Differential Activation of Phosphatidylinositol 3-Kinase Signaling during Proliferation and Differentiation of Lens Epithelial Cells

Gudiseva Chandrasekher and Dashetty Sailaja

PURPOSE. To investigate whether phosphatidylinositol 3-kinase (PI-3K) signaling is involved in lens epithelial cell proliferation and differentiation promoted by growth factors.

METHODS. Proliferation of rabbit lens epithelial cells grown in culture was measured with a DNA-binding fluorescent dye in a proliferation assay. Primary cultures of embryonic chicken lens epithelial cells that develop lentoids were used for differentiation-related studies, and δ-crystallin synthesis in these cultures was determined by metabolic labeling with [35S]methionine. Immunoprecipitation and immunoblot analyses were also used.

RESULTS. The PI-3K inhibitors wortmannin and LY294002 blocked the insulin, insulin-like growth factor (IGF)-1-, and fibroblast growth factor (FGF)-2–promoted cell proliferation in rabbit lens epithelial cells. Inhibition of PI-3K activity by these inhibitors unexpectedly increased the synthesis of early differentiation marker protein δ-crystallin in chicken lens epithelial cells. Insulin and IGF-1–stimulated activation of PI-3K in proliferating and differentiating cultures. FGF-2 showed no direct effect on PI-3K activation. Platelet-derived growth factor (PDGF) did not induce significant proliferation or increased expression of δ-crystallin, but stimulated PI-3K. The presence of FGF-2 in proliferating rabbit lens epithelial cells enhanced the IGF-1–, but not the PDGF-mediated PI-3K activation, suggesting a possible integration of FGF-2 signals with IGF-1. Whereas there was a gradual decrease in insulin/IGF-1–mediated activation of PI-3K and its downstream target Akt, with progression of differentiation in chicken lens epithelial cells, Erk2 phosphorylation induced by these growth factors was not decreased; rather, it remained increased in early stages of differentiation.

CONCLUSIONS. The results reveal significant differences in the modulation of PI-3K signaling by different growth factors during proliferation in rabbit lens epithelial cells and differentiation in chicken lens epithelial cells and demonstrate that regulation of the PI-3K pathway plays a key role in these processes. A balance between the nonactivation of PI-3K and the activation of Erk2 may be necessary during early stages of epithelial cell transformation. (Invest Ophthalmol Vis Sci. 2003;44:4400–4411) DOI:10.1167/iovs.03-0136

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Proliferation and differentiation are two fundamental cellular processes for the development and growth of the lens. Growth factors such as fibroblast growth factors (FGFs), insulin-like growth factor (IGF)-1, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin are shown to promote proliferation of epithelial cells and formation of fiber cells in many species, including human.1–6 Recently, bone morphogenic protein was shown to participate in fiber cell differentiation.7,8 The binding of growth factors to their cell surface receptors leads to the activation of the receptor tyrosine kinase, which is responsible for propagating the generated signals into cellular actions.9 These actions are mediated by a variety of signaling molecules, depending on the signaling cascades they trigger.10

The actual players in signal transduction cascades that are required for the regulation of lens epithelial cell proliferation and differentiation have not been clearly identified. Studies using growth factors in different in vivo and in vitro lens cell systems have produced some information regarding signaling molecules, such as the Erk MAP kinase, Jak/Stat, Smad, and PKC isoenzymes that are present in lens in vivo and in vitro lens cell proliferation and differentiation.5,11–14 Although many of the growth factors and cytokines affect both proliferation and differentiation,1,2 it is not known whether they trigger the same or different signal transduction cascades in these processes. Because coordination and synergism by growth factors appears to be key for the effective regulation of these very closely interrelated steps in lens growth,15,16 a cross-communication between the signal-transducing mediators activated by the growth factors is possible. Therefore, identification of a specific signaling intermediate that carries the signals generated by all growth factors is important. Phosphatidylinositol 3-kinase (PI-3K) is an intracellular signaling molecule involved in transducing signals of growth factor-activated membrane tyrosine kinase receptors.17–19 It is a heterodimeric protein comprising a 110-kDa catalytic subunit (p110) that phosphorylates the 3′ position of the inositol ring in phosphatidylinositol and an 85-kDa regulatory subunit (p85) with two SH2 domains that associate with the autophosphorylation sites of the activated membrane receptor. On its recruitment to the membrane, the activated PI-3K can generate 3′-phosphoinositides (3-PIs)—namely, PI-3P, PI-3,4P2, and PI-3,4,5P3. These 3-PIs serve as second messengers in the control of cellular functions such as mitogenesis, differentiation, vesicular trafficking, cell migration, tubule formation, and prevention of apoptosis.20,21 Studies from our laboratory have shown that insulin and IGF-1 activate PI-3K in bovine lens epithelial cells.22 In the present work, the involvement of PI-3K signaling in the regulation of lens epithelial cell proliferation and differentiation was explored by using PI-3K inhibitors. Two distinct lens cell culture systems—rabbit lens epithelial cells for proliferation and embryonic chicken lens epithelial cells for differentiation—were used for this study. Our studies demonstrate that PI-3K activation during proliferation is promoted by insulin and IGF-1 and that inhibition of PI-3K activity blocks growth factor–promoted proliferation. In a chicken lens cell culture
system, PI-3K activation induced by IGF-1 (50 nM) was higher in younger actively proliferating cells, but decreased significantly in older, more differentiated cultures. Further, inhibition of PI-3K activity promoted the synthesis of the differentiation marker protein δ-crystallin.

**Materials and Methods**

**Growth factors** (recombinant human IGF-1, FGF-2, and PDGF-Faa) were purchased from R&D Systems (Minneapolis, MN); insulin, Wortmannin, phosphatidylinositol, monoclonal antibodies for phospho-Erk and Erk from Sigma (St. Louis, MO); antibodies for phospho-tyrosine (anti-PY, polyclonal and monoclonal, 4G10), phospho-Akt (monoclonal) and Akt (polyclonal) from Upstate Biotechnology (Lake Placid, NY); anti-insulin receptor β-subunit (polyclonal) and horseradish peroxidase–conjugated species-specific secondary antibodies from Transduction Laboratories (Lexington, KY); and [32P]ATP and [35S]methionine from Amersham (Arlington Heights, IL). Silica gel (60 TLC) plates were purchased from Merck (Darmstadt, Germany); [3H]thymidine (25 mCi/mL) from Calbiochem (La Jolla, CA); a cell proliferation assay kit (CyQuant GR, Molecular Probes) was used to determine DNA content, as previously described.21 by measuring with a fluorescent dye that exhibits strong fluorescence enhancement when bound to DNA (excitation wavelength at 485 nm and emission at 536 nm), according to the manufacturer’s instructions. In preliminary experiments with the kit’s reagent, rabbit lens epithelial cells produced linear fluorescence intensity as a function of cell number. To corroborate the results were in some experiments, cultures (at the end of a 48-hour incubation) were trypsinized, and cell proliferation was determined by counting the number of cells with a hemocytometer. For these experiments cells were seeded in 24-well plates (20–25 × 10³ cells/well). Some cultures were also trypsinized to determine the initial number of cells in culture wells before starting treatments with various compounds.

**Chicken Lens Epithelial Cell Culture**

Chicken lens epithelial cell primary cultures were used for differentiation-related studies. Embryonic chicken eggs (10 days’ gestation) were supplied by the Texas A&M University Poultry Science Department (College Station, TX). Lenses from chicken embryos were carefully dissected and washed thoroughly in DMEM containing 0.1% gentamicin and 1% antibiotic-antimycotic solution. Epithelial cells were isolated according to the protocol described by Menko et al.22

Chicken lens epithelial cells were used in all proliferation-related experiments. Embryonic chicken lenses were dissected from 10-day-old embryos and the lenses were incubated overnight at 37°C in 5% CO₂ and 95% O₂. The next day, nonadherent cells were removed, and attached cells were incubated in DMEM containing 0.2% BSA, with or without growth factors or PI-3K inhibitors (wortmannin and LY294002) for 48 hours. After 2 hours, the cells were thawed and a cell proliferation assay kit (CyQuant GR, Molecular Probes) was used to determine DNA content, as previously described.21 by measuring with a fluorescent dye that exhibits strong fluorescence enhancement when bound to DNA (excitation wavelength at 485 nm and emission at 536 nm), according to the manufacturer’s instructions. In preliminary experiments with the kit’s reagent, rabbit lens epithelial cells produced linear fluorescence intensity as a function of cell number. To corroborate the results were in some experiments, cultures (at the end of a 48-hour incubation) were trypsinized, and cell proliferation was determined by counting the number of cells with a hemocytometer. For these experiments cells were seeded in 24-well plates (20–25 × 10³ cells/well). Some cultures were also trypsinized to determine the initial number of cells in culture wells before starting treatments with various compounds.

**Metabolic Labeling of Chicken Lens Epithelial Cells with [35S]methionine**

Chicken lens epithelial cells were seeded in 24-well plates (4 × 10⁵ cells/well) and grown in DMEM containing 10% FCS for 24 to 48 hours. After that, the medium was replaced with DMEM containing 0.2% BSA, with or without growth factors or PI-3K inhibitors, for 48 hours.

**Preparation of Cell Extracts and Immunoprecipitation of PI-3K**

All culture dishes at the end of each experiment were washed three times with ice-cold PBS, and cells were homogenized in lysis buffer (20 mM HEPES, 2 mM MgCl₂, 2 mM EGTA, 2 mM orthovanadate, 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM leupeptin, 150 mM NaCl, 1% Triton X-100, and 0.5% NP-40) and centrifuged at 14,000 rpm for 20 minutes. The supernatant was collected and protein concentrations were determined by dye reagent (Bio-Rad, Hercules, CA). All operations were performed at 4°C. Epithelial cell extracts containing 150 to 300 μg of protein were immunoprecipitated as described earlier23 using anti-PY antibody (4 μg). The extracts in a total volume of 0.5 mL of lysis buffer were incubated with antibody for 2 to 3 hours. Then, the immunocomplexes were recovered by incubation in the presence of protein A-agarose beads for 2 hours, and the immunocomplexes bound to the agarose beads were collected by centrifugation for 5 minutes at 5000 rpm. The beads were washed three times with lysis buffer and twice with PI-3K assay buffer (described later). The final pellet was suspended in PI-3K assay buffer and the enzyme activity determined.

**Assay of PI-3Kinase**

PI-3K activity in anti-PY immunoprecipitates was determined as previously described,24 using phosphatidylinositol (P1) as the substrate (37.5 μg).

The assay was performed in a total volume of 100 μL assay buffer containing 20 mM HEPES (pH 7.5), 0.4 mM EGTA, 0.4 mM orthovanadate, 0.2 mM PMSF, 0.2 mM leupeptin, 10 mM MgCl₂, 100 μM...
adensine triphosphate (ATP) containing 5–10 μCi [32P]ATP and 37.5 μg phosphatidylserine. The samples were incubated for 10 minutes at 30°C in a shaken water bath, and the enzyme reaction was stopped by adding 0.9 mL of chloroform, methanol, and 12 N HCl (200:100:1 vol/vol/vol). Lipids were extracted first with chloroform, methanol, and 0.1 N HCl (3:48:47 vol/vol/vol) followed by another extraction with chloroform, methanol, 0.01 N HCl (3:48:47 vol/vol/vol). The PI-3K reaction product, PI-3P, was separated by thin-layer chromatography (TLC) into silica gel plates precoated with 1% potassium oxalate. Methanol, chloroform, ammonia, and water (100:70:15:25 vol/vol/vol/vol) were used in the developing solvent. The plates were scanned and quantified for [32P]-PI-3P–labeled spots in an electronic autoradiography image analyzer (Instant Image Analyzer; Perkin Elmer-Packard).

Western Immunoblot Analysis

Cellular extracts (approximately 25 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis (10%) gel, then transferred to nitrocellulose membrane using a mini transblot electrophoretic transfer cell (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in TBS (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20) and probed with the antibodies specified in each experiment. The antibodies were diluted according to the manufacturers’ specifications. The membranes were washed six times at 10-minute intervals with TBS and 0.05% Tween 20 and incubated with species-specific secondary antibodies visualized by chemiluminescence. The mobilities of the proteins of interest were compared with the biotinylated protein molecular weight standards. The intensities of the protein bands were quantified by densitometric scanning (Molecular Analyst program; Bio-Rad) and recorded with a gel documentation system fitted with a white light conversion screen (Gel Doc-1000; Bio-Rad).

RESULTS

Effect of PI-3K Inhibitors on Mitogenic Activity of Insulin, IGF-1, and FGF-2 in Lens Epithelial Cells

Cultured rabbit lens epithelial cells were incubated with insulin, IGF-1, FGF-2, and PDGF in the presence and absence of PI-3K inhibitors. Cell proliferation was determined by analyzing the total cellular DNA content by fluororescence. A significant increase in cell proliferation was obtained after a 48-hour treatment with insulin, IGF-1, and FGF-2 (Fig. 1). No appreciable effect on proliferation was observed with PDGF. FGF-2 at a 10-ng/mL concentration was more effective than insulin (200 nM) and IGF-1 (50 nM) in mitogenic activity, and it increased cell proliferation in 48 hours by approximately 70% to 80% when compared with untreated cultures. With insulin and IGF-1, an approximate 40% to 50% increase in proliferation was observed. Stimulation with IGF-1 and FGF-2 together did not result in a synergistic increase in proliferation. Mitogenesis promoted by IGF-1 and FGF-2 was blocked significantly by both wortmannin (200 nM) and LY294002 (10 μM). In the presence of wortmannin and LY294002, IGF-1 (and insulin, data not shown)–induced cell proliferation was similar to control cultures. The mitogenic property of FGF-2 decreased by approximately 50% when PI-3K inhibitors were present. Similar data were obtained in separate experiments when the number of cells was determined after trypaninization and cell counting by hemocytometer (Table 1). Numbers of cells in cultures not treated with growth factors or inhibitors increased from $17.2 \pm 1.4 \times 10^3$ to $20.2 \pm 2.8 \times 10^3$ in 48 hours, indicating that control cultures incubated in the basal medium alone were viable and dividing. As shown in Table 1, the presence of growth factors increased the capacity of these cells to divide. Wortmannin and LY294002 alone did not exert a significant inhibitory effect on cell proliferation at the concentrations used in this study.

Stimulation of PI-3K/Akt Activation by Growth Factors

The inhibition of rabbit lens epithelial cell proliferation induced by insulin, IGF-1, and FGF-2 by PI-3K inhibitors indicates that PI-3K activation could be important during cell proliferation. Therefore, we examined the effects of these growth factors on PI-3K activation. Quiescent rabbit lens epithelial cell cultures were stimulated with insulin, IGF-1, FGF-2, and PDGF for 10 minutes. Because growth factor binding triggers the autophosphorylation of the receptor tyrosine kinase domain and association of activated PI-3K with receptor,22–26,27 the stimulated lens epithelial cell extracts were immunoprecipitated with anti-PY antibody and the PI-3K activity associated with these immunoprecipitates was determined (Fig. 2). Consistent with our previous findings in bovine lens epithelial cells,22 both insulin and IGF-1 stimulated PI-3K activation. Insulin and IGF-1 activated PI-3K by approximately 200% to 300% when compared with the untreated control. However, treatment of cells with FGF-2 had little or no effect on PI-3K activation. Although PDGF did not promote any significant proliferation, it stimulated PI-3K activity by more than 500%. Next, we examined the activation of Akt, an important downstream kinase of the PI-3K–signaling pathway. This kinase plays a prominent role in cell proliferation and prevention of apoptosis.21 Activation of Akt is necessary for cell-cycle progression.26,29 Phosphorylation of certain serine residues of this protein (ser308 and ser473) by upstream PI-3K–dependent kinases (PKDs) stimulate Akt.21 We determined Akt activation after stimulating rabbit lens epithelial cells for 10 minutes with growth factors, by Western immunoblot, using a phosphospecific Akt antibody. Insulin and IGF-1 caused an eight- to ninefold increase in the levels of phospho-Akt (pAkt; Figs. 3A,
compared with untreated control cultures. Consistent with its effect on PI-3K stimulation, FGF-2 induced very little activation of Akt. However, PDGF (50 ng/mL), which stimulated PI-3K significantly, as did insulin (200 nM) and IGF-1 (50 nM), was not as effective as insulin or IGF-1 in activating Akt. A twofold increase in Akt phosphorylation was observed in the presence of PDGF. Increased pAkt levels were also found in proliferating cells cultured in the presence of IGF-1 for 48 hours (Fig. 3C). The presence of wortmannin or LY294002 in proliferating cultures inhibited the IGF-1–induced activation of Akt, indicating that phosphorylation of Akt is dependent on PI-3K activation and that these compounds actually inhibited PI-3K activity throughout the entire assay period (up to 48 hours). It should be noted that in cultures treated with wortmannin, IGF-1–induced Akt activation was not completely blocked, perhaps because of the loss of inhibitory potency of wortmannin, as this compound is unstable in aqueous medium over a prolonged period. The changes in the levels of pAkt observed in the presence of IGF-1 and PI-3K inhibitors over a 48-hour period were not due to changes in the expression of the enzyme, because the amount of total Akt remained un-

### Table 1. Lens Epithelial Cell Proliferation in the Presence of Growth Factors and PI-3K Inhibitors

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IGF-1</th>
<th>IGF-1 + Wort</th>
<th>IGF-1 + LY</th>
<th>FGF-2</th>
<th>FGF-2 + Wort</th>
<th>FGF-2 + LY</th>
<th>Wort</th>
<th>LY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number (1 x 10^5)</td>
<td>20.2 ± 2.8</td>
<td>27.6 ± 1.6</td>
<td>21.2 ± 1.9</td>
<td>19.4 ± 1.5</td>
<td>35.5 ± 2.2</td>
<td>28.3 ± 1.4</td>
<td>24.2 ± 1.1</td>
<td>20.0 ± 1.6</td>
<td>18.6 ± 0.5</td>
</tr>
<tr>
<td>% Change over control</td>
<td>100</td>
<td>157</td>
<td>105</td>
<td>96</td>
<td>176</td>
<td>143</td>
<td>120</td>
<td>99</td>
<td>92</td>
</tr>
</tbody>
</table>

Rabbit lens epithelial cell proliferation after 48 hours of treatment with or without growth factors or PI-3K inhibitors was determined by counting the number of cells. The count before beginning various treatments was 17.2 ± 1.4 x 10^5. Wort, wortmannin (200 nM), LY: LY294002 (10 μM).

3B) compared with untreated control cultures. Consistent with its effect on PI-3K stimulation, FGF-2 induced very little activation of Akt. However, PDGF (50 ng/mL), which stimulated PI-3K significantly, as did insulin (200 nM) and IGF-1 (50 nM), was not as effective as insulin or IGF-1 in activating Akt. A twofold increase in Akt phosphorylation was observed in the presence of PDGF. Increased pAkt levels were also found in proliferating cells cultured in the presence of IGF-1 for 48 hours (Fig. 3C). The presence of wortmannin or LY294002 in proliferating cultures inhibited the IGF-1–induced activation of Akt, indicating that phosphorylation of Akt is dependent on PI-3K activation and that these compounds actually inhibited PI-3K activity throughout the entire assay period (up to 48 hours). It should be noted that in cultures treated with wortmannin, IGF-1–induced Akt activation was not completely blocked, perhaps because of the loss of inhibitory potency of wortmannin, as this compound is unstable in aqueous medium over a prolonged period. The changes in the levels of pAkt observed in the presence of IGF-1 and PI-3K inhibitors over a 48-hour period were not due to changes in the expression of the enzyme, because the amount of total Akt remained unchanged (Fig. 3D). These results suggest that activation of PI-3K/Akt could be important for rabbit lens epithelial cell proliferation promoted by insulin and IGF-1.

### Enhancement of IGF-1–Stimulated PI-3K Activity by FGF-2

Regulation of cell proliferation is a complex process, and cooperation among different growth factors that have mitogenic property may be necessary for cell division. The signal-transduction pathways activated by each growth factor during this cellular process could be different and may involve cross talk between these cascades. We examined whether there is any such interaction between IGF-1 and FGF-2, and PDGF and FGF-2 signaling in the activation of the PI-3K pathway. Proliferating epithelial cells were cultured in the presence of FGF-2 for 48 hours and stimulated with IGF-1 or PDGF for 10 minutes, and PI-3K activation was measured as has been described. The results obtained in these experiments are presented in Figure 4. In cells that were cultured in the absence of FGF-2, the PI-3K activity stimulated by IGF-1 was approximately 500% com-

![Figure 2](https://iovs.arvojournals.org/static/content/44/10/IOVS_12_1284_Fig_2.jpg)

**Figure 2.** Stimulation of PI-3K activity by insulin, IGF-1, and PDGF, but not by FGF-2. Rabbit lens epithelial cell cultures at 80% to 90% confluence were starved overnight in medium supplemented with 0.2% FCS and then stimulated with or without growth factors, insulin (200 nM), IGF-1 (50 nM), FGF-2 (10 ng/mL), and PDGF 50 ng/mL) for 10 minutes. The cells were washed with ice-cold PBS three times, and cellular proteins were extracted in lysis buffer. PI-3K associated with tyrosine-phosphorylated proteins in cell extracts (500 μg protein) under each condition was immunoprecipitated (IP) using anti-PY antibody, and PI-3K activity in immunoprecipitates was assayed. (A) Separation of the PI-3K product by thin-layer chromatography. (B) PI-3K activity is expressed as radiolabel (counts per minute) associated with PI-3P bands that were quantified in an instant imager. The data are the average results in four to six independent experiments.
PI-3K activation has been shown to promote differentiation in many cell types. Several cellular changes are known to occur during the transformation of chicken lens epithelial cells into fiber cells. An increase in δ-crystallin synthesis is one of the very large number of transcriptional and posttranscriptional changes associated with fiber formation and is a widely used marker of the early stages of epithelial-to-fiber maturation in the chick. Because inhibition of PI-3K activation blocked δ-crystallin synthesis and thus differentiation, for this study, early nondifferentiating chicken lens epithelial cell cultures (2 days after seeding) were incubated in the presence of growth factors or PI-3K inhibitors (wortmannin, 200 nM; LY294002, 10–20 μM) for 48 hours. These cultures were metabolically labeled with [35S]methionine (100 μCi/mL) during the last 4 hours of incubation. The presence of IGF-1 and PDGF resulted in increased incorporation of radiolabeled [35S]methionine into δ-crystallin protein (Fig. 5). With PDGF, no significant increase was detected. The presence of wortmannin or LY294002 in the cultures had no inhibitory effect on the differentiation process. Instead, these inhibitors produced an approximate 50% increase in δ-crystallin synthesis (Fig. 5). These results indicate that suppression of PI-3K activity coupled with the other signaling mechanisms regulated by growth factors could be essential in the differentiation process. Incubation of cells together with growth factors (IGF-1 or PDGF) plus PI-3K inhibitors (wortmannin or LY294002) did not result in a synergistic increase in δ-crystallin synthesis (data not shown).

Changes in IGF-1-Mediated PI-3K/Akt Activation During Epithelial Cell Differentiation

Because PI-3K inhibitors promote δ-crystallin synthesis and thus differentiation, regulation of PI-3K activity could be a determining factor in differentiation. Therefore, we examined the activation of PI-3K during differentiation. Primary cultures of embryonic chicken lens epithelial cells that differentiate and form lentoids have been used in the study of several biochemical and biological processes during differentiation. As shown in Fig. 6A, embryonic chicken lens primary epithelial cell cultures differentiated with time. Epithelial cell proliferation was very active during the first 2 to 3 days. After that cultures started to differentiate, and distinct lentoid bodies were developed throughout the cultures by 5 to 6 days. Lentoid formation and growth continued, and by 9 to 12 days these lentoids spread extensively and fused with neighboring lentoids.

Chicken lens epithelial cell cultures at different stages of differentiation were stimulated with IGF-1 (50 nM) for 10 minutes, and PI-3K activity was determined as described earlier. As observed in rabbit lens cultures, IGF-1 also induced PI-3K activity early in chicken lens cell cultures (2–3 days), where proliferation was very active. A 600% increase in enzyme activity was observed in these cultures after stimulation with IGF-1 (Figs. 6B, 6C). As differentiation progressed with time, there was a gradual decrease in PI-3K activation by IGF-1. The activity was decreased from 600% (in early cultures) to 200% to 250% in lentoid-developing 6-day cultures. In 10-day cultures with extensively spread lentoids, IGF-1-stimulated PI-3K activity was only approximately 100% compared with unstimulated cells. The low levels of activity in these cultures could be due to a small population of cells that undergo

**FIGURE 3.** Effect of growth factors on Akt activation. (A) Rabbit lens epithelial cell cultures at 80% to 90% confluence were starved overnight in medium supplemented with 0.2% FCS and then stimulated with or without growth factors: insulin (200 nM), IGF-1 (50 nM), PDGF (10 ng/mL), and PDGF (50 ng/mL) for 10 minutes. (C, D) Rabbit lens epithelial cells were plated in DMEM containing 10% FCS. Twenty-four hours after plating the media were removed, and the cells were further grown for 48 hours in the presence or absence of IGF-1 (50 nM) or PI-3K inhibitors (wortmannin, 200 nM; and LY, LY294002, 10 μM) dissolved in DMEM containing 0.2% FCS. The cells were washed with ice-cold PBS three times, and cellular proteins were extracted in lysis buffer. Protein extracts (25 μg each) were subjected to SDS-PAGE and Western blot. Phospho-Akt (pAkt) and Akt antibodies, respectively. (B) The band intensities in (A) were determined by densitometry and are presented in arbitrary units. The data are from one experiment. Similar results were obtained in two other independent experiments.
proliferation or to cells that have remained quiescent and undifferentiated that respond to IGF-1. We observed significant stimulation of PI-3K by IGF-1 in quiescent rabbit lens epithelial cells that did not undergo differentiation in the culture system (Fig. 2). Therefore, it is possible that decreased activation of PI-3K in response to IGF-1 in differentiated chicken cultures is not entirely related to the overall reduction in proliferation but to the differentiation status of the cells. These results clearly show that PI-3K activity induced by IGF-1 (50 nM) is down-regulated during transformation of chicken lens epithelial cells into fiber cells and are consistent with the possibility that decreased PI-3K activation may be a prerequisite for differentiation induced by IGF-1. Stimulation of PI-3K activity in lentoid-developing cultures by insulin and PDGF but not by FGF-2 (data not shown) was also detected. Next, we investigated whether the decrease in PI-3K activation could affect the downstream Akt kinase. Insulin- and IGF-1–induced activation of Akt also decreased significantly as the differentiation progressed (Figs. 7A, 7B). In proliferating 3-day cultures without any lentoid bodies, IGF-1 induced the phosphorylation of Akt

FIGURE 4. FGF-2 increased IGF-1–mediated stimulation of PI-3K activity in proliferating cultures. Rabbit lens epithelial cells were plated in DMEM containing 10% FCS and after 24 hours, cells were incubated with or without FGF-2 (10 ng/mL) in DMEM containing 0.2% FCS for another 48 hours. These cultures were then stimulated with IGF-1 (50 nM) or PDGF (50 ng/mL) for 10 minutes, and PI-3K activity in the cell cultures was determined as described in the legend for Figure 2 (A). Separation of PI-3K product by thin-layer chromatography. (B) PI-3K activity was expressed as radiolabel (counts per minute) associated with PI-3P bands that were quantified in an instant imager. Data presented were averages from two different experiments. Probabilities obtained for IGF-1 in FGF-2-preincubated samples were significantly lower than for samples not incubated with FGF-2 (P < 0.02).

FIGURE 5. PI-3K inhibitors increased the synthesis of δ-crystallin. Chicken lens epithelial cells were seeded in 24-well tissue culture plates and grown in DMEM containing 10% FCS for 48 hours. After that, the medium was replaced with DMEM containing 0.2% BSA, and the cells were incubated in the presence or absence of growth factors (IGF-1, 50 nM; FGF-2, 50 ng/mL; PDGF, 50 ng/mL) or PI-3K inhibitors (wortmannin, 200 nM or LY294002, 20 μM) for 48 hours. The cells were metabolically labeled with [35S]methionine during the last 4 hours of incubation. Cell extracts were prepared with lysis buffer and the protein quantities determined. Extracts containing equal amounts of protein (approximately 10 μg) were analyzed by SDS-PAGE for radiolabeled proteins (A). The radiolabel (counts per minute) incorporated into major protein bands was determined. The quantity of δ-crystallin ([35S]methionine synthesized in each experimental condition was calculated as the percentage of total radiolabeled protein and the increase in the synthesis of δ-crystallin was expressed as percentage over control. The values represent average of two to three experiments (for growth factors) and five to six experiments (for inhibitors). Probabilities were FGF-2 versus control (P < 0.009), IGF-1 versus control (P < 0.04), wortmannin versus control (P < 0.04), and LY294002 versus control (P < 0.002).
FIGURE 6. Downregulation of PI-3K activation in differentiating chicken lens epithelial cell cultures. (A) Phase-contrast micrographs of cells at different days in culture. Ten-day-old chicken embryo lens epithelial cells were prepared, and cells were plated at a density of $10^6$ cells/mL in 12-well plates. The cells were grown in DMEM containing 10% FCS. Distinct lentoid body formation was observed in 5- to 6-day cultures. (B, C) Decrease in stimulation of PI-3K activity mediated by IGF-1 during chicken lens epithelial cell differentiation. Differentiating chicken lens cultures similar to those shown in (A) were starved overnight in DMEM containing 0.2% FCS and stimulated with IGF-1 (50 nM) for 10 minutes. PI-3K activity in these cultures was determined as described in the legend to Figure 2. Approximately 150 μg cellular protein was used for immunoprecipitation. (B) Separation of PI-3K product by thin-layer chromatography. (C) PI-3K activity is expressed as radiolabel (counts per minute) associated with PI-3P bands that were quantified in an instant imager. The data presented are averages obtained from three independent experiments.

FIGURE 7. Insulin and IGF-1–mediated activation of the antiapoptotic factor Akt decreased during differentiation. Chicken lens cultures grown for different periods as shown in Figure 6A were starved overnight in DMEM containing 0.2% FCS and stimulated with insulin (200 nM) or IGF-1 (50 nM) for 10 minutes. Cellular protein extracts (25 μg each) were subjected to SDS-PAGE and Western blot. Phospho-Akt (pAkt; A, D) and Akt (B) were identified by immunoblot with specific anti-phospho (ser 473) Akt and Akt antibodies, respectively. (C) The band intensities in (A) were determined by densitometry and are presented in arbitrary units. (D). Four-day chicken cultures were preincubated with wortmannin (200 nM) or LY294002 (10 μM) for 30 minutes before stimulation with IGF-1 (50 nM) for 10 minutes. The results represent one experiment. Similar results were obtained in two other independent experiments.
by approximately 10-fold, whereas in 6-day cultures with lentoids, Akt phosphorylation decreased to three- to fivefold. In 10-day cultures that contained well-spread lentoids, the Akt phosphorylation decreased to one- to twofold. A similar pattern of Akt activation was also observed with insulin; however, insulin was less effective in inducing Akt phosphorylation than was IGF-1. As shown in Fig. 7C, the amount of total Akt in cell lysates remained the same. Wortmannin and LY294002 inhibited IGF-1-induced Akt phosphorylation (Fig. 7D). A complete block in the activation of Akt by these inhibitors was not achieved in these experiments. This could be due to exposure of cultures to inhibitors for only a short duration (30 minutes).

A progressive decrease in the ability of IGF-1 and insulin to stimulate PI-3K/Akt signaling during different stages of differentiation can occur if there is a reduction in insulin/IGF-1 receptor expression in these cultures. When insulin and IGF-1 bind to their cell surface receptors, the 96-kDa β-subunit, which possesses intrinsic tyrosine kinase activity, is autophosphorylated. A decrease in the number of receptors would result in reduced receptor protein tyrosine phosphorylation, decreased interaction of PI-3K with receptor and reduced activation of the enzyme. Therefore, we have assessed the phosphorylation level of the 96-kDa protein in differentiating chicken lens epithelial cells after IGF-1 treatment. Cultures were stimulated with IGF-1 for 10 minutes, protein extracts were subjected to SDS-PAGE, and protein tyrosine phosphorylation was analyzed by immunoblot with anti-PY antibody. As shown in Figure 8A, a 96-kDa protein band, which could be the IGF-1 receptor β subunit, was found to be phosphorylated in IGF-1-treated cultures. No significant differences in the phosphorylation level of this protein band was noticed after IGF-1 treatment in epithelial cultures grown for 3, 6, and 10 days (Fig. 8B). A similar pattern of tyrosine phosphorylation of 96-kDa protein was also observed with insulin (data not shown). In separate experiments using cultured chicken lens epithelial cell extracts, we identified the insulin receptor β subunit in the 96-kDa region after immunoprecipitation with an anti-insulin receptor β subunit antibody (Fig. 8C). These findings imply that insulin and IGF-1 receptor levels are not altered during different stages of differentiation, and the reduction in PI-3K activation in differentiating cultures may not be related to receptor expression.

Insulin and IGF-1–Induced Activation of MAP Kinase (Erk-2) during Early Stages of Differentiation

Although insulin- and IGF-1–mediated PI-3K signaling is decreased during differentiation of cultured chick lens epithelial cells, the ability of these growth factors and FGF-2 to induce an increase in δ-crystallin synthesis suggests that other signaling cascades activated by these compounds participate in the differentiation process. A recent study by Le and Musil has shown that FGF-2 and insulin/IGF-1 stimulate Erk activation in chicken embryonic epithelial cells and that inhibition of insulin/IGF-1–dependent activation of Erk significantly decreases δ-crystallin synthesis. We investigated whether the activation of Erk by insulin and IGF-1 changes with progression of differentiation, as seen with PI-3K. Chicken lens epithelial cell cultures at different stages of differentiation were stimulated with insulin and IGF-1, and activation of Erk was analyzed after Western immunoblot analysis with a phospho-specific Erk antibody. In proliferating-day cultures, phosphorylation of Erk2 by insulin and IGF-1 was increased by more than 200% compared with the unstimulated control (Figs. 9A, 9B). However, unlike PI-3K–mediated Akt phosphorylation, Erk2 phosphorylation did not decrease in early (6 day) differentiating cultures containing lentoids. Instead, the increase was sustained (approximately 300%–400%), but in 10-day cultures Erk2 activation decreased. Total Erk2 levels in all conditions did not change (Fig. 9C). These data indicate that Erk signaling may be important during early stages of differentiation. Inhibition of the PI-3K pathway by wortmannin did not increase the Erk2 activation induced by IGF-1 (Fig. 9D), whereas inhibition of the MAP kinase cascade by PD98059 significantly blocked Erk2 phosphorylation, indicating that activation of Erk2 by IGF-1/insulin is independent of PI-3K inhibition. In chicken lens epithelial cells, Erk2 appears to be the predominant form, because insignificant or barely visible immunostaining for Erk1 was observed (Figs. 9A, 9B, 9D).

**DISCUSSION**

The present study was designed to further the understanding of the involvement of PI-3K signal transduction in lens epithe-
PI-3K inhibitors blocked FGF-2 direct effect on PI-3K activation. This is surprising, because also a potent inducer of cell proliferation, did not show any insulin and IGF-1 stimulated PI-3K. However, FGF-2, which was demonstrated to be similar to the changes that occur during the differentiation of epithelial cells into fiber cells in lens in vivo. We used chicken embryo lens epithelial primary cultures for differentiation studies. Although, these cultures differentiate into lentoids, some of the epithelial cells continue to proliferate. To avoid overlap of the PI-3K activation between proliferation and differentiation stages, we did not conduct proliferation experiments with chicken lens cultures. Instead, we used rabbit lens epithelial cell cultures, which proliferate several times to fill the culture dish and then become quiescent. Unlike embryonic chicken lens cultures, these cells do not undergo differentiation and are therefore ideally suited for investigating signaling pathways associated with cell proliferation.

We observed an increase in lens epithelial cell proliferation with insulin, IGF-1, and FGF-2 in rabbit lens epithelial cell cultures. These findings are consistent with previously published reports. The inhibition of FGF-2-, insulin-, and IGF-1-promoted cell proliferation by wortmannin and LY294002 demonstrates that PI-3K activity is necessary for cell proliferation in response to these growth factors in rabbit lens epithelial cell cultures. This is further supported by the finding that both insulin and IGF-1 stimulated PI-3K. However, FGF-2, which was also a potent inducer of cell proliferation, did not show any direct effect on PI-3K activation. This is surprising, because PI-3K inhibitors blocked FGF-2-induced proliferation. Recently, Zatechka and Lou demonstrated a moderate increase in PI-3K activity in porcine lens epithelial layer obtained from organ-cultured lenses incubated in the presence of FGF-2 for 24 hours. It may be that lens epithelial cells in porcine species respond to FGF-2 differently. It is known that lens epithelial cells obtained from different species can respond quite differently to the same concentration of the same growth factor. It has been reported that cultured embryonic chick lens epithelial cells differentiate in response to exogenously added insulin or IGF-1, whereas rodent cells do not (unless exogenous FGF is also included). Moreover, there are also region-specific and age-specific differences in the response of lens epithelial cells to growth factors.

Our results show that FGF-mediated cellular functions such as proliferation can be blocked by PI-3K inhibitors, despite this growth factor’s inability to stimulate PI-3K. The decrease in proliferation promoted by FGF-2 in the presence of PI-3K inhibitors does not appear to be due to inhibition in basal PI-3K activity, as wortmannin (200 nM) and LY294002 (10 μM) did not exert significant effects on basal proliferation. The mechanism by which these inhibitors block FGF-2–promoted cell proliferation in rabbit lens epithelial cells is unclear. It has been reported that in rat L6 myoblasts, FGF-2 does not activate PI-3K, but wortmannin and LY294002 inhibit the p70 S6 kinase (another downstream target of PI-3K) activation required for proliferation. Activation of Akt also occurred in these cells in the presence of FGF-2. These investigators also showed that, in porcine aortic endothelial cells, FGF receptor–mediated activation of PI-3K is very inefficient, yet wortmannin inhibits the FGF receptor–mediated cell migration. However, it is not indicated in these reports whether PI-3K inhibitors had any effect on basal cellular levels of PI-3K. These investigators considered the possibility that, in their models, PI-3K inhibitors exert their effect on FGF-receptor–directed cell migration and FGF-2–stimulated p70 S6 kinase activation, by inhibiting a non-

![Figure 9](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933434/)
classic PI-3K or another unidentified kinase. Although we have not investigated the possibility in the present study, it may be that in rabbit lens epithelial cells, inhibition of proliferation promoted by FGF-2 by wortmannin and LY294002 is due to the inhibition of G-protein (βγ subunit)-induced p110γ-PI-3K activity. This type of PI-3K does not have the p85 regulatory subunit, which interacts with SH2 binding sites of the growth factor receptor tyrosine kinase. This wortmannin-sensitive kinase associates with a p101 regulatory subunit and also activates Akt. We have found a modest increase in pAkt level in rabbit lens epithelial cells in the presence of FGF-2, but further studies are needed to attribute any physiological significance to this observation.

Growth factor receptor-activated cell signaling pathways may converge at single or multiple points. Cross talk among these signaling pathways can occur through many signaling intermediates, and PI-3K could be one of those molecules. PI-3K activation may be essential for the interaction between FGF-2-mediated signals and other signaling cascades. An interesting finding was that, in proliferating rabbit lens epithelial cells incubated with FGF-2 for 48 hours, IGF-1-inducible PI-3K activation increased significantly, showing that FGF-2 does not have a direct effect on PI-3K but augments its activation by other growth factors that also promote proliferation. Further, the absence of a synergistic increase in proliferation with IGF-1 and FGF-2 (or insulin and FGF-2, or insulin and IGF-1, data not shown) suggests that, under in vivo conditions, all these growth factors could act in concert, rather than independently, to trigger mitogenesis. In addition, insulin and IGF-1 stimulated Erk kinase significantly during early stages of differentiation in chicken lens epithelial cells, indicating the participation of multiple signaling pathways activated by growth factors. PDGF-activated PI-3K signaling may be involved in cellular reactions other than proliferation and differentiation.
modulation of 3’phosphoinositide (PI-3,4,5P3)-dependent kinase 1 (PDK1). This kinase is responsible for the phosphorylation and activation of Akt and several other serine/threonine kinases, such as p70 S6, p90 S6, protein kinase A, and protein kinase C isoenzymes and some tyrosine kinases, such as Bruton’s tyrosine kinase. It is possible that, in rabbit lens epithelial cells, PDGF-mediated PI-3K/PDK1 activity is directed more toward regulating the activation of these serine/threonine kinases rather than Akt. The low levels of activated Akt produced in the presence of PDGF in rabbit lens epithelial cells may not be sufficient to stimulate significant proliferation but could promote some other cellular processes. No significant enhancement in proliferation was observed when rabbit lens epithelial cell cultures were treated with another form of this growth factor PDGF-ab data (not shown).

We found that PI-3K inhibitors promoted the synthesis of δ-crystallin, indicating that inhibition of PI-3K activation may be a requirement for the onset of differentiation. Further, IGF-1-stimulated PI-3K activation decreased considerably with the initiation of differentiation. It has been reported by Walker et al. that suppression of the src family of kinases (involved in the initiation of differentiation) also promotes the initiation of differentiation by inducing cadherin cell–cell junction assembly and reorganization of the actin cytoskeleton. The stimulation of PI-3K by IGF-1 in the early cultures is very high, as the cells are undergoing active proliferation and not committed to differentiation. PI-3K inhibitors arrest mitosis and cell-cycle progression to the G1/S phase and decrease the expression of cyclins. A decrease in PI-3K activation may slow proliferation and eventually lead the cell to differentiate.

Early cellular events, such as nuclear degeneration, which take place during lens epithelial cell transformation to fiber cells resemble programmed cell death or an apoptotic process. Cells may be susceptible to apoptosis or survival-signaling mechanisms are downregulated. Our data show that IGF-1/insulin-induced activation of Akt decreased significantly as differentiation progressed. It may be necessary for the lens epithelial cells to have much less activation of Akt so that apoptotic processes can be triggered during their transformation into fiber cells. Increased caspase activation and proapoptotic Bcl-2 family protein expression have been demonstrated in differentiating lens fibers. Akt activation blocks the participation of Bcl-2 family proteins in apoptosis. However, activation of Akt should be highest in proliferating cells, because they must survive to differentiate at an appropriate time. Activation of Akt also plays a role in cell cycle progression. Thus, downregulation of PI-3K/Akt activation could be very important in lens fibers and a determinant factor for fiber differentiation in lens in vivo. Unlike PI-3K/Akt, activation of Erk2 MAP kinase by insulin and IGF-1 remained increased during early stages of differentiation, suggesting that multiple signaling mechanisms control this process. As mentioned, a study by Le and Musil in chicken lens epithelial cells showed that Erk2 MAP kinase function is essential for stimulation of δ-crystallin synthesis in response to insulin and IGF-1. Association of α6-integrin with IGF-1 receptor and activation of Erk2 also occur in the early stages of chicken lens epithelial cell differentiation, indicating the activation of other signaling cascades through the IGF-1 receptor.

Other studies revealed significant differences in the modulation of PI-3K signaling by different growth factors during proliferation and differentiation. The involvement of PI-3K signaling in lens epithelial cell proliferation and differentiation promoted by insulin, IGF-1, and FGF-2 is depicted in Figure 10. Whereas inhibition of PI-3K signaling increased the synthesis of δ-crystallin, an early differentiation marker protein in chicken lens epithelial cells, its activation by insulin and IGF-1 promoted proliferation in rabbit lens epithelial cells. A gradual decrease in the activation of PI-3K/Akt by insulin and IGF-1 also occurred with the progress of differentiation in chicken lens cultures. FGF-2 which also promotes proliferation and differentiation, however, showed no direct effect on PI-3K stimulation. Thus, signaling mechanisms that are involved in lens cell proliferation and differentiation promoted by insulin/IGF-1 and FGF-2 could be different. Our current studies suggest that a coordination or cross talk between different growth factor-mediated signaling pathways is essential in the proliferation and differentiation processes. As seen with PDGF, all growth factors that activate PI-3K may not play a role in proliferation and/or differentiation. Differential activation of PI-3K/Akt and Erk-2 pathways by insulin and IGF-1 indicate that a balance between MAP kinase activation and PI-3K nonactivation may also be required for differentiation.

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References


