stationary cultures, actinomycin D and cycloheximide produced a clear inhibition in the activity of alkaline phosphatase but no appreciable change in the activity of γ-glutamyl transpeptidase. The concentrations of actinomycin D and cycloheximide used in this study inhibited more than 75% of the incorporation of [3H]uridine and more than 90% of the incorporation of [14C]proline, respectively, into the
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Effect of cell density on the alkaline phosphatase and γ-glutamyl transpeptidase activity in LLC-PK₁ monolayers

LLC-PK₁ cells were plated at different densities on 10-cm diameter plastic tissue culture dishes in the presence of 10 mM thymidine to avoid any cell growth in the nonconfluent cultures. Seventy-two hours after plating, the monolayers were washed with ice-cold phosphate-buffered saline, scraped from the culture dish, and homogenized in a Sonifer cell disruptor for 5 s at maximal intensity.

### TABLE V

| Condition      | Cell density | Alkaline phosphatase | γ-Glutamyl transpeptidase |
|----------------|--------------|----------------------|---------------------------|
|                | µg DNA cm⁻² | unit µg⁻¹ DNA        | unit µg⁻¹ DNA             |
| Confluent      | 3.46 ± 0.11  | 0.68 ± 0.02          | 0.86 ± 0.05               |
| Nonconfluent   | 0.08 ± 0.004 | 0.71 ± 0.04          | 1.00 ± 0.06               |

Alkaline phosphatase and γ-glutamyl transpeptidase activity in LLC-PK₁ cells grown on a series of Hydron substrates of graded thickness

A 24-well tray was coated with dilutions of poly(2-hydroxyethyl methacrylate) (Hydron) as described under "Materials and Methods." LLC-PK₁ cells were plated at a density of 5 × 10⁵ cells cm⁻² to saturate the area for growing. Forty-eight hours after plating, the adherent cells were scraped into the culture medium with a rubber policeman. The cell suspensions were centrifuged for 10 min at 1,000 × g. The cell pellets were resuspended in 0.5 ml phosphate-buffered saline and sonicated for 2 min in a Sonifer cell disruptor set at maximal intensity.

### TABLE VI

| Hydron density µg cm⁻² | Alkaline phosphatase unit µg⁻¹ DNA | γ-Glutamyl transpeptidase unit µg⁻¹ DNA |
|------------------------|-----------------------------------|--------------------------------------|
| Control                | 0.61 ± 0.02                       | 0.86 ± 0.05                          |
| 3 × 10⁻⁴               | 0.65 ± 0.13                       | 0.77 ± 0.05                          |
| 3 × 10⁻⁴               | 0.48 ± 0.14                       | 0.63 ± 0.04                          |
| 3 × 10⁻⁴               | 0.28 ± 0.02                       | 0.52 ± 0.03                          |

The concentration of tunicamycin used in this study inhibited more than 80% of the incorporation of [³H]fucose but only 16% of the incorporation of [¹⁴C]proline into the trichloroacetic acid-precipitated material. In confluent monolayers prepared with cells in exponential growth, actinomycin D inhibited not only the increase in the activity of alkaline phosphatase but also the increase in the activity of γ-glutamyl transpeptidase.

Effect of Cell Density on Alkaline Phosphatase and γ-Glutamyl Transpeptidase—Table V shows the effect of cell density on alkaline phosphatase and γ-glutamyl transpeptidase in LLC-PK₁ cells. Although the nonconfluent cultures were plated at a density 40 times lower than confluent monolayers, the activity of the alkaline phosphatase and γ-glutamyl transpeptidase determined 48 h after plating was almost identical in both groups. Light microscopy of the nonconfluent monolayers confirmed that each cell remained totally isolated and independent of the neighboring cells.

Effect of Cell-Substratum Adhesion on the Activity of Alkaline Phosphatase and γ-Glutamyl Transpeptidase—Progressive decreases in cell-substratum adhesion, induced by growing the LLC-PK₁ cells in a series of poly(2-hydroxyethyl methacrylate) substrates of graded thickness, produced progressive decreases in the activity of alkaline phosphatase and γ-glutamyl transpeptidase (Table VI). The reduction of the cell-substratum adhesion is almost complete at a polymer density of 30 mg cm⁻². This is shown by the total absence of cells attached to the substratum 48 h after plating. At the lowest polymer density (0.3 µg cm⁻²), however, all the cells attached without any perceptible morphological difference with the control monolayers.

### DISCUSSION

The results of this study indicate that confluent monolayers of LLC-PK₁ cells contain high levels of alkaline phosphatase.
and γ-glutamyl transpeptidase activities. These enzymes are specifically distributed in the apical membrane of the epithelial cells. 

Alkaline phosphatase has been shown to exist in a number of different isoenzyme forms named for the tissue in which they predominate. These different forms have been identified on the basis of electrophoretic mobility, inhibition studies, thermal stability, and immunological reactivity (17). Genetic studies also support the presence of the different isoenzyme forms. In humans, for example, there are at least three gene loci involved in the determination of the different forms of alkaline phosphatase. One locus codes for the term-placental form, another for the enzyme from intestine, and one or more for the enzymes from liver, bone, and kidney (18). The various forms of alkaline phosphatase have been demonstrated in several continuous cell lines (19, 20). The enzymes expressed in these cell lines, however, do not always reflect the characteristics of the tissue enzyme from which the cell line originated (21). Therefore, it is necessary to perform a complete characterization of the alkaline phosphatase activity before concluding that a particular cell line is capable of expressing enzyme activity consistent with the characteristics of its tissue of origin. A test commonly used in characterizing the term-placental isoenzyme is based on the resistance of the enzyme to heat inactivation at 60 °C. All other alkaline phosphatases are rapidly inactivated at this temperature. The thermolability of alkaline phosphatase activity in LLC-PK1 monolayers indicates that the enzyme is not the heat-stable, term-placental type. Our results also show that the alkaline phosphatase activity of LLC-PK1 cells is different from the heat-labile intestinal form. Thus, whereas L-phenylalanine markedly inhibits the activity of the intestinal form (22), it produces only a small inhibition in the enzymatic activity of this kidney cell line. The results with the other two inhibitors presented above confirm the similarity of the alkaline phosphatase activity of LLC-PK1 cells, with the so-called liver-bone-kidney enzyme. L-Homoarginine (23) and Levamisole (24), two specific inhibitors of the kidney form, strongly inhibit the alkaline phosphatase activity in LLC-PK1 cells.

A large fraction of the brush-border alkaline phosphatase in intestinal mucosa is also present in the supernatant fraction (25). In LLC-PK1 monolayers, however, the fractionation studies carried out by differential centrifugation of the total homogenate show that practically all of the alkaline phosphatase and γ-glutamyl transpeptidase activities are recovered in the microsomal fraction and are totally absent from the 48,000 × g supernatant. More specifically, a method described for the separation of brush-border and basolateral membranes from the microsomal fraction of kidney homogenate indicates a preferential distribution of both enzymes in the fractions that correspond to the brush-border membranes. The presence of γ-glutamyl transpeptidase activity in LLC-PK1 monolayers confirms previous results obtained in the same cell line (26, 27).

The specific location of alkaline phosphatase and γ-glutamyl transpeptidase in the apical membrane of LLC-PK1 cells, as shown in the histochemical studies presented in Figs. 1 and 2, concurs with the localization of both enzymes in the brush-border membranes of the intestinal mucosa and renal proximal tubule (7, 8).

Although a possible relationship between renal alkaline phosphatase and phosphate transport has been suggested in rat kidney (28), the functional implications of the enzyme and its polarized distribution are totally unknown. γ-Glutamyl transpeptidase is an enzyme that catalyzes the transfer of the γ-glutamyl moiety of glutathione and other γ-glutamylamides to a variety of acceptors, forming γ-glutamyl peptides (29). It has been postulated that the transpeptidase activity of the enzyme is integral to the transport of amino acids into the cell via the so-called γ-glutamyl cycle (30). The association, however, between the enzyme and the amino acid transport system present in the apical membrane of LLC-PK1 cells (3) remains to be defined.

Based on the different behavior of the γ-glutamyl transpeptidase and the transport of alanine and leucine during cell growth, it was concluded that this enzyme is not an important mediator of neutral amino acid transport (27). Our results indicate, however, that although the conclusion may be correct the arguments are not necessarily related to each other. The transport systems for alanine and leucine are localized at the opposite side of the enzyme in the basolateral side of the cells (31).

Although its mode of action has not been elucidated, cell density is one of the several factors that modulate the levels of alkaline phosphatase (20) and γ-glutamyl transpeptidase activity (27, 32) in cultured cells. The significant reduction in the activity of alkaline phosphatase and γ-glutamyl transpeptidase observed in LLC-PK1 cells in exponential growth agree with these observations. The reduction persists as long as the cells remain in exponential growth. The addition of 10 mM thymidine to the culture medium blocks the synthesis of DNA and produces an increase in the activity of alkaline phosphatase and γ-glutamyl transpeptidase to control levels. This effect is observed even though the cultures were scarce enough to continue in exponential growth in the absence of thymidine (results not shown). These results suggest that the low levels of alkaline phosphatase and γ-glutamyl transpeptidase in exponentially growing LLC-PK1 cells are directly associated with continuous cell division and not with the absence of cell-to-cell interaction.

Another interesting observation presented in Table II is the specific inhibition of alkaline phosphatase activity that occurs after treating the cells with trypsin-EDTA solution for 15 min. γ-Glutamyl transpeptidase is only partially affected by this treatment. The results obtained after treatment of the monolayers with trypsin alone indicate that it is the presence of the chelating agent in the mixture which is responsible for the inhibition of the alkaline phosphatase activity. Escherichia coli alkaline phosphatase is a dimeric zinc metalloenzyme (33) and it has been reported that the pig kidney alkaline phosphatase is similarly constructed (34). Since the activity of this enzyme is totally restored by the presence of 2 mM Zn²⁺ in the incubation medium, we can conclude that the effect of EDTA is due, in part, to the removal of Zn²⁺ from the enzyme molecule.

Alkaline phosphatase and γ-glutamyl transpeptidase activities are localized in the proximal tubule and are absent from the rest of the nephron (8, 35). The presence of both enzymes in LLC-PK1 monolayers together with our previous studies on Na⁺-dependent sugars (3), amino acids (5), and phosphate (4) transport systems strongly suggest that the proximal tubule is the nephron segment from which this cell line most probably originates.

Changes in the concentration of inorganic phosphate in the rat kidney produced by a diet lacking in phosphate are followed by a 2-fold increase in alkaline phosphatase activity due to the formation of new enzymes (36). In agreement with that observation, the results presented in Table III indicate that the activity of alkaline phosphatase in LLC-PK1 monolayers is also modulated by the inorganic phosphate concentration in the incubation medium. 

The increase in the activity of alkaline phosphatase and γ-glutamyl transpeptidase observed after plating LLC-PK1 cells at saturation density does not reach its apex until several
hours after the synthesis and assembly of the occluding junctions reach a steady state. The alkaline phosphatase activity follows essentially the same time course irrespective of the origin of the cells. The increase in the γ-glutamyl transpeptidase activity follow a time course different from that observed in cells seeded at low density. In actively growing cells, it is not until the cultures have reached saturation density that the enzyme reaches its maximal value (27). The slow increase observed in actively growing cultures probably represents the progressive arrest of cell division as the monolayers become more confluent. This possibility is supported by the results presented in Table V and discussed later in this section. On the other hand, the activity of γ-glutamyl transeptidase shows a clear difference between monolayers prepared with cells from stationary versus cells from actively dividing cultures. Since treatment of the stationary cultures with trypsin-EDTA solution does not affect appreciably the level of γ-glutamyl transpeptidase, the de novo synthesis of this enzyme (see later) can only be attained in monolayers prepared with cells from exponentially growing cultures where the activity of the enzyme is originally low. Under these conditions, γ-glutamyl transpeptidase synthesis follows essentially the same time course as alkaline phosphatase. The changes in activity observed after plating raises some questions about the regulatory processes involved in the synthesis of these enzymes. Alkaline phosphatase and γ-glutamyl transpeptidase consist of two dimeric glycoproteins anchored to the brush-border membrane of epithelia like the renal proximal tubule and intestinal mucosa (37, 38). Modulation of the enzyme capacity may be caused by changes in the amount of enzyme protein, alterations in the catalytic capacity of existing enzyme molecules, or a combination of these two variables. The possibility that the increase in enzyme activity is mediated by recycling or activation of a pre-existing protein must be discarded on the basis of the results presented in Table IV, which show that the response is dependent on the de novo synthesis of RNA and protein. The inhibitory effect of actinomycin D and cycloheximide agree with similar results obtained on the synthesis of alkaline phosphatase in the rat kidney induced by a low phosphate diet (39) and supports the conclusion that the activity of both enzymes in LLC-PK1 monolayers is also regulated at the transcriptional level. Due to the glycoprotein nature of these enzymes, however, the results obtained with actinomycin D and cycloheximide do not rule out the possibility that the modulation of both enzymes could also occur as a post-translational phenomenon, during the glycosylation of the proteins. Synthesis of active alkaline phosphatase and γ-glutamyl transpeptidase, however, is only moderately reduced by tunicamycin, an inhibitor of dolichol-mediated protein glycosylation (40, 41). These results suggest that glycosylation is not necessary for the synthesis of these enzymes since almost the total inhibition of [3H]glucose incorporation causes a reduction in the enzyme level no greater than that accounted for by a moderate inhibition of general protein synthesis caused by this agent. From these results, we infer that glycosylation is not a significant step in the modulation of alkaline phosphatase or γ-glutamyl transpeptidase.

The increase in the transepithelial electrical resistance is an expression of the development of the occluding junctions, the belt-like structures that seal the intercellular space at the apical end (1, 42). In addition to functioning as a permeability barrier, the occluding junctions also behave as a fence to establish the limit between the apical and basolateral membrane in epithelial cells (43). The delay in the development of the two enzymatic markers of the apical membrane with respect to the occluding junctions indicates that the different membrane components are inserted after the limits of the membrane have been established. The results presented in Fig. 5 support this possibility. They show that alkaline phosphatase activity appears directly in the apical membrane of the epithelial cells after the limits of the membrane have already been established by the synthesis and assembly of the occluding junctions. We have not observed a preferential distribution of the newly synthesized enzymes near the cell-to-cell contact region of the apical membrane as was observed in the distribution of the enzyme aminopeptidase in Madin-Darby kidney cells monolayers (44). This discrepancy, however, could be explained by the presence of different distribution mechanisms or by the different methodologies used in visualizing the enzymes.

The segregation of the two membrane domains is maintained by the continuity of the occluding junctions that form a transmembrane barrier between the apical and basolateral membrane. Removal of Ca2+ (45, 46) or the complete dissociation of the epithelial membrane into individual cells, for example (47), destroys the surface heterogeneity that differentiates the apical from the basolateral membrane of epithelial cells. In agreement with these observations, the results presented in Fig. 6 show that after the monolayers have been transferred to a Ca2+-free medium, the alkaline phosphatase activity is no longer restricted to the apical domain of the cell membrane. After 6 h of incubation, the enzyme has migrated over the cell surface, until they cover the cell membrane at

FIG. 6. Histochemical localization of alkaline phosphatase in confluent monolayers exposed for different times to a Ca2+-free culture medium. Confluent monolayers of LLC-PK1 cells grown for 7 days in Dulbecco’s culture medium were transferred to minimal essential medium for suspension cultures. Cultures exposed for 0 h (upper left), 6 h (upper right), and 24 h (lower left) to the Ca2+-free medium were fixed and processed for the histochemical localization of alkaline phosphatase. Note the progressive migration of the activity, first to the cell membrane facing the intercellular space and finally to cover all the cell surface. × 7500.
the level of the intercellular space. After 24 h, the migration of the enzyme is even more evident, covering by this time practically the entire surface of the cell.

An essential requirement in the formation of the occluding junctions is that the epithelial cells make contact with each other so that their membrane borders come into close apposition. It is not clear whether cell-to-cell contact is required as well to initiate the synthesis of alkaline phosphatase and γ-glutamyl transpeptidase. The results presented in Table V indicate that the synthesis of both enzymes can occur even in the absence of any contact between the cells. These observations suggest that the occluding junction in LLC-PK1 monolayers is involved in the polarized distribution of, but not in the modulation of, the synthesis of both enzymes.

The relationship between epithelial cell attachment and polarization is poorly understood. Studies performed in cultured thyroid cells, however, show that the culture substrate has a clear influence on epithelial cell differentiation (48) and on the orientation of epithelial cell polarity (49). In agreement with these observations, the experiment presented in Table VI indicates that a progressive reduction in cell-substrate adhesion is associated with a progressive decrease in the activity of the enzyme even more evident, covering by this time practically the entire surface of the cell.

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