Regulatory Role of PI 3-Kinase on Expression of Cdk4 and p27, Nuclear Localization of Cdk4, and Phosphorylation of p27 in Corneal Endothelial Cells

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PURPOSE. FGF-2 is a potent mitogen of rabbit corneal endothelial cells (CECs). This study was undertaken to investigate whether PI 3-kinase participates in cell cycle regulation in response to stimulation with FGF-2 in CECs.

METHODS. Cell proliferation was assayed by counting the cells. Subcellular localization of proteins was determined by immunofluorescent staining and expression of cyclin-dependent kinase 4 (Cdk4), p27kip1 (p27), phosphatidylinositol 3 (PI 3)-kinase, protein kinase B/Akt (Akt), and β-actin was analyzed by immunoblot. PI 3-kinase activity was determined by measuring production of phosphatidylinositol-3-phosphate. LY294002 was used to inhibit PI 3-kinase.

RESULTS. CEC required prolonged and continuous exposure to FGF-2. FGF-2 at 10 ng/mL markedly stimulated PI 3-kinase enzyme activity, and stimulation with FGF-2 also caused activation of Akt. LY294002 inhibited both cell proliferation and PI 3-kinase activity in a concentration-dependent manner. The role of PI 3-kinase in cell cycle stimulation was determined: FGF-2 markedly upregulated expression of Cdk4 and stimulated translocation of Cdk4 into nuclei, whereas LY294002 blocked the upregulation of Cdk4 expression, and the inhibitor facilitated nuclear export of Cdk4. In contrast, FGF-2 significantly downregulated expression of p27 and facilitated phosphorylation of p27. LY294002 completely blocked the action of FGF-2 on the expression and phosphorylation of p27.

CONCLUSIONS. These data indicate that PI 3-kinase ultimately leads to activation of the cell cycle machinery in response to FGF-2. It does so by upregulating expression of Cdk4, facilitating the nuclear import of Cdk4, and sequestering Cdk4 in the nuclei as it simultaneously downregulates expression of p27 and facilitates the translocation of the molecule by phosphorylation.

Corneal endothelium is a monolayer of differentiated cells located in the posterior portion of the cornea that is essential for maintaining corneal transparency. Maintenance of corneal transparency requires an intact endothelial layer. If many cells are lost, a decline in corneal transparency ensues. One major pathway for regeneration of corneal endothelium after injury is migration and spreading of the cells from the wound’s edge. Cell division is a rare occurrence in this system. In contrast, corneal endothelial cells (CECs) in vivo convert to fibroblast-like cells in response to certain pathologic conditions. The morphologically modulated cells then resume their proliferation ability and begin to produce fibrilar collagens, leading to formation of a fibrillar extracellular matrix (ECM) in the basement membrane (Descemet’s membrane) environment. One clinical example of such phenotypic modulation of CECs is the development of a retrocorneal fibrous membrane in Descemet’s membrane, the presence of which blocks vision, thereby causing blindness. FGF-2 is a potent mitogen of CECs but the direct mediator for endothelial mesenchymal modulation. We have shown that FGF-2 is not only the potent mitogen of CECs but the direct mediator for endothelial mesenchymal modulation.

FGF-2 has diverse roles in regulating cell development, differentiation, regeneration, proliferation, migration, and angiogenesis. The biological actions of the 18-kDa FGF-2 (ECM isoform) are mediated through transmembrane cell surface receptors that possess tyrosine kinase activity. In normal cornea, the 18-kDa FGF-2 isoform is a component of Descemet’s membrane that may be necessary for wound repair. In a previous study, we have shown that stimulation of CECs with FGF-2 facilitates the association of the SH3 domain of phosphoinositide (PI)-specific phospholipase C (PLC) γ1 and vinculin. The cytoskeleton-associated PLC-γ1 is involved in mitogenesis. The mitogenic signaling pathway through PLC-γ1 accounts for approximately 20% of the FGF-2-mediated cell proliferation as determined by a number of experimental approaches: cytochalasin B, which disrupts the association of PLC-γ1 with cytoskeleton inhibits cell proliferation mediated by FGF-2 by approximately 20%; PLC-γ1-specific antisense oligonucleotide primers demonstrate an approximate 15% decrease in FGF-2-stimulated cell proliferation; neutralizing PLC-γ1 antibody blocks both the enzyme activity and cell proliferation by 20%. These findings suggest that the mitogenic signaling pathway through PLC-γ1 may not be a major pathway for the 18-kDa FGF-2 in CECs. In contrast, the phosphatidylinositol 3 (PI 3)-kinase inhibitor LY294002 was able to inhibit the mitogenic activity of FGF-2 in CECs by more than 50%

Activation of PI 3-kinase has been shown to be required for DNA synthesis in response to several mitogens. PI 3-kinase is further known to participate directly in the cell cycle progression: in rat embryo fibroblasts, PI 3-kinase activation sufficiently promotes the entry of quiescent cells into the cell cycle by activating G1 and G0/S phase cyclin-cyclin dependent kinase (Cdk) complexes and induces DNA synthesis. PI 3-kinase is required for α-thrombin–stimulated DNA synthesis in Chinese hamster embryonic fibroblasts; PI 3-kinase and its downstream target, protein kinase B/Akt, are also known to downregulate p27kip1 (p27) in response to the BCR/ABL oncogene. Although the importance of the PI 3-kinase pathway in cell proliferation is well established, its role in cell cycle regulation is not fully understood.
In our previous study,\textsuperscript{14} we showed that Cdk4 and p27 are involved in FGF-2-stimulated mitogenesis. We therefore investigated in the present study whether PI 3-kinase is directly involved in cell cycle progression by regulating the expression and translocation of Cdk4 and the expression and phosphorylation of p27. We present evidence that PI 3-kinase is the major signaling molecule in FGF-2-mediated cell proliferation. Furthermore, we show that the enzyme exerts its activity not only by regulating expression of Cdk4 and p27 but by effecting the events that occur after synthesis, such as translocation and phosphorylation, as well.

**MATERIALS AND METHODS**

FGF-2 was purchased from Intergen (Purchase, NY); the radiochemicals from ICN (Irvine, CA); anti-p85 subunit of PI 3-kinase antibody and anti-cyculin A antibody from BD Biosciences (San Diego, CA); anti-Akt and anti-phosphorylated Akt (Ser\textsuperscript{473}) antibodies from Cell Signaling Technology (Beverly, MA); monoclonal antibodies against Cdk4, p27, and β-actin and LY294002 from Sigma (St. Louis, MO); anti-phosphorylated p27 (Thr\textsuperscript{157}) from Zymed Laboratories Inc. (South San Francisco, CA); fluorescein isothiocyanate (FITC)- and rhodamine-conjugated secondary antibodies from Chemicon (Temecula, CA); and biotinylated secondary antibodies from Vector Laboratories (Burlingame, CA).

**Cell Cultures**

Isolation and establishment of rabbit CECs were performed as previously described.\textsuperscript{23} Briefly, the Descemet’s membrane-corneal endothelial complex was treated with 0.2% collagenase and 0.05% hyaluronidase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37°C. Cultured cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 50 μg/mL gentamicin (DMEM-10) in a 5% CO\textsubscript{2} incubator. First-passage CECs were used in all experiments. For subculture, concent rate the resultant supernatant was assessed with a Bradford reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK), and immunoblot analysis was performed using SDS-PAGE. The conditions of electrophoresis were as described by Laemmli.\textsuperscript{25} Thirty micrograms of protein was electrophoresed on a 12% SDS-polyacrylamide gel under the reduced condition. The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and immunoblot analysis was performed using a commercial avidin-biotin complex (ABC) kit (Vectastain; Vector Laboratories, Inc.) as previously described.\textsuperscript{14} Non-specific binding sites of nitrocellulose membrane were blocked by 5% nonfat milk. The incubations were performed with primary antibodies (1:1000 dilution) for 1 hour, with biotinylated secondary antibody (1:5000 dilution) for 1 hour, and with ABC reagent for 30 minutes. The membrane was treated with the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the ECL-treated membrane was exposed to ECL film.

**Confocal Microscopy and Image Analysis**

Antibody labeling was examined by laser scanning confocal microscopy (LSM-510; Zeiss, Thornwood, NY). The 1.8-μm optical slices and harvested by scraping into 300 μL of cold lysis buffer (20 mM Tris-HCl [pH 7.4], 10 mM NaF, 100 mM NaN\textsubscript{3}, 1 mM sodium orthovanadate, 1 mM MgCl\textsubscript{2}, 10% [vol/vol] glycerol, 1% [vol/vol] Nonidet P-40, 1 mM PMFS, 1 μg/mL leupeptin, and 1 mM aprotinin). Enzyme assays were performed as previously described\textsuperscript{14} with a slight modification. The lysates were sonicated briefly, and the insoluble material was pelleted by centrifugation at 14,000 g for 10 minutes. The p85 subunit of PI 3-kinase (p85) was immunoprecipitated from lysates containing 500 μg of protein by incubation with monoclonal anti-p85 antibody at 4°C for 2 hours, followed by incubation with protein-G agarose (Sigma) at 4°C for 1 hour. The p85 immune complexes were pelleted by centrifugation and washed three times with lysis buffer and once with PI 3-kinase assay buffer (20 mM HEPES [pH 7.4], 100 mM NaCl, 2 mM EGTA, and 12.5 mM MgCl\textsubscript{2}). The immune complex was resuspended in 20 μL of assay buffer and mixed with 20 μL of lipid-adenosine triphosphate (ATP) mix containing 500 μg/mL phosphatidylinositol, 80 μM ATP, 200 μM adenosine (PI-4-kinase inhibitor), 10 μg of γ\textsuperscript{32}P-ATP (3000 Ci/mmol), 20 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM EGTA, and 12.5 mM MgCl\textsubscript{2}. Samples were incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 80 μL of 1 N HCl. The phospholipids were extracted with 160 μL of chloroform-methanol (1:1, vol/vol). Phosphatidylinositol monophosphate was used in organic phase was separated by borate thin-layer chromatography (TLC) on aluminum-backed plates (Silica Gel 60; Fisher Scientific, Pittsburgh, PA), as previously described.\textsuperscript{24} Phosphatidylinositol-3-phosphate (PI(3-P)) was detected using autoradiography. Phosphatidylinositol-4-phosphate was used as a standard for TLC resolution of the lipid and visualized by iodine vapor. The relative density of the PI(3-P) spots was estimated using a one-dimensional image analyzer.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis**

The conditions of electrophoresis were as described by Laemmli.\textsuperscript{25} Thirty micrograms of protein was electrophoresed on a 12% SDS-polyacrylamide gel under the reduced condition. The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and immunoblot analysis was performed using a commercial avidin-biotin complex (ABC) kit (Vectastain; Vector Laboratories, Inc.) as previously described.\textsuperscript{14} Non-specific binding sites of nitrocellulose membrane were blocked by 5% nonfat milk. The incubations were performed with primary antibodies (1:1000 dilution) for 1 hour, with biotinylated secondary antibody (1:5000 dilution) for 1 hour, and with ABC reagent for 30 minutes. The membrane was treated with the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the ECL-treated membrane was exposed to ECL film.

**Immunofluorescent Staining**

The conditions of immunologic staining were described previously.\textsuperscript{14} Cells were fixed and permeabilized by 30 minutes of cold lysis buffer followed by blocking with 2% bovine serum albumin. Cells were incubated with the primary antibodies (1: 200 dilution) for 1 hour at 37°C and then incubated with FITC-conjugated secondary antibody (1:200 dilution) for 1 hour at 37°C in the dark. After extensive washing, the slides were mounted in a drop of antifade mounting medium (Vectashield; Vector Laboratories, Inc.) to reduce photobleaching. Control experiments, performed in parallel with the omission of the primary antibodies, showed negative staining in all experiments. For double staining, cells were simultaneously incubated with both primary antibodies at 37°C for 1 hour and then rinsed. Cells were then simultaneously incubated with FITC-conjugated secondary antibody (1:100 dilution) and rhodamine-conjugated secondary antibody (1:200 dilution) for 1 hour at 37°C in the dark.
were made perpendicular to the cell monolayer (apical to basal orientation). A 488-nm argon laser and the 543-nm helium neon laser were used as described previously.14 Simultaneous images of FITC or rhodamine were captured from the same optical section. The captured images were then pseudocolored: red for rhodamine and green for FITC. Regions of colocalization appear in yellow, reflecting the additive effect of superimposing green and red pixels. Image analysis was performed using the standard system operating software provided with the confocal microscope. All illustrations were assembled and processed digitally (Photoshop, ver. 5.5; Adobe, San Diego, CA).

RESULTS

Effect of FGF-2 on Cell Proliferation of CECs

We have reported that CECs stimulated with FGF-2 for 30 minutes, for two duplicated 30-minute exposures with an 8-hour interval, or for a continuous 8-hour exposure showed no proliferative activity.14 We therefore determined whether CECs require a prolonged and continuous stimulation with FGF-2 in CECs (Fig. 1). The serum-starved cells were stimulated with FGF-2 for 1, 8, 16, or 24 hours. At the end of the treatment with FGF-2, cells were further maintained in serum-free medium for up to 24 hours. Cells stimulated with FGF-2 for up to 8 hours did not increase in number, cells treated for 16 hours showed a slight increase in number, and cells treated with FGF-2 for 24 hours demonstrated a marked increase in number, suggesting that cell proliferation requires a prolonged and continuous exposure to FGF-2 in CECs.

Activation of PI 3-Kinase by FGF-2

We have shown that both PLC-γ1 and PI 3-kinase are signaling molecules in the mitogenic pathway mediated by the exogenously added 18-kDa FGF-2 in CECs.13,15 Furthermore, our previous study suggested that the mitogenic signaling pathway through PLC-γ1 may not be a major pathway when CECs are stimulated with FGF-2. The maximum inhibition through the PLC-γ1 signaling pathway reached only 20%.13,14 We therefore investigated whether PI 3-kinase was involved in the major mitogenic signaling pathway in response to stimulation with FGF-2. The serum-starved CECs were treated with FGF-2 for 24 hours in concentrations ranging from 0.01 to 10 ng/mL. Cell extracts were immune-precipitated with anti-PI 3-kinase (p85 subunit) antibody. They were then assayed for PI 3-kinase by measuring the phospholipid product PI-3-P (Fig. 2A). Cell extracts obtained from FGF-2 treatment at 10 ng/mL contained high levels of PI 3-kinase activity; the lower concentrations of FGF-2 did not stimulate PI 3-kinase. Because cell proliferation of CECs required prolonged and continuous exposure to FGF-2, we examined whether similar kinetics of PI 3-kinase activation in response to stimulation by FGF-2 were observed in CECs. Activation of PI 3-kinase enzyme was observed in the cells treated for 16 hours, and much higher enzyme activation was observed when cells were treated for 24 hours (Fig. 2B). When the amount of the p85 subunit of PI 3-kinase was determined under the same conditions, the expression of the regulatory subunit of PI 3-kinase remained constant, suggesting that FGF-2 does not upregulate p85 expression at the protein level (Fig. 2B). The activation of PI 3-kinase in response to FGF-2 was further confirmed with PI 3-kinase–mediated Akt phosphorylation. The amount of total Akt determined by immunoblotting was similar in all cells, regardless of the duration of stimulation.

FIGURE 1. Effect of FGF-2 on cell proliferation. The serum-starved cells were stimulated with FGF-2 for 1, 8, 16, or 24 hours. At the end of the treatment with FGF-2, cells were further maintained in serum-free medium for 24 hours, cells treated for 1 hour were further maintained in serum-free medium for 23 hours, and so on. At the end of the treatment with FGF-2, cells were counted. Experiments were performed in triplicate and repeated three times. The results of all three experiments were combined and plotted as the mean ± SE.

FIGURE 2. Effect of FGF-2 on PI 3-kinase enzyme activity and Akt phosphorylation. The serum-starved CECs were treated either with FGF-2 in concentrations ranging from 0.01 to 10 ng/mL for 24 hours (A) or with FGF-2 (10 ng/mL) for up to 24 hours (B, C). PI 3-kinase enzyme activity was then measured. Cell extracts were examined by immunoblot for Akt expression and phosphorylated Akt. Autoradiography of PI-3-P spots (B) and Western blot of Akt and phosphorylated Akt (C, p-Akt) at Ser473 are representative of three experiments. The relative density demonstrates the combined results of all three experiments plotted as the mean ± SE.
with FGF-2 (Fig. 2C). When active Akt was measured by antibody specific for phosphorylated Akt (Ser735), there were two waves of Akt activation: an early event between 5 and 15 minutes after stimulation with FGF-2, and a late event 24 hours after stimulation with FGF-2.

We further confirmed that FGF-2-mediated cell proliferation was the action of PI 3-kinase, as determined by the PI 3-kinase-specific inhibitor LY294002. Cells were simultaneously treated with FGF-2 and LY294002 for 24 hours in concentrations ranging from 2 to 40 μM. At 2 μM, the inhibitor showed approximately 25% inhibition of FGF-2–mediated cell proliferation, at 20 μM, the inhibitor showed 40% inhibition of FGF-2–mediated cell proliferation, and at 40 μM, the inhibitor showed approximately 45% inhibitory action on FGF-2–stimulated cell proliferation (Fig. 3A). LY294002 (20 μM) alone, in the absence of FGF-2, did not affect cell proliferation. The high degree of inhibitory action of LY294002 suggests that PI 3-kinase may be the major signaling molecule in FGF-2–stimulated mitogenesis. A parallel experiment was performed to determine the effect of LY294002 on PI 3-kinase activity. The enzyme activity was markedly elevated in the cells treated with FGF-2 alone. At 2 μM, LY294002 markedly inhibited PI 3-kinase activity, and at 20 μM, LY294002 strongly inhibited enzyme activity, reaching the maximum inhibitory effect on PI 3-kinase activity (Fig. 3B). LY294002 (20 μM) alone did not exert any effect on PI 3-kinase activity, suggesting that LY294002 alone has no inhibitory activity on any basal functions of CECs. These data also indicate that there are good parallel inhibitory actions of LY294002 on both cell proliferation and PI 3-kinase activity.

**FIGURE 3.** The inhibitory effect of LY294002 on FGF-2–mediated cell proliferation and PI 3-kinase enzyme activity. Serum-starved CECs were treated with LY294002 in concentrations ranging from 0 to 40 μM in the presence or absence of FGF-2 (10 ng/mL) for 24 hours. (A) Cell proliferation was then assayed. The inhibitory effect is presented as a percentage of the control, in which cells were maintained in serum-free DMEM for 24 hours. Experiments were performed in triplicate and repeated four times; the results of all experiments were combined and plotted as the mean ± SE. (B) PI-3-P formation was assayed. Autoradiography of PI-3-P spots is representative of four experiments. The relative density of PI-3-P demonstrates the combined results of all experiments plotted as the mean ± SE.

**FIGURE 4.** Effect of FGF-2 and LY294002 on the expression of Cdk4 and p27. The serum-starved cells were treated with FGF-2 (10 ng/mL) in the presence or absence of LY294002 (20 μM) for 24 hours (A) or with FGF-2 (10 ng/mL) in the presence of LY294002 in concentrations ranging from 2 to 40 μM for 24 hours (B). Cell extracts were examined by immunoblot analysis for expression of Cdk4 and p27. β-Actin was used for control of protein concentration on Western blot. Data are representative of three experiments.

### Role of PI 3-Kinase in Expression of Cdk4 and p27

Data obtained from a number of cell systems have shown that PI 3-kinase is directly involved in G1/S progression by regulating the expression of G1 cell cycle regulatory proteins during mitogen-stimulated cell growth.17,26,27 Our previous study demonstrated that FGF-2 regulates the expression of Cdk4 and subcellular localization of p27.14 We therefore wanted to determine whether the expression of Cdk4 and p27 was regulated by PI 3-kinase in response to stimulation with FGF-2. Cells were treated with FGF-2 (10 ng/mL) for 1, 8, 16, or 24 hours. The 24-hour incubation of cells with FGF-2 was performed in the presence or absence of LY294002. The basal level of Cdk4 expression in the mitogen-deprived cells was very low. Cells treated with FGF-2 demonstrated an elevated level of Cdk4 expression in a time-dependent manner. Cells treated for 16 or 24 hours expressed high levels of Cdk4, whereas LY294002 completely abolished the elevated expression of Cdk4 mediated by stimulation with FGF-2, suggesting that this inhibition was attributable to PI 3-kinase (Fig. 4A). In contrast, the mitogen-deprived cells demonstrated a high level of p27 expression. FGF-2 downregulated p27 expression in a time-dependent manner. Cells treated for 16 or 24 hours demonstrated markedly reduced levels of p27, whereas, in the cells simultaneously treated with FGF-2 and LY294002, the inhibitor was able to block the effect of FGF-2 on the downregulation of p27 expression (Fig. 4A). To further confirm that expression of Cdk4 is regulated by PI 3-kinase, their expression was examined in the presence of LY294002 in concentrations rang-
ing from 2 to 40 μM (Fig. 4B). There was a dose-dependent downregulation of Cdk4 and upregulation of p27 at 2 μM, LY294002 did not downregulate expression of Cdk4, but it upregulated expression p27, causing an inhibition of cell proliferation and PI 3-kinase activity. These data suggest that FGF-2-stimulated PI 3-kinase activity promotes proliferation of CECs through Cdk4 and p27.

**Role of PI 3-Kinase on Translocation of Cdk4**

Subcellular compartmentalization of cell cycle regulatory proteins plays a key role in regulating cell cycle progression. Translocation of these proteins from the cytoplasmic biosynthetic site to the nuclear action site is essential in many cellular events. Therefore, we asked whether translocation of Cdk4 is present in the cells stimulated with FGF-2 and whether such translocation is regulated at the level of PI 3-kinase. To document the translocation process, cells were treated with FGF-2 for 1, 8, 16, or 24 hours. LY294002 was used only in the cells treated with FGF-2 for 24 hours. Cells maintained in DMEM-0 for 24 hours showed no nuclear Cdk4 staining (Fig. 5A). Cells treated for 1 hour began to show faint nuclear Cdk4 staining in a few cell cultures (Fig. 5B). The nuclear Cdk4 staining was increased in cells treated for 8 hours, although the staining potential remained weak (Fig. 5C). All cells treated for 16 (Fig. 5D) and for 24 (Fig. 5E) hours demonstrated a strong positive nuclear staining and faint cytoplasmic staining. Simultaneous treatment of cells with FGF-2 and LY294002 for 24 hours demonstrated a faint cytoplasmic staining in the absence of nuclear staining (Fig. 5F). Although the inhibitor markedly inhibited Cdk4 expression, as shown in Figure 4, the absence of nuclear Cdk4 staining and the positive cytoplasmic Cdk4 staining in the cells treated with the inhibitor suggest that LY294002 is involved in the nuclear import of Cdk4, and that the translocation event of Cdk4 is therefore directly regulated by PI 3-kinase. This result is in agreement with reported findings in Chinese hamster embryonic fibroblasts (IC9), in which LY294002 inhibits the translocation of Cdk2 to nuclei.28 We also examined whether PI 3-kinase is further involved in nuclear sequestration of Cdk4. Cells stimulated with FGF-2 for 16 hours were treated with LY294002 for 1, 2, 4, or 8 hours in the absence of FGF-2. Cells maintained in serum-free medium demonstrated a very low level of Cdk4 in the cytoplasm (Fig. 6A), whereas cells treated with FGF-2 for 16 hours demonstrated dual staining of the nuclear and cytoplasmic Cdk4 (Fig. 6B). When cells were treated with the inhibitor for 1 hour, the dual location of Cdk4 was not altered (Fig. 6C), but when the cells were treated with the inhibitor for 2 (Fig. 6D) or 4 (Fig. 6E) hours, LY294002 facilitated the translocation of Cdk4 from nuclei to cytoplasm. Cells treated with the inhibitor for 8 hours demonstrated the absence of nuclear Cdk4, but they showed a cytoplasmic and perinuclear Cdk4 (Fig. 6F). Because expression of Cdk4 is not induced in the absence of FGF-2 and the nuclear export of Cdk4 is mediated by LY294002, this observation suggests that PI 3-kinase is involved in the sequestration of Cdk4 in the nuclei.

**Role of PI 3-Kinase on Phosphorylation of p27**

Our previous report14 demonstrates that FGF-2 induces nuclear export of p27 to the cytoplasm, albeit at a low level. Nuclear p27 is phosphorylated at the residue of threonine (Thr187) before nuclear export into the cytoplasm, where the phosphorylated p27 is subjected to degradation, either by the ubiquitin-proteasome pathway29,30 or by ubiquitin-independent proteolytic cleavage.31 The loss of staining potential of nuclear p27 was examined while exploring whether PI 3-kinase is involved in phosphorylation of p27 in FGF-2-mediated mitogenesis. For this purpose, cells treated with FGF-2 were double-stained with anti-p27 and anti-phosphorylated p27 antibodies. The anti-phosphorylated p27 antibody is specific to the Thr187 phosphorylated form of p27 and does not react with unphosphorylated p27. Cells maintained in DMEM-0 showed strongly positive staining for nuclear p27, whereas anti-phosphorylated p27 antibody did not stain the p27-positive cells (Fig. 7, T0). Cells treated with FGF-2 for 1 hour had a strong positive staining for nuclear p27, and these cells were not positive for the phosphorylated p27 (Fig. 7, T1h). Cells treated for 8 hours began to lose the staining potential of nuclear p27, and some of these cells demonstrated positive staining for phosphorylated p27 (Fig. 7, T8h). These staining profiles were more apparent in the cells stimulated for 16 hours (Fig. 7, T16h). All the cells stimulated for 24 hours demonstrated strongly positive staining for phosphorylated p27, whereas most of these cells lost their staining potential for nuclear p27 (Fig. 7, T24h). When the images of both p27 and phosphorylated p27 were merged, less than 20% of the cells demonstrated coincidental staining with the two antibodies, regard-

**Figure 5.** Subcellular localization of Cdk4 in response to stimulation with FGF-2. The serum-starved CECs were treated with FGF-2 (10 ng/mL) for the designated time and stained for Cdk4. (A) CECs maintained in DMEM-0. (B-E) CECs treated with FGF-2 for 1 (B), 8 (C), 16 (D), or 24 (E) hours. (F) CECs simultaneously treated with FGF-2 and LY294002 (20 μM) for 24 hours. Data are representative of four experiments. Bar, 20 μm.

**Figure 6.** Effect of LY294002 on translocation of Cdk4. The serum-starved CECs were treated with FGF-2 (10 ng/mL) for 16 hours. Cells were then treated with LY294002 (20 μM) in the absence of FGF-2 for up to 8 hours. Cells were stained for Cdk4. (A) CECs maintained in DMEM-0. (B) CECs treated with FGF-2 for 16 hours; (C-F) CECs treated with LY294002 for 1 (C), 2 (D), 4 (E), or 8 (F) hours. Data are representative of two experiments. Bar, 20 μm.
progression requires the continuous presence of the growth factor. Similar to previous reports, mitogen-stimulated cell cycle progression requires the continuous presence of the growth factor in CECs: 24-hour exposure to FGF-2 requires prolonged and continuous exposure of cells to the growth factor in CECs.

Our present study showed that cell proliferation stimulated by FGF-2 is directly regulated by PI 3-kinase. Stimulation with FGF-2, suggesting that phosphorylation of p27 is completely blocked in response to stimulation with FGF-2. LY294002 blocked more than 40% of proliferation of CECs in response to stimulation with FGF-2. The kinetics of PI 3-kinase activation is similar to that of CEC proliferation in response to stimulation with FGF-2. These data suggest that PI 3-kinase may play a major role in the mitogenic signaling pathway of FGF-2 in CECs.

Recent observations from several independent studies suggest that PI 3-kinase regulates mitogen-induced G1 transit by linking to the cell cycle regulatory machinery: Diehl et al. have reported a direct link between PI 3-kinase/Akt activation and stabilization of cyclin D1. Another study reported that PI 3-kinase is required for cyclin D1 accumulation at a protein level independent of the ERK pathway in α-thrombin–stimulated DNA synthesis in Chinese hamster embryonic fibroblasts (IC9). It has also been suggested that the PI 3-kinase-mediated pathway has a role in mitogen-mediated p27 downregulation because LY294002 restores expression of p27. Thus, our results in the present study were basically compatible with those of previous reports. Upregulation of Cdk4 was observed in CECs within 8 hours after stimulation with FGF-2, and maximum Cdk4 expression and accumulation were reached in those cells treated with FGF-2 for 16 hours. Such elevated expression mediated by FGF-2 was inhibited by LY294002 in a dose-dependent manner. In contrast, in parallel experiments FGF-2 downregulated p27 expression in a time-dependent manner and that LY294002 restored p27 expression. These data indicate that PI 3-kinase is directly involved in the regulation of Cdk4 and p27 expression at the protein level in CECs. However, it should be noted that p27 was not downregulated on stimulation with FGF-2 in our previous study. The growth stage of CECs may have contributed to the differences between the results in the two studies. In the present study, we used CECs that were almost confluent. It appears that the contact-inhibited cells that already have high p27 levels are much less sensitive to stimulation with FGF-2.

A recent study demonstrated that treatment of IIC9 cells with LY294002 inhibits α-thrombin–mediated nuclear translocation of Cdk2. Those findings suggest that PI 3-kinase is involved in nuclear import of Cdk2, and they indicate another role for PI 3-kinase, in addition to the regulation of Cdk4 expression. Therefore, we investigated whether PI 3-kinase facilitates the nuclear translocation of Cdk4 in addition to regulating expression of Cdk4. To document the time course of the translocation event, the mitogen-deprived cells were stimulated with FGF-2 for different times. Nuclear translocation of Cdk4 was observed 8 hours after stimulation with FGF-2, and dual subcellular localization of Cdk4 was observed 16 hours after stimulation. At that time, Cdk4 expression was markedly upregulated at the protein level. Most Cdk4 appears to be localized in the nuclei in cells stimulated for 24 hours. Nuclear localization is completely abolished by LY294002, but the inhibitor does not completely abolish the cytoplasmic Cdk4 staining, suggesting that PI 3-kinase is directly involved in the nuclear translocation of Cdk4. Translocation of Cdk4 is prerequisite for activation of the Cdk4-cyclin D complex. Nuclear translocation of Cdk2 is reportedly associated with com-
plexes containing active ERK. In this scheme, Cdk2-cyclin E associates with active ERK. It is carried into the nucleus along with ERK, which acts as a nuclear transport factor. It is thus important to examine whether Cdk1-cyclin D associates with active PI 3-kinase and is carried into the nucleus along with PI 3-kinase. However, this scenario may not occur in CECs, because immunofluorescent staining of PI 3-kinase demonstrates the absence of the nuclear staining of the enzyme even after mitogen-activation (Kay EP, unpublished data, 1996). Another interesting finding in the present study is that PI 3-kinase appeared to sequester Cdk4 in the nuclei in response to stimulation with FGF-2. In the absence of FGF-2, under the condition that no further induction of Cdk4 occurred, LY294002 facilitated the nuclear export of Cdk4 in a time-dependent manner, and Cdk4 was present in the cytoplasm, but not in the nuclei, 8 hours after treatment with the inhibitor. Together, these data suggest that PI 3-kinase facilitates the nuclear translocation and sequestration of Cdk4 in response to stimulation with FGF-2.

In contrast to these findings regarding the subcellular compartmentalization of Cdk4 and to our previous data showing that FGF-2 slightly induces nuclear export of p27, FGF-2 did not alter the subcellular localization of p27. Instead, it largely altered the staining potential of nuclear p27. The mitogen-deprived cells showed strong nuclear staining of p27. Within 8 hours of stimulation with FGF-2, the staining potential of nuclear p27 was markedly weakened. Cells stimulated for 24 hours demonstrated weakly positive staining of nuclear p27 in a few cell cultures, whereas LY294002 completely restored the p27 staining potential. These data confirm the immunoblot analysis in which FGF-2 downregulated p27 expression and that LY294002 blocked the effect of FGF-2 on p27 expression. These data led us to investigate whether PI 3-kinase plays a role in the degradation pathway of p27. The initial step for p27 degradation is phosphorylation at the Thr187 residue of p27. The phosphorylation of p27, suggesting that PI 3-kinase is involved in this event that is a prerequisite for p27 degradation. These data, taken together, suggest that PI 3-kinase regulates protein expression and the posttranslational modulation of Cdk4 and p27 expression: upregulation and nuclear sequestration as for Cdk4 and downregulation and phosphorylation as for p27. The dual activity of PI 3-kinase is in accordance with Gp1/S transition and subsequent cell proliferation in the response to FGF-2 in CECs. Of great interest, our previous report demonstrates that PLC-γ1 also utilizes Cdk4 and p27 while exerting the mitogenic signal.14 Our studies using antimotogens (TGF-β2 and cyclic adenosine monophosphate [AMP]) demonstrate that the two antimotogens also use Cdk4 and p27 for their actions.57,58 Together, these data suggest that CECs use Cdk4 and p27 to regulate cell proliferation regardless of the diverse cytoplasmic signaling pathways. CECs are thus able to tightly regulate cell proliferation events using a few well-defined regulators of cell cycle progression.

References


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