Inhibition of cell differentiation by Gαq in the renal epithelial cell line LLC-PK₁

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Department of Cell Biology and Anatomy, The University of North Carolina at Chapel Hill, Chapel Hill 27599; Department of Biological Sciences, Campbell University, Buies Creek, North Carolina 27506; and Department of Physiology and Biophysics, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Sun, Li, Hyun, Debra J. Weaver, Kurt Amsler, and Ellen R. Weiss. Inhibition of cell differentiation by Gαq in the renal epithelial cell line LLC-PK₁. Am. J. Physiol. 274 (Cell Physiol. 43): C1030–C1039, 1998.—LLC-PK₁, an epithelial cell line derived from the kidney proximal tubule, was used to study the ability of the G protein α-subunit, Gαq, to regulate cell differentiation. A constitutively active mutant protein, αqQ209L, was expressed using the LacSwitch-inducible mammalian expression system. Induction of αqQ209L expression with isopropyl-β-D-thiogalactopyranoside (IPTG) enhanced phospholipase C activity maximally by 6- to 7.5-fold. Increasing concentrations of IPTG progressively inhibited the activity of two differentiation markers, Na⁺-dependent hexose transport and alkaline phosphatase activity. Induction of αqQ209L expression also caused a change from an epithelial to a spindle-shaped morphology. The effects of αqQ209L expression on cell differentiation were similar to those observed with 12-O-tetradecanoylphorbol 13-acetate (TPA) treatment. However, protein kinase C (PKC) levels were downregulated in TPA-treated cells but not in αqQ209L-expressing cells, suggesting that the regulation of PKC by Gαq may be different from regulation by TPA. Interestingly, the PKC inhibitor GF-109203X did not inhibit the effect of IPTG on the development of Na⁺-dependent hexose transport in αqQ209L-expressing cells. These data implicate PKCα and PKCε in the pathway used by Gαq to block the development of Na⁺-dependent hexose transport in IPTG-treated cells.

Phospholipase C; kidney; proximal tubule; protein kinase C; G protein

The renal epithelial cell line LLC-PK₁ has been used extensively as a model for investigating the regulation of kidney differentiation in the proximal tubule (2–4, 28, 32, 43). These cells display the characteristic morphology of a polarized renal epithelium on reaching confluence (8, 9). They progressively acquire several properties typical of the brush border of the kidney proximal tubule, such as expression of Na⁺-dependent hexose transport, γ-glutamyltranspeptidase, and alkaline phosphatase activities (7, 20, 43). The development of Na⁺-dependent hexose transport activity is accelerated in response to compounds that raise cAMP levels and is significantly decreased in LLC-PK₁ mutants deficient in cAMP-dependent protein kinase (3, 4). Phorbol esters, such as 12-O-tetradecanoylphorbol 13-acetate (TPA), which activate protein kinase C (PKC), can prevent or drastically delay the expression of several of these differentiation markers, including the Na⁺-dependent hexose transporter (3, 8, 37), γ-glutamyltranspeptidase, and alkaline phosphatase (5, 7). The conversion from undifferentiated, actively growing cells to a culture expressing proximal tubule-specific traits and the ability to manipulate the expression of these differentiation markers make these cells particularly useful for studying the regulation of epithelial cell differentiation. The inhibitory influence of TPA on the expression of differentiation markers in this cell line has suggested the participation of PKC in their regulation. However, the effect of physiological activation of PKC through the stimulation of phospholipase C (PLC) has not been studied. Members of the Gαq family of G proteins are known to mediate the activation of phosphoinositide (PI)-specific PLCβ in response to stimulation of a variety of G protein-coupled receptors (31). PI-PLCβ hydrolyzes phosphatidylinositol bisphosphate (PIP₂), generating diacylglycerol (DAG), and inositol trisphosphate (IP₃). Both of these metabolic products play critical roles in cell signaling; DAG is known to be a physiological activator of PKC, and IP₃ stimulates the release of Ca²⁺ from intracellular stores (34). Therefore these pathways may be important in the regulation of cell differentiation.

The introduction of constitutively active mutants of G protein α-subunits into cells has been used by many laboratories to define the signaling pathways regulated by a particular G protein without the necessity of activating the appropriate G protein-coupled receptor (17). The mutations that generate constitutive activation are in the GTP binding domain of the α-subunit, resulting in loss of the ability to hydrolyze GTP. These mutants have been found to constitutively activate their downstream effectors. In fibroblasts, expression of constitutively active mutants of members of the Gαq family has profound effects, inducing mitogenesis and transformation in NIH-3T3 cells (10, 11, 18) and inhibiting the proliferation of Swiss-3T3 cells (29). The present study represents the first investigation of the regulation of growth control and differentiation in epithelial cells by a member of the Gαq family.

The GTPase-deficient constitutively active mutant protein αqQ209L (containing a mutation of Gln-209 to Leu) was stably expressed in LLC-PK₁ cells using the LacSwitch-inducible system, which allows for the stable integration of this gene into cells without disturbing their normal pattern of growth and differentiation. The use of an inducible promoter circumvents the problem of lethality (29) or possible compensation in cells due to constitutive expression of GTPase-deficient mutants (22). In the present study, we show that induction of αqQ209L expression enhances PLC activity and results in inhibition of cell differentiation in LLC-PK₁ cells. Although αqQ209L expression exerts effects on cell...
differentiation similar to treatment with TPA, total PKC activity is downregulated in TPA-treated cells but not in induced \( \alpha_Q209L \)-expressing cells. Therefore the activation of the PLC pathway by its physiological regulator, \( G_\text{q} \), may have effects that are distinct from those observed with TPA.

**MATERIALS AND METHODS**

Cell culture The LLC-PK1 cell line was received from the American Type Culture Collection. This cell line is maintained in \( \alpha \)-MEM supplemented with 10% fetal bovine serum in 5% \( \text{CO}_2 \)-95% air at 37°C. Medium is replenished every 3–4 days. When cells reach confluence, routine passage is carried out by washing cell monolayers in \( \text{Ca}^{2+}, \text{Mg}^{2+} \)-free PBS (in mM: 137 \( \text{NaCl} \), 2.7 \( \text{KCl} \), 4.3 \( \text{Na}_2\text{HPO}_4 \), and 1.4 \( \text{KH}_2\text{PO}_4 \), pH 7.4), followed by trypsin digestion (0.25% trypsin and 1 mM EDTA) in Hank’s balanced salt solution (HBSS).

Stable transfection of LLC-PK1 cells. The LacSwitch-inducible mammalian expression system (Stratagene) was used. The Lac repressor vector (p3SS) was transfected into the LLC-PK1 cells by calcium phosphate precipitation (16). Cells (10/10-cm dish) were transfected with 10 \( \mu \)g of the plasmid DNA. Stable clones were selected for resistance to 1 mg/ml hygromycin (LC Laboratories) and examined for the expression of the Lac repressor by indirect immunofluorescence using a polyclonal antibody to the Lac repressor (Stratagene). A clone that showed the highest expression of the Lac repressor based on the brightness of nuclear staining was selected. This clone was stably transfected with the pOPRSVI operator vector alone or pOPRSVI containing the cDNA for mutant \( \alpha_Q209L \) (a gift from Dr. Gary L. Johnson, National Jewish Medical and Research Center, Denver, CO) using calcium phosphate precipitation. For the second transfection, 2 \( \times \) 10\(^6\) cells were transfected with 1 \( \mu \)g of the specific DNA and 10 \( \mu \)g of pSP72 carrier DNA per 10-cm dish. G418 (GIBCO BRL) at 1 mg/ml was added into the media to select for clones containing the lac operator vector.

Screening of clones by RT-PCR. To measure the expression of \( \alpha_Q209L \) in the presence of isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG, Stratagene), hygromycin- and G418-resistant clones were screened by RT-PCR. For each clone, 5 mM IPTG was added to a 10-cm dish of cells for 12 h. Total RNA was isolated from each clone. After reverse transcription with Moloney murine leukemia virus RT (Boehringer Mannheim), cDNA was amplified by PCR with Taq DNA polymerase (Promega) using a 31-base 5'-primer corresponding to nucleotides 739–769 in the \( \alpha_q \) cDNA and a 31-base 3'-primer corresponding to the 5'-end of the thymidine kinase poly(A) consensus sequence in pOPRSVI. The predicted size of the PCR product is 351 bp.

PLC assay. Cells were plated at a density of 2 \( \times \) 10\(^9\)/35-mm dish in hygromycin- and G418-free media and allowed to grow for the indicated times in culture. One day before measurement, cells were washed with PBS and labeled with myo-[2\(^3\)H]inositol (Amersham) at 1 \( \mu \)Ci/ml for 16–24 h in serum-free \( \alpha \)-MEM containing 0.1% BSA at 37°C. After labeling, cells were washed twice with serum-free \( \alpha \)-MEM-0.1% BSA and once with serum-free \( \alpha \)-MEM-0.1% BSA containing 20 mM LiCl. Cells were then incubated for 30 min in \( \alpha \)-MEM-0.1% BSA containing 20 mM LiCl to prevent degradation of inositol phosphates (IPS). Different concentrations of IPTG were added as described in the text. At the end of the incubation, cells were fixed in 1 ml of ice-cold acidified methanol (methanol-HCl, 100:1), scraped, and transferred into a tube containing 2 ml of chloroform, 1 ml of \( \text{H}_2\text{O} \), and 1 ml of acidified methanol. After mixing and centrifugation, the aqueous phase containing IPS was passed through an A1G-148 (200–400 mesh, formate form, Bio-Rad) anion-exchange column. Total IPS were eluted with 0.1 M formic acid-1.0 M ammonium formate (6). Aliquots were assayed for radioactivity by liquid scintillation spectroscopy. Total \([\text{H}]\text{inositol incorporation into lipid was determined by measuring the radioactivity in the organic phase. The IPS produced were calculated as a percentage of the total labeled lipids.}\n
Na\(^+\)-dependent hexose transport activity. To measure the activity of the Na\(^+\)-dependent hexose transporter, uptake of the nonmetabolizable glucose analog, methyl-\( \alpha\)-D-glucopyranoside (\( \alpha\)-MeG) was performed as described by Amsler and Cook (3). Cells were plated at a density of 2 \( \times \) 10\(^9\)/35-mm dish in hygromycin- and G418-free media. Different concentrations of IPTG were added as described in the figure legends. Where indicated, cells were also incubated with 2 \( \mu \)M GF-109203X (GFX), 50 \( \mu \)g/ml of oleyl-2-acetylglycerol (OAG), or 100 mM A-23187, a Ca\(^{2+}\) ionophore. At the desired points, the medium was aspirated, and cells were incubated in HBSS without glucose (in mM: 137 \( \text{NaCl} \), 5 \( \text{KCl} \), 1.3 \( \text{CaCl}_2 \), 0.4 \( \text{MgSO}_4 \), 7\( \text{H}_2\text{O} \), 0.5 \( \text{MgCl}_2 \)-6\( \text{H}_2\text{O} \), 0.3 \( \text{Na}_2\text{HPO}_4 \), 0.3 \( \text{KH}_2\text{PO}_4 \), and 20 mM HEPEs, pH 7.2) at 37°C for 2 min. The uptake assay was initiated by adding 1 ml of HBSS containing 100 \( \mu \)M \( \alpha\)-[\(^{14}\)C]MeG (0.2 \( \mu \)Ci/ml; \( \alpha\)-[\(^{14}\)C]MeG was from New England Nuclear; \( \alpha\)-MeG was from Sigma), and dishes were incubated at 37°C for 60 min. The cells were washed by aspiration of the solution, and dishes were rinsed three times in ice-cold Tris-buffered saline (15 mM Tris·HCl (pH 7.4)-0.15 M \( \text{NaCl} \)). Cell monolayers were solubilized in 0.2% SDS, and radioactivity was determined by liquid scintillation spectroscopy. Protein was measured using a modified Lowry assay (Bio-Rad) at 750 nm, with BSA as a standard.

Alkaline phosphatase activity. Alkaline phosphatase activity was measured using a modification of the method described by Amsler (2). Cells were plated at a density of 2 \( \times \) 10\(^9\)/35-mm dish in hygromycin- and G418-free media. IPTG at different final concentrations (0.005, 0.05, 0.5, and 5 mM) or TPA (0.1 \( \mu \)M) was added the following day and replaced every other day. At the desired time points, cells were rinsed three times with ice-cold HBSS and solubilized in the assay buffer (in mM: 150 \( \text{NaCl} \), 5 \( \text{KCl} \), 1 \( \text{CaCl}_2 \), 1 \( \text{MgCl}_2 \), and 10 Tris·HCl, pH 10) containing 1% Triton X-100. Approximately 200–300 \( \mu \)g of cell protein per sample were incubated in the assay buffer containing 10 mM p-nitrophenyl phosphate (Sigma) for 60 min at 37°C. The reaction was stopped by the addition of 0.2 N NaOH. Activity was determined by measurement of optical density at 405 nm using p-nitrophenol (Sigma) as a standard. Protein was determined from the same dishes using the Bradford assay.

Counting cells. Cells were plated at a density of 2 \( \times \) 10\(^9\)/35-mm dish, and IPTG at a final concentration of 5 mM was added the following day. Cells were trypsinized on various days indicated in the legend to Fig. 2 and diluted into \( \alpha\)-MEM. Trypan blue was added to a final concentration of 0.04%, and the number of viable cells was determined using a hemocytometer.

Microscopy. Cells were plated at the density of 5 \( \times \) 10\(^5\)/35-mm dish, and IPTG at a final concentration of 5 mM was added the next day. Morphological changes induced by IPTG were viewed with a Zeiss IM35 phase-contrast light microscope containing a \( \times 16 \) Zeiss lens (0.35 NA). Photographs were taken with an Olympus OM-2 camera.

PKC assay. Cells were plated at the density of 1.4 \( \times \) 10\(^9\)/10-cm plate in hygromycin- and G418-free media. IPTG at different final concentrations (0.005, 0.05, 0.5, and 5 mM) or TPA (0.1 \( \mu \)M) was added the following day (day 0) and...
replaced every 2 days throughout the experiments. The kinase assay was performed on day 7 using the PKC Assay System (GIBCO BRL) according to the manufacturer’s protocols. Briefly, total cellular PKC was extracted and partially purified by DEAE-cellulose ion-exchange column chromatography. Kinase reactions were performed by measuring the phosphorylation of an acetylated synthetic peptide from myelin basic protein in the presence of 20 µM [γ-32P]ATP (5 µCi/ml, Amersham) at 30°C for 5 min. TPA at a final concentration of 10 µM was included in the reaction to maximally activate PKC. The specificity of the reaction for PKC was confirmed using the PKC pseudosubstrate inhibitor peptide. Aliquots of the reaction mixtures were spotted onto phosphocellulose disks, washed twice in 1% phosphoric acid and twice in water. The radioactivity was determined by liquid scintillation spectroscopy. Proteins were precipitated with 10% TCA and dissolved in 0.5 M NaOH for quantification using the modified Lowry assay as described above.

Statistical analysis. Statistics were calculated using the computer programs Statview and SuperANOVA from Abacus Concepts.

RESULTS

Expression of constitutively active αqQ209L increases PLC activity. To investigate the effect of Goq expression on cell differentiation, clones stably expressing the GTPase-deficient mutant protein, αqQ209L, were established. Although the expression of mRNA for αqQ209L could be detected by RT-PCR in cells treated with IPTG, protein could not be detected in whole cell homogenates (data not shown). This may be due to the inability of the antibody to detect small increases in protein expression against a background of endogenous Goq in these cells. Because αqQ209L is constitutively active, high levels of expression may not be necessary to cause a significant increase in Goq activity. PLC activity was assayed to determine the induction of αqQ209L expression. A time course (Fig. 1A) demonstrated a maximal increase in IP accumulation after a 12-h incubation in the presence of IPTG in one of the clones, αqQ2. No IP accumulation was observed in cultures incubated in the absence of IPTG. In contrast, a clone transfected with the vector alone (v1) demonstrated no increase in IP accumulation in the presence or absence of IPTG. PLC activity measured after a 12-h incubation with IPTG was selected to screen for IPTG inducibility in all clones found to express αqQ209L mRNA by RT-PCR. Among different αqQ209L-expressing clones, functional inducibility by IPTG varied from no induction to 6- to 7.5-fold induction (data not shown). Two clones, αqQ1 and αqQ2, demonstrated approximately two- and sixfold increases in IP accumulation, respectively (Fig. 1B). These clones were selected for further study because, in the absence of IPTG, both of them demonstrated PLC activity comparable to that of the vector control clone (v1), suggesting that no expression of αqQ209L occurred in uninduced cells. In addition, they represent two different levels of αqQ209L activity.

αqQ209L expression inhibits Na+ dependent hexose transport activity. Na+-dependent hexose transport occurs at the apical membrane of proximal tubule cells and is a biochemical marker for the differentiation of LLC-PK1 cells (8, 9, 21). To study the effect of αqQ209L expression on differentiation in this cell line, the development of Na+ dependent hexose transport activity in the absence or presence of IPTG was compared over a 14-day culture period (Fig. 2A). In the absence of IPTG, both v1 and the αqQ209L-expressing clone, αqQ2, ac-
quired transport activity progressively over time. Incu-

bation with IPTG blocked the development of this

activity in \( \alpha_q Q_2 \), but not in v1. Transport activity was

also plotted as a function of protein amount, as an

indicator of cell density (Fig. 2 (B)). In clones v1 (in

either absence or presence of IPTG) and \( \alpha_q Q_2 \) (in the

absence of IPTG), the ability to transport \( \alpha -\)MeG was

apparent when the cells reached 400–500 µg of protein/dish. In

contrast, IPTG-treated \( \alpha_q Q_2 \) cells exhibited almost no

acquisition of transport activity even at cell densities

far above those of untreated \( \alpha_q Q_2 \) and v1 cells. Furth-

ermore, the \( \alpha_q Q_2 \) clone was shown to reach higher cell

densities in the presence of IPTG compared with unin-
duced cells (Fig. 2C). Na\(^{+}\)-dependent hexose transport

activity was also examined in \( \alpha_q Q_1 \), the \( \text{G}_{\alpha_q} \)-expressing

cell line that showed lower inducibility of IP accumulation

by IPTG (Fig. 2D). After a 7-day exposure to IPTG, Na\(^{+}\)-
dependent hexose transport activity was reduced to a lesser

extent than observed in the \( \alpha_q Q_2 \) clone. Because TPA

has been shown to drastically delay the development of

Na\(^{+}\)-dependent hexose transport activity in wild-type

LLC-PK_1 cells (3), its effect was also examined in the

\( \alpha_q Q_1 \) cells. As shown in Fig. 2D, 0.1 µM TPA caused a greater inhibition of the development of
transport activity in \( \alpha_q Q1 \) compared with IPTG induction, ruling out the possibility that transport activity is less sensitive to PKC activation in this clone. These results are consistent with the lower level of IP accumulation when this clone is treated with IPTG (Fig. 1B). The data suggest that \( \alpha_q Q209L \) expression inhibits the development of Na\(^{+}\)-dependent hexose transport activity and that different levels of expression induced by IPTG, demonstrated by the differences in IP accumulation in the two \( \alpha_q \)-expressing clones, result in different extents of inhibition of Na\(^{+}\)-dependent hexose transport activity.

On the basis of the results shown in Fig. 2, we tested the hypothesis that varying IPTG concentrations would induce different levels of \( \alpha_q Q209L \) expression, resulting in modulation of the development of Na\(^{+}\)-dependent hexose transport activity. As shown in Fig. 3A, there is a progressive increase in IP accumulation with increasing concentrations of IPTG. IP accumulation reached a maximum of approximately fivefold over basal activity at 2 mM IPTG. The half-maximal increase was at 0.03 mM IPTG. A corresponding decrease in Na\(^{+}\)-dependent hexose transport activity to \(~50\%\) of maximum at an IPTG concentration of 0.01 mM was observed (Fig. 3B). At 0.05 mM IPTG, transport activity declined to \(~13\%\) of maximal levels and reached its lowest level at 2 mM IPTG. These results suggest that cell differentiation is modulated in an IPTG concentration-dependent manner, presumably by regulating the level of \( \alpha_q Q209L \) expression in a manner directly proportional to the induction of PLC activity.

Expression of \( \alpha_q Q209L \) inhibits alkaline phosphatase activity. To examine whether IPTG-dependent expression of \( \alpha_q Q209L \) can inhibit other differentiation markers, the activity of alkaline phosphatase in \( \alpha_q Q2 \)-expressing cells was assayed. Alkaline phosphatase is another differentiation marker in LLC-PK\(_1\) cells that demonstrates increased expression at the apical surface over time in culture (20, 30, 43). As shown in Fig. 4, alkaline phosphatase activity was higher on day 7 than on day 1 in both the control cell line v1 and \( \alpha_q Q2 \) cells in the absence of IPTG. TPA treatment reduced acquisition of this differentiated function in both v1 and \( \alpha_q Q2 \) cells on day 7. Similarly, increasing concentrations of IPTG reduced alkaline phosphatase activity in \( \alpha_q Q2 \) cells. There was also a gradual decrease in alkaline phosphatase activity with increasing concentrations of IPTG. Therefore regulated expression of \( \alpha_q Q209L \) also inhibited alkaline phosphatase activity.

Morphological changes in \( \alpha_q Q209L \)-expressing LLC-PK\(_1\) cells. The morphology of uninduced and induced \( \alpha_q Q2 \) cells was examined at the light-microscopic level (Fig. 5). Uninduced subconfluent cells exhibit the island-like clusters of closely apposed cells typical of cultured epithelia, which are indistinguishable from the control v1 clone. In the presence of IPTG, these cells acquire a spindle-shaped morphology, extending long, spiny processes with ruffled edges, and appear more loosely associated. In larger islands, uninduced cells form a monolayer of rounded cells, whereas IPTG-treated cells are more disorganized and appear to overgrow each other in multicellular aggregates. Interestingly, Mullin et al. (27) observed that PKC\(_{\alpha}\) is expressed preferentially in LLC-PK\(_1\) cells in areas that are multilayered. Untreated confluent monolayers of these cells were able to form domes, whereas IPTG-treated cells did not. Similar morphological changes were observed in five different clones that demonstrated at least a twofold induction in IP accumulation by IPTG. These morphological changes could be observed within 12 h of IPTG incubation (data not shown).
αqQ209L expression does not downregulate PKC activity. The activation of Gαq stimulates PLC-mediated hydrolysis of PIP2, leading to the release of DAG and IP3. Because DAG stimulates PKC, the effect of IPTG-induced expression of αqQ209L on the levels of PKC was examined. Prolonged treatment with phorbol esters has been shown to downregulate PKC, a process attributed to the proteolytic degradation of the activated enzymes (39). We were therefore interested in the levels of total PKC activity in cells incubated with IPTG compared with cells chronically exposed to TPA. As illustrated in Fig. 6, chronic TPA treatment caused downregulation of PKC activity in both control (v1) and αqQ209L-expressing cells (αqQ2), as reported previously for LLC-PK1 cells (26). However, αqQ209L expression induced by IPTG did not downregulate PKC. The total PKC activity in αqQ2 cells treated with IPTG was comparable to that of untreated αqQ2 cells and of untreated and treated v1 cells.

Effect of PKC activators and inhibitors on Na\(^+\)-dependent hexose transport activity. The activation of PLC by Gαq results in the generation of DAG and IP3. Both DAG and IP3 can stimulate downstream signaling pathways; DAG activates members of the PKC family of kinases and IP3 induces the release of Ca\(^{2+}\) from intracellular stores (34). To determine the ability of these pathways acting separately or together to influence the development of Na\(^+\)-dependent hexose transport, αqQ2 cells were incubated with the DAG analog OAG and the Ca\(^{2+}\) ionophore A-23187 (Fig. 7). After a 7-day incubation, cells treated with OAG showed ~50% decrease in the development of Na\(^+\)-dependent hexose transport, whereas cells treated with A-23187 were not...
affected, suggesting that PKC activation, but not increased cytosolic Ca\(^{2+}\), is important in the regulation of Na\(^{+}\)-dependent hexose transport. The addition of A-23187 together with OAG had no greater effect than OAG alone. The role of PKC was further investigated using the inhibitor GFX. At 2 \(\mu\)M, this inhibitor blocks the morphological changes observed in these cells (Sun and Weiss, unpublished observations). However, GFX had no effect on the ability of cells to develop Na\(^{+}\)-dependent hexose transport activity in the presence or absence of IPTG in either v1 or \(\alpha_\text{Q}Q2\) cells (Fig. 8).

**DISCUSSION**

We have described for the first time the inducible expression of a constitutively active mutant of G\(\alpha_\text{q}\) in a renal epithelial cell to investigate its participation in the regulation of proximal tubule-specific markers. Our results demonstrate that the expression of \(\alpha_\text{Q}Q209L\) inhibits the acquisition of Na\(^{+}\)-dependent hexose transport and alkaline phosphatase activities, both markers for the kidney proximal tubule. The inhibition of differentiation was regulated in a concentration-dependent manner by IPTG, suggesting that the level of active G\(\alpha_\text{q}\) directly affects the extent of inhibition. In addition, expression of constitutively active G\(\alpha_\text{q}\) induced a conversion from an epithelial to a spindle-shaped morphology. The cells became more disorganized and reached a higher cell density than those not treated with IPTG. The morphological changes observed in \(\alpha_\text{Q}Q209L\)-expressing cells are similar to those reported previously on exposure to TPA (3, 26). OAG was also found to inhibit the development of Na\(^{+}\)-dependent hexose transport activity, further implicating PKC in this regulatory pathway. Surprisingly, the PKC inhibitor GFX, which blocks the morphological changes observed in our cells and is reported to inhibit the TPA-induced disruption of tight junctions (23), had no effect on the inhibition of Na\(^{+}\)-dependent hexose transport by IPTG. It has been shown that LLC-PK\(_1\) cells possess the PKC isoforms \(\alpha, \beta, \delta, \epsilon,\) and \(\zeta\) (5, 25). PKC\(\alpha\), PKC\(\beta\), and PKC\(\gamma\) are most sensitive to GFX, with IC\(_{50}\) values ranging from 0.01 to 0.02 \(\mu\)M. In contrast, the IC\(_{50}\) values are an order of magnitude higher (0.21 and 0.13 \(\mu\)M, respectively) for PKC\(\delta\) and PKC\(\epsilon\) and 5.8 \(\mu\)M for PKC\(\zeta\) (24, 36). Therefore GFX is unlikely to be an effective inhibitor of \(\delta, \epsilon,\) and \(\zeta\) in our experiments. Because PKC\(\zeta\) is not regulated by DAG (12), PKC\(\delta\) and PKC\(\epsilon\) are the most likely candidates for the regulation of Na\(^{+}\)-dependent hexose transport activity by \(\alpha_\text{Q}Q209L\) in LLC-PK\(_1\) cells.

The most significant difference between TPA treatment and expression of constitutively active \(\alpha_\text{Q}\) was on the levels of cellular PKC. Chronic TPA treatment downregulated PKC levels in both v1 and \(\alpha_\text{Q}Q2\) cells, consistent with previous reports in both LLC-PK\(_1\) and other cell types (13, 26). In contrast, long-term treatment of \(\alpha_\text{Q}Q2\) cells with IPTG did not significantly alter PKC levels, suggesting that physiological activation of PLC by \(\alpha_\text{Q}Q209L\) expression does not result in down-
regulation of PKC. It should be noted that our experiments do not measure the activity of PKC in TPA-treated or Gαq-expressing cells. Designing an assay that preserves and measures actual PKC activity in a cell under a given set of experimental conditions is difficult, since breaking open the cells changes compartmentalization, Ca\(^{2+}\) levels, and probably the state of activation of PKC. The reason for the difference in PKC levels observed for TPA treatment and IPTG induction is not clear but may be due to differences in the metabolism of DAG and TPA. DAG, the endogenous activator, is rapidly metabolized and appears to cause only transient activation of PKC. On the other hand, TPA is degraded very slowly in cells, causing persistent activation of PKC (39) and demonstrating greater potency than DAG in inducing the differentiation of macrophages (1, 40). Incubation with TPA has generally been shown to cause the complete loss of PKC, whereas this has been reported not to occur with DAG in the monoblastoid cell line U937 (19). In previous studies, phorbol ester and DAG displayed differences in phosphorylation substrates, the activation of specific enhancer elements, and the stimulation of cell differentiation (35, 38, 40). These data suggest that the down-regulation of PKC observed in TPA-treated cells may not be a critical step in preventing the expression of proximal tubule-specific traits. Interestingly, TPA was previously shown to reduce the expression of PKCa but enhanced the expression of PKCb and had no effect on PKCc (5). Therefore the levels of PKCs whose activities are known to be regulated by TPA in vitro may be differentially regulated in vivo. This may be due in part to compartmentalization of PKC isoforms within the cell as well as compartmentalization of the various factors that influence the levels of expression of these proteins.

The mechanism for the progressive, concentration-dependent inhibition of differentiation by Gαq is unknown. The expression of Na\(^{+}\)-dependent hexose transport activity that begins at confluence has been correlated with an increase in the synthesis of its mRNA and protein (41, 42). A 1-h treatment with TPA induces a rapid loss of transporter mRNA in postconfluent, differentiated LLC-PK\(_1\) cells, a phenomenon attributed to posttranscriptional degradation of the mRNA (33). These data are consistent with the observation that activation of PKC by phorbol esters or DAG analogs inhibits Na\(^{+}\)-dependent hexose transport activity in renal proximal tubular primary cell culture through a decrease in maximal enzyme reaction rate (V\(_{max}\)) (14), suggesting a change in the level rather than the activity of this protein. Phorbol esters can also block the expression of tissue-specific markers in the intestinal epithelial cell line, HT-29 (15). Therefore PKC is implicated in regulating the formation of the differentiated phenotype in both kidney and intestinal epithelia.

The expression of α\(_q\)Q209L has been found to cause transformation in fibroblasts in a cell type-specific manner (10, 18). The expression of constitutively active mutants of the Gαq family was either growth stimulatory or inhibitory in different fibroblasts (11, 29). With the inducible expression of α\(_q\)Q209L, we were able to show a growth-stimulatory effect, indicated by an increase in the amount of protein per dish and in the number of cells, in these epithelial cells. Taken together with the data for cell differentiation, inducible expression of constitutively active α\(_q\)Q209L resulted in stimulation of growth and inhibition of differentiation in LLC-PK\(_1\) epithelial cells. Future studies will include transformation assays to test whether these cells are transformed by the expression of α\(_q\)Q209L. The mechanism whereby Gαq-mediated pathways affect cell growth and differentiation remains to be elucidated. PKC phosphorylates a number of cellular substrates that could lead to changes in expression of proximal tubule markers in the LLC-PK\(_1\) cell line. Investigating the downstream events regulated by PKC in this cell line is in progress in the laboratory.

In summary, we have successfully expressed α\(_q\)Q209L under the control of an inducible expression system in LLC-PK\(_1\) kidney epithelial cells. In an IPTG concentration-dependent manner, the expression of constitutively active Gαq inhibited differentiation of these cells, as indicated by measurement of both Na\(^{+}\)-dependent hexose transport and alkaline phosphatase activity, and caused changes in the epithelial morphology of these cells. From morphological changes occurring in the α\(_q\)Q209L-expressing cells, cell-cell contacts seem to
be altered in cells treated with IPTG (Sun and Weiss, unpublished observations). The inhibition of differentiation resulting from αQ209L expression could be due to impaired cell-cell contacts induced by IPTG. Changes in cell-cell contacts, such as the adherens junctions, between these epithelial cells are currently under investigation.

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