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Expression of Gamma-Glutamyl Transpeptidase by Renal Epithelial Cells Occurs on a Cell-by-Cell Basis and Is Inhibited by Chronic TPA Treatment

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Abstract Upon attaining a confluent density, populations of the renal epithelial cell line, LLC-PK_1, express progressively many properties characteristic of the renal proximal tubule cell, including gamma-glutamyl transpeptidase activity. Expression of transpeptidase activity was inhibited reversibly by chronic treatment with the phorbol ester tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). TPA treatment inhibited expression of transpeptidase activity regardless of whether added prior to or following appearance of the activity. Increased transpeptidase activity in postconfluent cell populations was due to an increased enzyme V_{max} with no change in substrate K_m. TPA-treated cell populations exhibited a low V_{max} similar to subconfluent populations. Detection of transpeptidase activity at the individual cell level by enzyme histochemistry demonstrated that near-confluent cell populations possessed few transpeptidase activity–positive cells. Progressive expression of transpeptidase activity in the cell population was due to an increasing proportion of cells in the population possessing transpeptidase activity. There was a parallel increase in the proportion of cells expressing transpeptidase protein, detected by immunofluorescence. TPA treatment inhibited appearance of both transpeptidase activity and transpeptidase protein in virtually all cells of the population. These results demonstrate that expression of transpeptidase activity in populations of LLC-PK_1 cells occurs on a cell-by-cell basis and reflects expression of transpeptidase protein. Chronic treatment with TPA inhibits reversibly expression of transpeptidase activity and protein, suggesting a role for protein kinase C in regulating expression of this proximal tubule–specific property. © 1995 Wiley-Liss, Inc.

Key words: proximal tubule cell, LLC-PK_1 cell, differentiation, brush border membrane, phorbol ester, protein kinase C

During fetal renal development presumptive renal epithelial cells undergo both morphological and functional differentiation. Functional differentiation is defined as the progressive expression of segment-specific properties. Differentiation of the renal proximal tubule cell is characterized by expression of multiple properties, including gamma-glutamyl transpeptidase (gGT) activity [Curto et al., 1988]. Renal tissue samples from several renal disease states exhibit decreased levels of gGT activity [Wilson et al., 1986], suggesting that disruption of renal epithelial cell differentiation is a feature of some renal diseases. Despite the central importance to both renal development and disease of understanding how the renal proximal tubule cell regulates expression of proximal tubule–specific properties, this process is poorly understood. We have employed a renal epithelial cell line, LLC-PK_1 [Hull et al., 1976], as a model system in which to elucidate both the process of proximal tubule cell differentiation and its regulation.

Confluent populations of the LLC-PK_1 cell exhibit many morphological and functional properties characteristic of the renal proximal tubule cell, including high levels of gGT activity [Amsler et al., 1991; Rabito et al., 1984; Sepulveda et al., 1982; Yoneyama and Lever, 1984]. Subconfluent populations, in contrast, express low gGT activity. Upon attaining a confluent density, gGT activity in cell homogenates increases progressively over days to weeks. We have recently proposed a model for expression of proximal tubule–specific properties by the LLC-PK_1 cell [Amsler, 1994]. This model predicts that expression of proximal tubule–specific properties will occur on a cell-by-cell basis. Histochemical stud-

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ies demonstrated that gGT activity was expressed by only some of the cells in a population [Rabito et al., 1984], consistent with this prediction. To date, however, independent confirmation of this finding has not been reported. Nor has it been directly examined whether changes in gGT activity in a cell population are correlated with changes in the number of gGT activity–positive cells. Further, it is not known how heterogeneity in expression of gGT activity relates to expression of the gGT protein. Progressive expression of activity could reflect either progressive expression of active gGT protein or, alternatively, progressive activation of preexisting protein.

A second major area of interest in the field is identification of modulators of expression of differentiated properties, such as gGT activity, by renal proximal tubule cells. A role for protein kinase C in modulating expression of proximal tubule–specific properties by LLC-PK_1 cell populations has been suggested [Peng and Lever, 1993] but remains uncertain. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a phorbol ester tumor promoter which activates multiple protein kinase C isoforms [Nishizuka, 1988]. Effects produced by treatment with TPA have been interpreted as implicating the involvement of protein kinase C in a process. Chronic treatment of LLC-PK_1 cell populations with TPA inhibited expression of another proximal tubule–specific property, Na-hexose symport activity [Amsler and Cook, 1982; van den Bosch, 1991]. It is not known whether chronic TPA treatment affects only expression of symport activity or whether expression of other proximal tubule–specific properties, such as gGT activity, are also inhibited by chronic TPA treatment. In addition, it is not known whether TPA-mediated inhibition of expression of proximal tubule–specific functional activities reflects posttranslational modulation of the activity of existing proteins or modulation of protein expression.

MATERIALS AND METHODS

Cell Culture

Stock cultures of Cl4 cells, a clone of the LLC-PK_1 cell line [Amsler and Cook, 1985], were maintained at a subconfluent cell density in complete medium (alpha modification of Eagle's Minimal Essential Medium supplemented with 10% fetal bovine serum) at 37°C in a 5% CO_2 atmosphere. Cells were detached with trypsin-EDTA solution and reseeded routinely at a 1:10 dilution. Medium was replenished every 2–3 days.

For experiments, Cl4 cells were seeded onto 35 mm tissue culture dishes or permeable membrane filter inserts in complete medium and incubated as above. Two days after seeding, medium was aspirated and replaced with complete medium without or with 100 nM TPA. Medium was replenished every 2–3 days.

gGT Enzyme Assay

gGT activity was measured essentially as described by Sepulveda et al. [1982]. Cl4 cell populations were rinsed three times with ice-cold balanced salt solution and solubilized by addition of 1 ml 1% Triton X-100. Approximately 5 μg cell protein, quantitated according to Bradford [1976], was added to 0.45 ml assay solution (150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.5) and 0.5 ml substrate solution (assay buffer containing 5 mM L-glutamyl-p-nitroanilide and 100 mM glycylglycine). Triton X-100 was added to a final concentration of 0.05%, and final volume was 1 ml. Samples were incubated at room temperature for the desired period. Reaction was stopped by addition of 2 ml 10% acetic acid. Light absorption at 405 nm was measured, and p-nitroaniline content was determined by comparison to the absorbance of known standard p-nitroaniline solutions. Using these conditions, reaction rate was linear for at least 15 min. A standard assay time of 10 min was routinely used. Data are expressed as mean ± standard deviation of triplicate samples taken from the same cell homogenate.

When the gGT inhibitor, acivicin (L-[alpha S, 5S]-alpha-amino-3-chloro-4,5-dihydro-5-isoxazol-acetic acid [AT-125]), was used, cell homogenates were preincubated for 30 min at 37°C with 0.45 mM acivicin in assay solution [Reed et al., 1980]. Identical results were obtained regardless of whether acivicin-containing solution was present during the reaction or was removed prior to the reaction.

gGT Histochemistry

Localization of gGT activity in populations of intact cells was performed essentially as described by Glenner and Folk [1961]. Cl4 cells seeded on either 35 mm tissue culture dishes or filter supports were rinsed twice with room tem-

perature phosphate-buffered saline solution (PBS). To the apical or basolateral compartment was added 2 ml of reaction solution (0.5 ml of a stock solution containing 4 mg/ml (13 mM) L-glutamyl-1-naphthylamide monohydrate in acetone, 0.5 ml of a stock solution containing 20 mg/ml (0.15 M) glycylglycine in 100 mM Tris-assay buffer, pH 7.2, 1 ml of a stock solution containing 15 mg/ml stable diazomium salt of o-aminoazotoluene (fast garnet GBC), and 38 ml 100 mM Tris-HCl, pH 7.2). For populations on filters the compartment not receiving the reaction solution received PBS. Populations were incubated at 37°C for 30 min, and reaction solution was aspirated. Cells were fixed by addition of 1% formaldehyde solution and incubation at 4°C for 30 min. Fixed cell populations were rinsed several times with PBS and maintained in PBS on ice until viewing and photography using a Zeiss IM-35 microscope with camera attachment.

For quantitation of positively staining cells, a total of 600–700 cells were counted in five separate fields at 10× magnification on each of two cell populations. The mean ± standard deviation of the number of stained cells and the total number of cells per field were calculated. Data are expressed either as the raw counts or as the ratio of the number of stained cells divided by the total number of cells counted.

gGT Immunofluorescence

Cl4 cell populations maintained on permeable membrane filters were rinsed three times with Hanks' Balanced Salt Solution and then fixed by addition of 95% methanol and incubation at −20°C for 30 min. Methanol was aspirated, and cells were rinsed three times with PBS (pH 8.0) and incubated in PBS containing 0.2% BSA and 0.2% gelatin for 1 h. Populations were then incubated in PBS containing BSA and gelatin plus a rabbit polyclonal anti-gGT antibody (a gift from Dr. R. Hughey, University of Pittsburgh School of Medicine, Pittsburgh, PA) at a 1:20 dilution for 30 min. Populations were rinsed four times with PBS and then incubated in PBS plus BSA and gelatin containing fluorescein-conjugated anti-rabbit IgG (1:200 dilution) (Vector Laboratories) for 30 min. Following four rinses with PBS, cells were viewed using a Zeiss IM-35 microscope equipped for epifluorescence and photographed.

RESULTS

Progressive Expression of gGT Activity and Inhibition by Chronic TPA Treatment

Our initial experiments determined if chronic TPA treatment inhibited expression of gGT activity by a clone (Cl4) of the LLC-PK_1 cell line. Cl4 cells were seeded at a subconfluent density. Two days later populations were refed with complete medium or complete medium containing 100 nM TPA. Medium was replenished every 2 days, and gGT activity was quantitated periodically in cell homogenates as described in Materials and Methods.

As demonstrated previously [Amsler et al., 1991; Sepulveda et al., 1982], Cl4 cell populations expressed progressively gGT activity upon attaining confluence, day 1 of this experiment (Fig. 1). Populations maintained in 100 nM TPA exhibited a much smaller increase in gGT activity throughout the course of the experiment. Greater than 90% of the gGT activity of control Cl4 cell homogenates was inhibited by the irreversible gGT inhibitor, acivicin [Reed et al., 1980], demonstrating that virtually all of the measured activity reflected true gGT activity (data not shown).

TPA Inhibits gGT Expression Regardless of When Added

TPA inhibited expression of gGT activity when added prior to the initial appearance of this activity. We then asked if addition of TPA following appearance of gGT activity would still in-

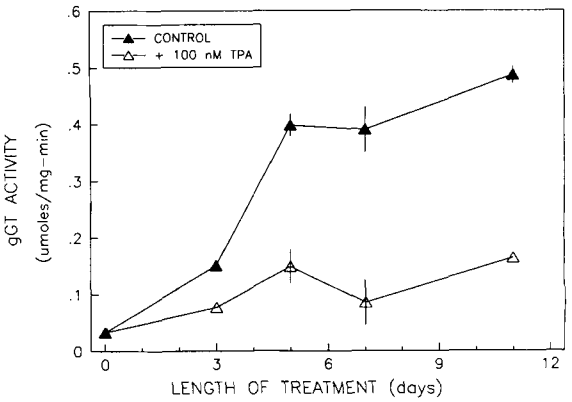

Fig. 1. Expression of gGT activity in Cl4 cell populations maintained in the absence or presence of 100 nM TPA as a function of time in culture. Activity was measured in total cell homogenates as described in Materials and Methods and is expressed as mean ± standard deviation of triplicate samples taken from the same homogenate.

hibit further expression of gGT activity. Cl4 cell populations were maintained in complete medium for the first 7 days of the experiment, at which time the populations had expressed substantial gGT activity (Fig. 2). Populations were then refed with either complete medium or complete medium containing 100 nM TPA. gGT activity in cell homogenates was measured periodically thereafter.

Populations maintained in complete medium continued to show a progressive acquisition of gGT activity through the end of the experiment. However, 3 days after refeeding these populations with complete medium containing 100 nM TPA, gGT activity had decreased from the level observed at the time of addition. gGT activity continued to a basal level, attained within 5 days after TPA addition. Thus, not only did addition of TPA halt further expression of gGT activity, but it also reversed the expression which had occurred prior to TPA addition. This result suggests that expression of gGT activity in Cl4 cells is reversible under certain circumstances.

Reversibility of TPA Inhibition

To determine if the TPA-mediated inhibition of gGT expression was reversible, Cl4 cell populations were maintained in complete medium plus 50 nM TPA for either 6 or 10 days and then refed with complete medium without TPA. gGT activity in cell homogenates was measured periodically during this experimental period (Fig. 3).

As demonstrated previously, chronic TPA treatment inhibited the progressive expression of gGT activity by Cl4 cell populations. When TPA was removed either on day 6 or day 10 following initial exposure, the cell populations subsequently initiated expression of gGT activity. The time course for expression of gGT activity was similar to that displayed by control cell populations which were never exposed to TPA. This result indicates that the TPA effect is completely reversible. That is, upon TPA removal the cells revert completely to their normal behavior pattern.

TPA Concentration Dependence

The concentration dependence for TPA-mediated inhibition of gGT activity expression was assessed by maintaining Cl4 cell populations in complete medium plus various TPA concentrations (0.1–100 nM) for 7 days. gGT activity was then determined in all cell homogenates (Fig. 4). There was little effect of TPA at concentrations of 1 nM and below. Above 1 nM there was a concentration-dependent decrease in homogenate gGT activity. Half-maximal inhibition of expression of gGT activity was observed at about 10 nM. Maximal inhibition was attained at between 50 nM and 100 nM. Preexposure of control cell homogenates to 100 nM TPA for 60 min

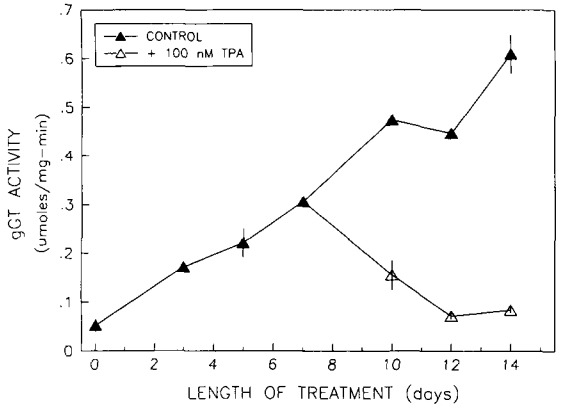

Fig. 2. Effect of adding 100 nM TPA to postconfluent Cl4 cell populations (already expressing substantial gGT activity) on subsequent expression of gGT activity. Cell populations were maintained in complete medium for 7 days. Then half of the populations were refed with complete medium and half were refed with complete medium + 100 nM TPA. Cell homogenate gGT activity was measured periodically during the experiment as described in Materials and Methods. Data are expressed as mean ± standard deviation of triplicate samples taken from the same homogenate.

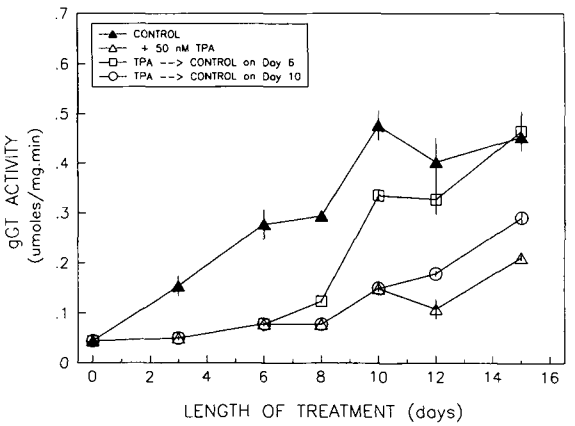

Fig. 3. Reversibility of TPA-mediated inhibition of expression of gGT activity. Cl4 cell populations were maintained in the absence or presence of 100 nM TPA. On day 6 and day 10 of this experiment, TPA-treated cell populations were refed with and maintained thereafter in complete medium without TPA. gGT activity in cell homogenates was measured periodically during throughout the experiment as described in Materials and Methods. Data are expressed as mean ± standard deviation of triplicate samples taken from the same homogenate.

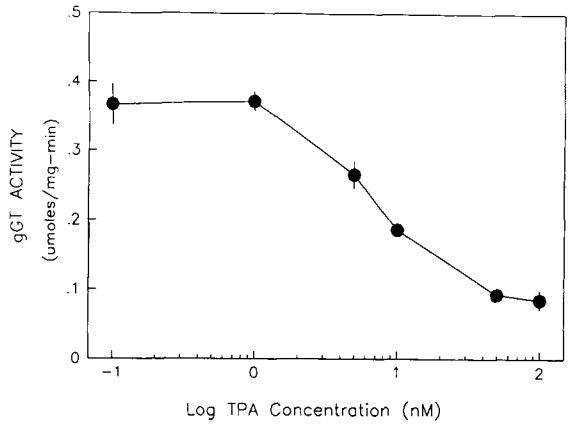

Fig. 4. Concentration dependence of TPA-mediated inhibition of expression of gGT activity. Cl4 cell populations were maintained in complete medium containing varying concentrations of TPA (0.1–100 nM) for 7 days. At the end of this period, gGT activity in cell homogenates was measured as described in Materials and Methods. Data are expressed as mean ± standard deviation of triplicate samples taken from the same homogenate.

did not affect the activity, demonstrating that TPA did not directly inhibit gGT activity (data not shown).

Differentiation and TPA Affect V_{max}

We next turned our attention to the basis for the progressive increase in gGT activity observed following confluence and to the decreased gGT activity produced by chronic TPA treatment. Differences in gGT activity could reflect differences in either or both of substrate affinity (K_m) or maximal enzyme velocity (V_{max}). To determine which of these parameters is affected during development and by chronic TPA treatment, kinetic analyses of gGT activity were performed on subconfluent cell populations, postconfluent populations, and postconfluent populations maintained in the presence of 100 nM TPA (Fig. 5). Glycylglycine concentration (the acceptor in the enzyme reaction) was held constant at a supersaturating concentration to simplify the kinetic analysis. The K_m measured for each set of cell populations was similar, arguing that substrate affinity was not altered either during development or by chronic TPA treatment. In contrast, the maximal enzyme velocity was markedly different in the cell populations. Subconfluent cell populations (Fig. 5A) and postconfluent populations treated with TPA (Fig. 5C) exhibited a relatively low V_{max}. Postconfluent cell populations (Fig. 5B) exhibited a much greater V_{max}, approximately five- to sixfold greater. These results demonstrate that differences in cell homogenate gGT activity reflect differences in the maximal enzyme activity in the populations rather than differences in the characteristics of the expressed enzyme.

Histochemical Localization of gGT Activity

Changes in gGT activity in a cell population could reflect either changes in the gGT activity expressed by all cells of the population or changes in the proportion of cells expressing gGT activity. To determine which of these two possibilities is operative during expression of gGT activity by Cl4 cells and to determine how chronic TPA treatment interferes with this process, gGT activity was localized on a cell-by-cell basis by a histochemical technique [Glenner and Folk, 1961; Rabito et al., 1984]. The number of gGT activity–positive cells was quantitated and correlated with the gGT activity measured in cell homogenates.

Cell populations were seeded and maintained in culture as usual. Cultures were periodically stained for gGT activity as described in Methods and viewed with a light microscope using bright field illumination (Fig. 6). In near-confluent cell populations exhibiting low gGT activity, there were almost no stained cells (Fig. 6A). Just-confluent cell populations exhibited a small number of stained cells (Fig. 6B). The number of gGT activity–positive cells increased progressively with increasing numbers of days after confluence (Fig. 6D,F), as did the proportion of positive cells in the population (Table I). In a single cell population there was a wide range of staining intensity (see, e.g., Fig. 6D) from the absence of staining to very dark staining. Pretreatment with acivicin virtually eliminated the cell staining (data not shown), confirming the specificity of the histochemical staining reaction. Cell populations maintained in the presence of TPA exhibited little staining for gGT activity throughout the experiment (Fig. 6C,E,G).

A good quantitative correlation was observed between the proportion of stained cells and the gGT activity measured in cell homogenates (Table I). Control cell populations exhibited a parallel increase in cell homogenate gGT activity and the percentage of gGT activity–positive cells. TPA-treated cell homogenates exhibited a consistently low level of gGT activity, and there was a correspondingly low proportion of gGT activity–positive cells.

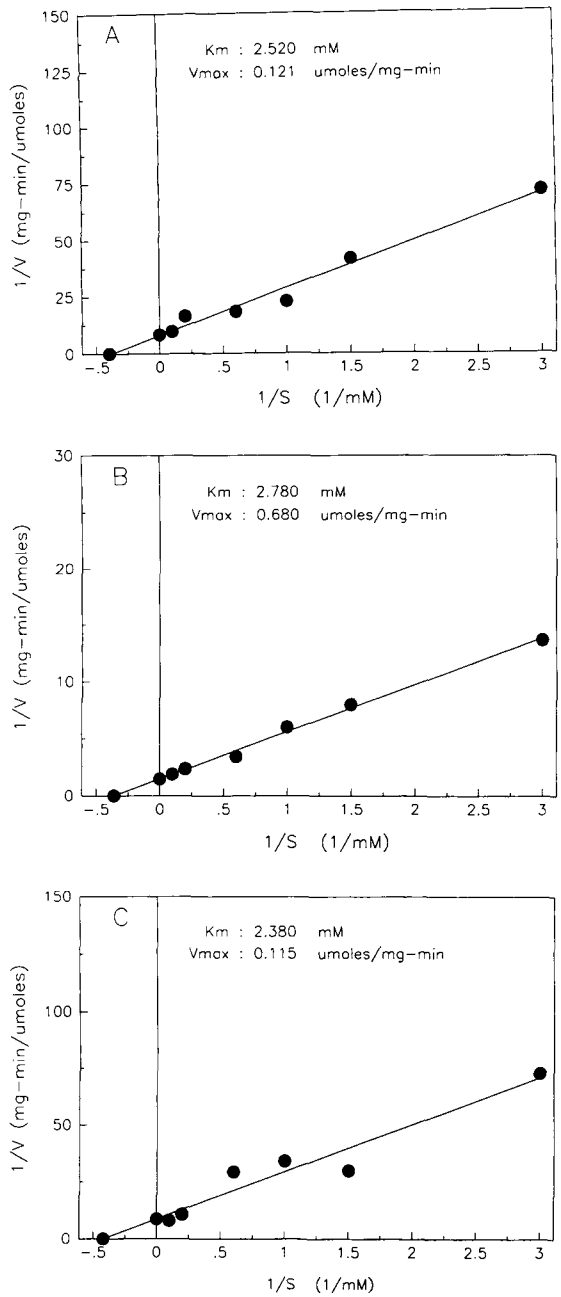

Fig. 5. Kinetic analysis of gGT activity in Cl4 cell populations. gGT activity was quantitated as a function of gamma-glutamyl-p-nitroanilide concentration on subconfluent cell populations (**A**), postconfluent cell populations (**B**), and postconfluent cell populations maintained in the presence of 100 nM TPA (**C**). Data are expressed as mean ± standard deviation of triplicate samples taken from the same homogenate. Lines were drawn and calculated values for K_m and V_{max} were derived by linear regression analysis.

Expression of gGT Protein

Expression of gGT activity by cells in the population could reflect either cell-by-cell expression of the corresponding gGT protein or activation of preexisting gGT protein on a cell-by-cell basis. To determine which of these mechanisms is operative during development of gGT activity in Cl4 cells and at which step TPA acts, Cl4 cell populations (just-confluent, 10 days postconfluent, and 10 days postconfluent treated with TPA) were reacted with anti-gGT antibody. Bound antibody was localized by a fluorescein-labelled antirabbit IgG antibody.

Just-confluent cell populations expressed little detectable gGT protein (Fig. 7A). This result correlated well with the near absence of stained cells by histochemistry and the low maximal enzyme velocity measured in these cell homogenates. Postconfluent cell populations exhibited marked binding of gGT antibody (Fig. 7B). The binding was highly heterogenous, from some cells which were not labelled to other cells which were strongly labelled. This correlates well with the histochemical staining pattern and the relatively high maximal enzyme velocity described above. TPA-treated postconfluent cell populations exhibited virtually no staining (Fig. 7C), correlating well with the absence of stained cells by histochemistry and the low maximal enzyme velocity measured in cell homogenates.

Overexposure of a photograph of a just-confluent cell population (Fig. 7D) revealed the presence in a few cells of perinuclear vesicles which stain for gGT protein. These cells likely represent the first cells within these populations which are initiating expression of gGT protein. The perinuclear localization of the gGT protein may be the first stage in the process leading to appearance of mature gGT protein at the apical surface.

DISCUSSION

The experiments described above address two points about expression of gGT activity in Cl4 cell populations. First, our experiments have definitively demonstrated that expression of gGT activity by a cell population reflects a progressive increase in the proportion of cells in the population which express active gGT protein. The combination of the kinetic analysis, demonstrating a change in V_{max} but no change in K_m, and demonstration of cell-by-cell expression of

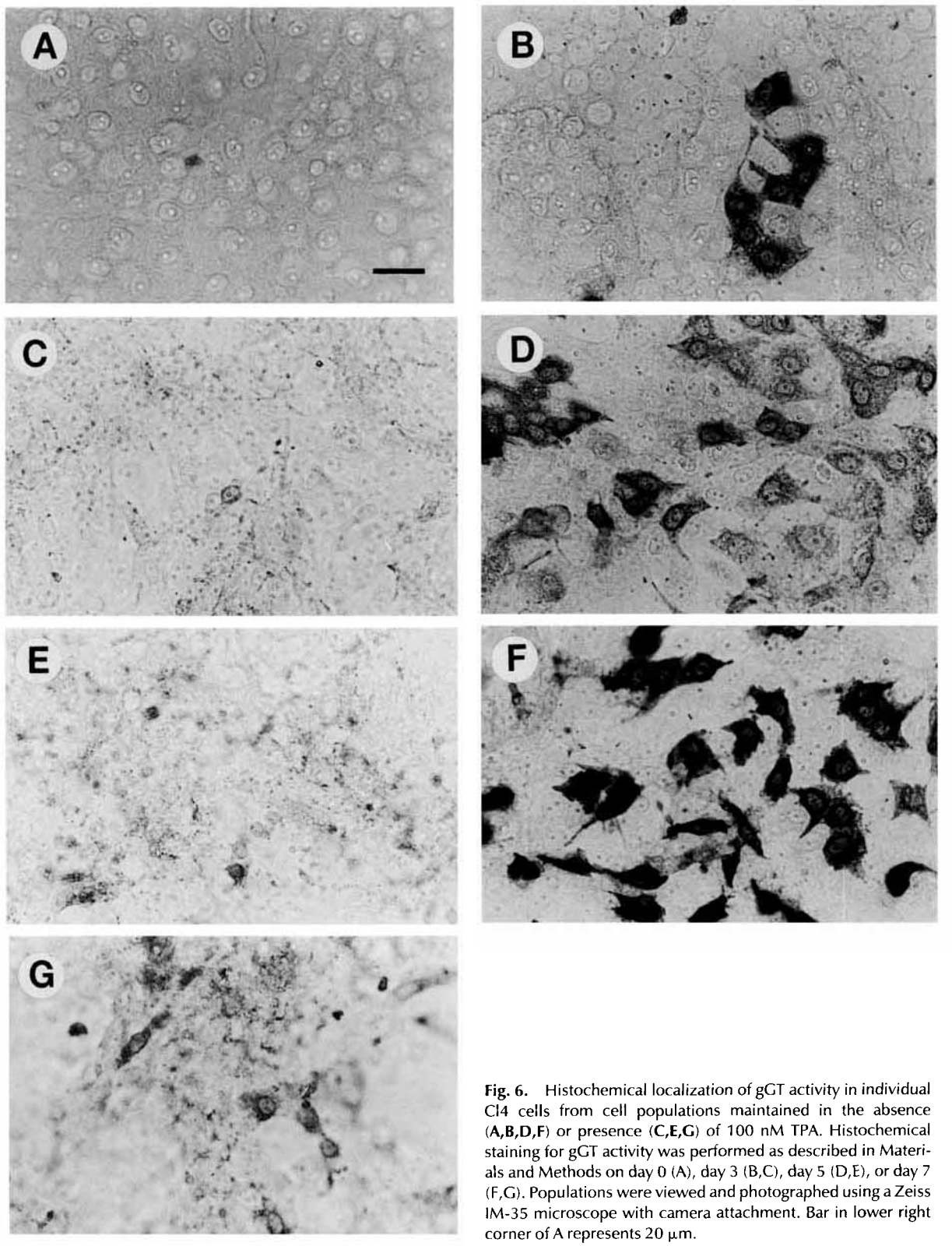

Fig. 6. Histochemical localization of gGT activity in individual Cl4 cells from cell populations maintained in the absence (**A,B,D,F**) or presence (**C,E,G**) of 100 nM TPA. Histochemical staining for gGT activity was performed as described in Materials and Methods on day 0 (A), day 3 (B,C), day 5 (D,E), or day 7 (F,G). Populations were viewed and photographed using a Zeiss IM-35 microscope with camera attachment. Bar in lower right corner of A represents 20 μm.

TABLE I. Histochemical Quantitation of gGT Activity–Positive Cells in Cl4 Cell Populations Maintained in the Absence or Presence of 100 nM TPA*

Sample	gGT activity (micromoles/mg − min)	Stained cells (%)
Day 0	0.042 ± 0.002	1.9
Day 3	0.209 ± 0.010	9.4
Day 3 + TPA	0.077 ± 0.001	1.1
Day 5	0.483 ± 0.015	23.6
Day 5 + TPA	0.149 ± 0.001	1.3
Day 7	0.385 ± 0.011	32.2
Day 7 + TPA	0.085 ± 0.001	1.7

*A total of 600–700 cells were counted in each of five separate fields in two separate cell populations at 100× magnification. The mean ± standard deviation of the number of stained cells and the total number of cells per field was calculated. Data are expressed as the ratio of the number of stained cells divided by the total number of cells counted.

both gGT activity and gGT protein provide definitive evidence that the primary, if not the sole, mechanism by which LLC-PK_1 cell populations express progressively gGT activity is through the cell-by-cell expression of functionally mature gGT protein. The results argue strongly against the possibility that appearance of gGT activity is due to progressive activation of existing gGT protein. The cell-by-cell appearance of gGT activity and protein suggests that Cl4 cells initiate independently expression of proximal tubule–specific properties.

The cell-by-cell expression of gGT protein is strongly supported by Figure 7D. In many days postconfluent cell populations, gGT protein was found primarily at the cell surface (see Fig. 7B) and exhibited a staining pattern consistent with staining of apical microvilli. In contrast, the few stained cells in just-confluent cell populations (see Fig. 7D) did not exhibit this microvillar-like staining pattern but rather possessed perinuclear vesicles which stained strongly for gGT protein. In these populations, the stained cells likely represent the first cells which have begun expressing gGT protein. The fact that the protein was localized primarily in these intracellular vesicles suggests that the cells were not yet transporting the synthesized gGT protein to the apical surface. This may be due to the absence of the cellular machinery required to transport gGT proteins to this surface or to some regulatory process which allows apical transport only at the appropriate time (e.g., when the apical surface structure has been assembled). Modulation of the transport of proteins to the apical surface of Cl4 cells has been previously demonstrated [Amsler, 1993].

We have previously shown that both cell growth state and cell-cell contact modulate expression of proximal tubule–specific properties by LLC-PK_1 cell populations [Amsler, 1994]. A model was proposed in which cells expressed proximal tubule–specific properties only upon growth arrest at a specific arrest point, termed G_D. Increasing levels of "appropriate" cell-cell contact (as yet undefined) increased the likelihood that an individual cell would arrest at this G_D point rather than at the standard G_0 point. This model would predict that progressive expression of a proximal tubule–specific property, such as gGT activity, in the cell population would reflect the progressive accumulation of cells in the population expressing the property. Our results presented here confirm this prediction. They further demonstrate that appearance of gGT activity in cells of the population reflects expression of gGT protein on a cell-by-cell basis.

The second portion of this study investigated the effect of chronic treatment with TPA on expression of gGT activity and protein. TPA treatment strongly inhibited expression of gGT activity. This inhibition was manifested in all cells of the population and reflected a decreased expression of gGT protein. Previous studies have demonstrated that chronic TPA treatment also inhibited expression of Na-hexose symport activity [Amsler and Cook, 1982; van den Bosch et al., 1991], supporting the hypothesis that chronic TPA treatment interferes with expression of the entire cassette of proximal tubule–specific properties by the LLC-PK_1 cell.

The inhibition of expression of gGT activity produced by TPA is reversible. If TPA inhibited a late event in the process leading to expression of gGT activity, then it would be expected that removal of TPA would result in a rapid appearance of gGT activity to the level observed in many days postconfluent cell populations. This was not observed. Rather, following TPA removal gGT activity in the cell population increased with a time course similar to that of control populations upon attaining a confluent density. This suggests that TPA interferes with an early step in the process, perhaps entry of cells into the G_D state.

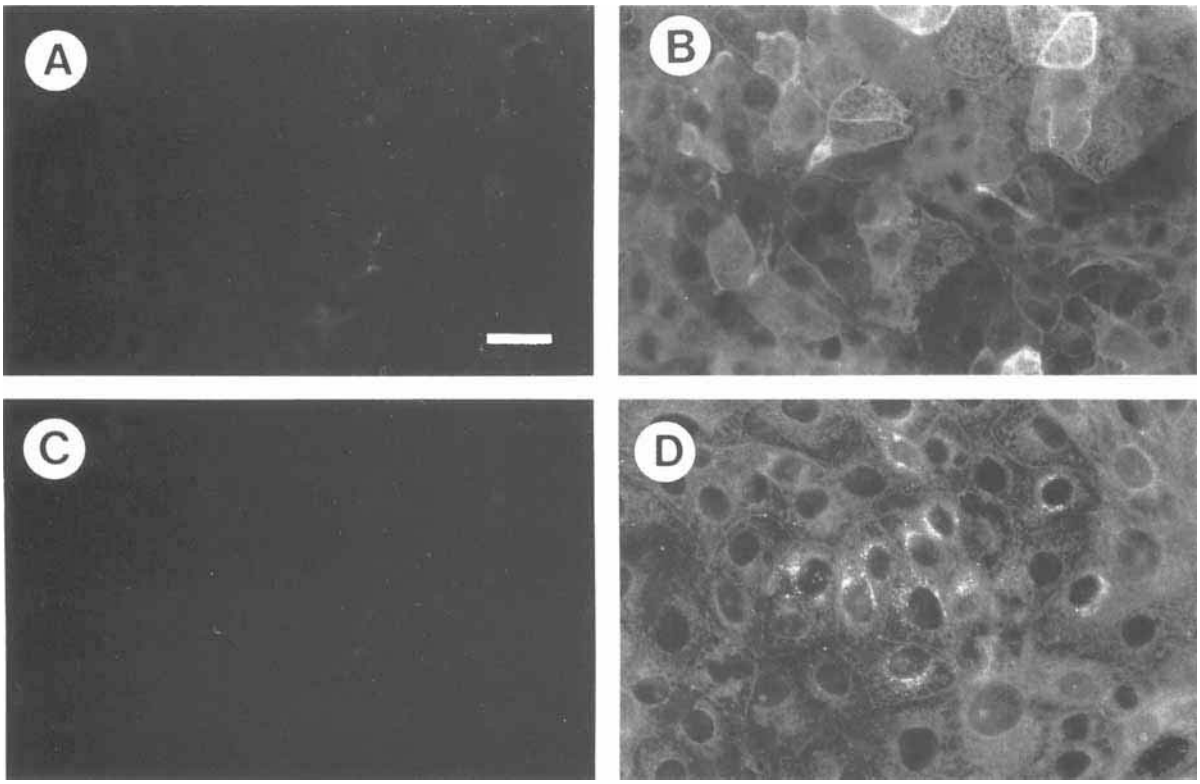

Fig. 7. Localization of gGT protein in individual Cl4 cells by immunofluorescence microscopy. Cell populations were fixed and labelled with anti-gGT antibody as described in Materials and Methods when they had just reached confluence (**A**) and when they were 10 days postconfluent. Postconfluent cell populations had been maintained in the absence (**B**) or presence (**C**) of 100 nM TPA. Populations were viewed and photographed using a Zeiss IM-35 microscope equipped for epifluorescence with camera attachment. **D:** An overexposure of a just-confluent cell population (similar to A) to reveal the presence in a few cells of perinuclear vesicles containing immunoreactive protein. Bar in lower right corner of A represents 20 μm.

Interestingly, addition of TPA to partially differentiated cell populations not only blocked further increases in gGT activity, but also decreased the level of gGT activity expressed by the population. The level eventually declined to that of chronic TPA-treated cell populations. This implies that maintenance of the differentiated state by LLC-PK_1 cells requires some factor(s) which is affected by chronic TPA treatment or is reversed by some factor(s) which is produced by chronic TPA treatment.

Finally, protein kinase C is the major TPA receptor in mammalian cells [Nishizuka, 1988]. Effects produced by TPA are most likely due to modulation of PKC activity. The TPA concentration dependence for inhibition of expression of gGT activity ($K_{I,0.5}$ = 10 nM) is consistent with the affinity of the TPA interaction with protein kinase C. Since chronic TPA treatment also inhibited expression of Na-hexose symport activity [Amsler and Cook, 1982; van den Bosch et al., 1991], these results suggest that protein kinase C is involved in regulating expression of the entire cassette of proximal tubule–specific properties by LLC-PK_1 cells.

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