PI 3-Kinase/Rac1 and ERK1/2 Regulate FGF-2–Mediated Cell Proliferation through Phosphorylation of p27 at Ser10 by KIS and at Thr187 by Cdc25A/Cdk2

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PURPOSE. To determine the mechanism of p27 phosphorylation through common and differential pathways triggered by FGF-2 in corneal endothelial cells (CECs).

METHODS. A GTP pull-down assay was performed to identify Rac1-GTP. Expression and activation of protein were analyzed by immunoblotting. Cell proliferation was measured by an MTT assay. Transfection of CECs with kinase-interacting stathmin (KIS) siRNA was performed.

RESULTS. FGF-2 activated Rac1 through Akt, and Rac1 inhibitor greatly inhibited the FGF-2–stimulated cell proliferation. Rac1 inhibitor reduced p27 phosphorylation at both serine 10 (Ser10) and threonine 187 (Thr187). ERK1/2 was also involved in FGF-2–stimulated CEC proliferation and phosphorylation of p27 at Ser10 and Thr187 in parallel to phosphatidylinositol (PI) 3-kinase. In both PI 3-kinase/Rac1 and ERK1/2 pathways, Ser10 of p27 is phosphorylated by KIS, confirmed by siRNA to KIS, which subsequently hampered the FGF-2–stimulated cell proliferation, while Thr187 of p27 was phosphorylated through Cdk2 activated by Cdc25A. Cdc25A inhibitor blocked activation of Cdk2, phosphorylation of p27 at Thr187, and cell proliferation. FGF-2 induced both KIS and Cdc25A during the G₁ phase; the maximum KIS expression was observed 4 hours after FGF-2 stimulation, while the maximum Cdc25A expression was observed at 12 hours. Blockade of ERK1/2 and Rac1 greatly reduced KIS and Cdc25A expression.

CONCLUSIONS. Results suggest that FGF-2 uses both PI 3-kinase/Rac1 and ERK pathways for cell proliferation; two signals employ common pathways for phosphorylating p27 according to the sites (KIS for Ser10 and Cdc25A/Cdk2 for Thr187) with their characteristic kinetics (early G₁ for Ser10 and late G₁ for Thr187). (*Invest Ophthalmol Vis Sci.* 2011;52:417–426) DOI: 10.1167/iovs.10-6140

Human corneal endothelial cells (CECs) remain arrested at the G₁ phase of the cell cycle throughout their lifespan.^{1,2} Such characteristic behavior of cell proliferation dictates most of the wound-healing processes occurring in the corneal endothelium: CECs do not use cell division to replace the lost cells but use migration and attenuation to cover the denuded area. On the other hand, in nonregen-

erative wound healing, CECs are transformed into mesenchymal cells that subsequently produce a fibrillar extracellular matrix (ECM) in the basement membrane environment. Thus, corneal fibrosis represents a significant pathophysiological problem, one that causes blindness by physically blocking light transmittance. One clinical example of corneal fibrosis observed in corneal endothelium is the development of a retrocorneal fibrous membrane (RCFM) in Descemet's membrane.^{3,4} We established an animal (rabbit) RCFM model, and we reported that CECs in RCFM are converted to fibroblast-like cells: The contact-inhibited monolayer of CECs is lost, resulting in the development of multilayers of fibroblast-like cells.^{5,6} These morphologically altered cells simultaneously resume their proliferation ability and deposit a fibrillar ECM in Descemet's membrane. Furthermore, our in vitro model using rabbit CECs (rCECs)^{7–10} elucidated the molecular mechanism of RCFM formation and demonstrated that fibroblast growth factor-2 (FGF-2) directly mediates the endothelial mesenchymal transformation (EMT) observed in rCECs. We reported that, among the phenotypes altered during EMT, FGF-2 signaling regulates cell cycle progression through phosphorylation of p27^{Kip1} (p27) by the action of phosphatidylinositol (PI) 3-kinase. Our kinetic studies^{11,12} demonstrated that phosphorylation of p27 at serine 10 (Ser10) occurred much earlier than phosphorylation of p27 at threonine 187 (Thr187) and that the subsequent polyubiquitination of the two phosphorylated p27s was carried out in the different subcellular localizations under the differential kinetics: phosphorylated p27 at Ser10 (pp27Ser10) is exported from nucleus to cytoplasm, followed by degradation through the KPC1/2 ubiquitin-proteasomal machinery in the cytoplasm, whereas phosphorylated p27 at Thr187 (pp27Thr187) is degraded through nuclear ubiquitin E3 ligase complex, Skp1-Cul1-F-box protein (SCF^{Skp2}), in the nucleus.¹² Thus, at least two respective populations of p27 undergo phosphorylation; each population functions at a different stage of the G₁ phase of the cell cycle in response to mitogenic signals.^{11,12}

The PI 3-kinase and the extracellular signal-regulated kinase (ERK) pathways are centrally involved in cell proliferation.^{13,14} The ERK signaling pathway regulates the subcellular localization of cyclin-dependent kinase 2 (Cdk2) to the nucleus and is necessary for Cdk activation through phosphorylation of Tyr160. The ERK signaling is also involved in upregulation of cyclin D1 and downregulation of p27.^{15–19} Likewise, the importance of p27 as a regulator of PI 3-kinase-mediated cell cycle progression is well established.^{11,13,20–24} Protein kinase B (commonly known as Akt) is an important downstream effector of the PI 3-kinase pathway. Akt has been shown to directly phosphorylate p27 on Ser-10, Thr-157, and Thr-198.^{25,26} Ser-10, which is the major phosphorylation site of p27, is also phosphorylated by kinase-interacting stathmin (KIS), a nuclear

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serine-threonine kinase.^{27,28} We have shown that phosphorylation of p27 at Ser-10 takes place in the nuclei within 2 hours after stimulation with FGF-2. The maximum p27 phosphorylation at Ser-10 occurred in the nucleus 6 hours after FGF-2 stimulation; nuclear export of pp27Ser10 was observed for up to 12 hours after FGF-2 stimulation. We further demonstrated that phosphorylation of p27 at Ser-10 is the major mechanism for FGF-2-mediated-G₁/S transition leading to cell proliferation, while phosphorylation of p27 at Thr-187 acts as the second major mechanism of FGF-2-stimulated cell proliferation. We have shown that these actions of FGF-2 are mediated by PI 3-kinase.¹¹

Because ERK1/2 is another mechanism for cell proliferation observed in many different cells, we decided to test whether this is the case in CECs stimulated with FGF-2. We also determined the downstream effector molecules for the distinctive phosphorylation events of p27. This study shows that PI 3-kinase uses Rac1 as the downstream to Akt and that FGF-2 employs the PI 3-kinase and ERK1/2 pathways in parallel for cell proliferation. We further showed that FGF-2 greatly induces both KIS and Cdc25A through PI 3-kinase/Rac1 and ERK1/2 pathways. Subsequent phosphorylation of p27 at Ser-10 is mediated by KIS, while phosphorylation of p27 at Thr-187 is mediated by Cdk2 activated by Cdc25A, a phosphatase that activates Cdk2.²⁹ This study, thus, shows that phosphorylation of p27 is regulated by multiple mechanisms, but that it occurs within the context of the already determined pathway.

MATERIALS AND METHODS

Materials

LY294002, U0126, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), monoclonal antibody against Rac1, α -tubulin, and β -actin and peroxidase conjugated secondary antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Anti-phospho-histone H1 antibody and purified histone H1 protein were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-lamin B antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pp27Ser10 and anti-pp27Thr187 were obtained from Zymed Laboratories, Inc. (South San Francisco, CA). Anti-Cdk2 antibody was obtained from BD Biosciences (San Jose, CA). FGF-2, Y27632, NSC23766, U0126, BN82002, and Akt inhibitor were purchased from Calbiochem (San Diego, CA). Anti-Akt, phospho-Akt (Ser473), Cdc25A, ERK1/2 antibodies, and anti-phospho-ERK1/2 antibody were purchased from Cell Signaling Technology (Danvers, MA). Anti-KIS antibody was obtained from Abgent (San Diego, CA).

Cell Culture

Rabbit eyes were purchased from Pel-Freez Biologicals (Rogers, AR). Isolation and establishment of rabbit CECs were performed as previously described.^{11,30} All details of culture conditions and procedures have been reported previously.^{11,30} In some experiments, pharmacologic inhibitors were used in the presence of FGF-2 stimulation: Y27632 (10 μ M), NSC23766 (100 μ M), LY294002 (20 μ M), Akt inhibitor (10 μ M), U0126 (1 μ M), or BN82002 (5 μ M).

A

B

FIGURE 1. Involvement of Rac1 in the FGF-2-stimulated cell proliferation through PI 3-kinase. **(A)** Cell proliferation was determined by an MTT assay. The serum-starved cells were pretreated with Y27632 for ROCK inhibition and NSC23766 for Rac1 inhibition for 2 hours and then maintained in DMEM with FGF-2 for 24 hours. At the end of incubation, an MTT assay was performed. Data were normalized to cells maintained in DMEM without serum (D-0). The graphs represent the mean $\pm$ SEM from three independent experiments. **(B)** For Rac1 pull-down assay, CECs were cultured in the indicated media condition and time. LY294002 for PI 3-kinase inhibition was pretreated for 2 hours and then maintained in DMEM with FGF-2 for the designated time. The amount of activated Rac1 was normalized to the total amount of Rac1 in the cell lysates to compare Rac1 activity (level of GTP bound Rac1) in different cell lysates. GTP γ S and GDP treatment served as positive and negative controls, respectively, for the pull-down assay. The relative fold differences were then compared with the values of stimulated CECs with FGF-2 for 1 hour. The graphs represent the mean $\pm$ SD from three independent experiments. Y, Y27632; NSC, NSC23766; F-2, FGF-2; LY, LY294002.

Cell Proliferation Assays

An MTT assay was used to measure cell proliferation as described previously.³¹ Briefly, cells were seeded in 96-well tissue culture plates at a concentration of 4×10^3 cells/well. When cells reached approximately 70% confluence, the medium was changed from DMEM-15 to DMEM for serum starvation and maintained for 24 hours. The serum-starved cells were then maintained for 24 hours in each culture condition. At the end of culture, the MTT (50 μ g/mL) was added and further maintained for 2 hours at 37°C. The MTT-containing medium was discarded, and 100 μ L of undiluted dimethyl sulfoxide was added to the cells. After 30 minutes incubation at room temperature, absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 650 nm using a spectrophotometric plate reader (Benchmark Plus Microplate Spectrophotometer; Bio-Rad Laboratories, Hercules, CA).

Cytoplasmic and Nuclear Fractionation, Protein Preparation, Protein Assay, SDS-PAGE, and Western Blotting Analysis

All details of methods and procedures have been presented previously.^{11,31–33} The following gel concentrations were used to sepa-

rate proteins: 12.5% gel for phospho-p27, Cdk2, Rac1, ERK1/2, KIS, and phospho-histone H1, and 10% gel for Akt, actin, Cdc25A, α -tubulin, and lamin B.

Active Rac1 Pull-Down Assay

Rac1 activity was quantified by measuring the amounts of Rac1 precipitated in a pull-down reaction from cell lysates, with the GTPase-binding domain of PAK-PBD (p21 binding domain) used as bait, using the active Rac1 pull-down detection kits (Pierce, Rockford, IL), according to the manufacturer's instructions. Briefly, CECs cultured in the designated culture condition were washed twice with sterile phosphate-buffered saline (PBS), followed by incubation in enzyme-free cell dissociation solution (Chemicon, Temecula, CA) for 3 minutes at room temperature. Cells were detached by scraping, transferred to microcentrifuge tubes, and pelleted at 5000g for 1 minute. The harvested cells were then lysed with lysis/binding/wash buffer containing 1 mM PMSF, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, and 1 μ g/mL pepstatin supplied by the manufacturer. The total 6 mg of cleared cell lysates were split into three equal aliquots. As negative and positive controls for the pull-down, two of the aliquots were added to 100 μ M GDP or GTP γ S, respectively, and incubated for 15 minutes at 30°C with agitation to deplete or enrich Rac1-GTP. The third aliquot remained un-

FIGURE 2. FGF-2-mediated Rac1 activation through Akt. The serum-starved cells were pretreated with Akt inhibitor or NSC23766 for 2 hours and then maintained in DMEM with FGF-2 for the designated time. At the end of treatment, cells were lysed. Nuclear and cytoplasmic fractions were prepared. Nuclear (A) and cytoplasmic (B) fractions were immunoblotted with the respective antibody, or a Rac1 pull-down assay was performed as described in Figure 1B. The purity of fractions was also controlled with lamin B for nuclear and α -tubulin for cytoplasmic fraction, respectively. The results represent data obtained in three independent experiments.

FIGURE 3. Involvement of Rac1 in phosphorylation of p27 at both the Thr-187 and Ser-10 sites. The serum-starved cells were pretreated with NSC23766 for 2 hours and then maintained in DMEM with FGF-2 for the designated time. The cell lysates were immunoblotted with (A) anti-pp27Ser10 and (B) pp27Thr187 antibody, respectively. Relative density of immunoblotting bands was determined using a gel documentation system, and actin was used to control protein concentration on immunoblot analysis. The relative fold differences were then compared with the values of nonstimulated CECs (0 hours). The graphs represent the mean $\pm$ SD from three independent experiments.

treated and was kept on ice while the controls were loaded. Two milligrams of cell lysates were transferred to the spin cup containing 100 μ L of glutathione resin and 20 μ g of glutathione S-transferase-PAK-PBD, and the reaction mixture was then incubated for 1 hour at 4°C with gentle agitation. After the pull-down reaction, the supernatants were removed by brief centrifugation, and the precipitated proteins bound to the beads were washed two times with lysis/binding/wash buffer. To discover the activated Rac1, 50 μ L of 2 $\times$ protein sample buffer was added to the spin cup and collected by centrifugation. Eluted protein samples were subjected to immunoblot analysis with monoclonal antibody to Rac1. The total amount of Rac1 was also determined by Western blot analysis of total cell lysates and used to normalize the protein concentration of each lane.

Small Interference RNA Transfection

After cloning of partial cDNA of rabbit KIS gene (GenBank accession no. GU815100), small interference RNA (siRNA) against rabbit KIS was

obtained from Ambion (Austin, TX) in deprotected and desalted form. The chemically synthesized, double-stranded siRNA, with 19-nt duplex RNA and 2-nt 3'-dTdT overhang siRNA sequences (sense) targeting respective rabbit KIS mRNA, is listed: 5'-AGCAGUGGUGAAUGCCGCA-3'. Transient transfections of siRNA were performed using transfection reagent (Lipofectamin RNAiMAX; Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Briefly, CECs were seeded on 6-well plates and maintained in culture until they reached 60–70% confluence. These cells were transiently transfected with 50 nM of the siRNA double-strand RNA and 5 μ L of the complex obtained from Invitrogen. After the 6 hour incubation, medium containing transfection reagent was removed, and the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with or without FGF-2 for an additional 30 hours. To verify specificity of the knockdown effect, we used an siRNA (Silencer Select Negative Control; Ambion) with no known mammalian target as nonspecific siRNA. There was no cytotoxic effect, and the transfection efficiency was 50–60% under these transfection conditions. The transfection efficiency was detected using fluorescein-conjugated nonspecific siRNA by a fluorescence microscope.

Protein Phosphorylation Assay In Vitro

Phosphorylation of histone H1 by activated Cdk2-associated kinase activity was detected as described previously¹¹ with some modifications. After transfection with KIS siRNA, the cell lysates were immunoprecipitated with anti-Cdk2 antibody. Immune-complexes precipitated with anti-Cdk2 antibody were washed three times with PBS and resuspended in 50 μ L of kinase buffer (50 mM Tris-HCl, pH 7.2, 10 mM MgCl₂, 1 mM DTT) containing 100 μ M ATP. One microgram of commercially purified histone H1, as a substrate of Cdk2 (Upstate Biotechnology, Inc.), was then added to the immunoprecipitates, and the mixtures were incubated for 1 hour at 30°C. The reaction mixtures were resolved by SDS-PAGE, and phosphorylated histone H1 was detected by immunoblotting with anti-phospho-histone H1-specific antibody. For positive control experiments, cell lysates were immunoprecipitated with anti-Cdk2 antibody, and the immune complex was detected with anti-Cdk2 antibody.

Statistics

All experiments were performed at least three times, and the results are presented as the mean and SD or SEM. Statistical differences were

FIGURE 4. Involvement of ERK1/2 and synergistic effect of Rac1 and ERK1/2 on cell proliferation stimulated by FGF-2. Cell proliferation was determined by an MTT assay as described in Figure 1A. The serum-starved cells were pretreated with U0126 for ERK1/2 inhibition, NSC23766, or both for 2 hours and then maintained in DMEM with FGF-2 for 24 hours. At the end of incubation, an MTT assay was performed. Cell proliferation was completely blocked by treatment of both Rac1 and ERK1/2 inhibitors. Data were normalized to unstimulated cells. The graphs represent the mean $\pm$ SEM from four independent experiments. U, U0126.

analyzed with the aid of statistical software (Excel; Microsoft, Redmond, WA) using a paired Student's *t*-test, as indicated in figure legends.

RESULTS

Involvement of Rac1 in p27 Phosphorylation at Both Thr-187 and Ser-10 Sites

A recent study in which a Rho kinase (ROCK) inhibitor, Y-27632, promoted cell proliferation of monkey CECs³⁴ prompted us to test whether the same is true in rabbit CECs. The serum-starved cells were pretreated with either Rac1 inhibitor (NSC23766) or ROCK inhibitor (Y-27632) for 2 hours and then stimulated with FGF-2 for 24 hours. The Rac1 inhibitor greatly blocked the FGF-2-stimulated cell proliferation, whereas ROCK inhibitor neither blocked it nor stimulated it (Fig. 1A), unlike the findings obtained from monkey CECs by Okamura et al.³⁴ Figure 1B further shows that Rac1 activation is mediated by FGF-2 in a time-dependent manner, while a specific inhibitor of PI 3-kinase (LY294002) completely abolished the Rac1 activation. We then determined whether Rac1 is the downstream effector of the PI 3-kinase/Akt pathway. Since the major role of Rac involves the organization of actin cytoskeleton, for which its subcellular localization may be crucial for the cellular activity, nuclear and cytoplasmic fractions were prepared for this particular experiment. The nuclear fraction, confirmed by the presence of lamin B and the absence of α -tubulin, demonstrated that phosphorylation of Akt at Ser-473, the downstream product of activated PI 3-kinase, was observed 4 hours after FGF-2 stimulation, and the maximum activation was reached

at 8 hours (Fig. 2A). Similarly, Rac1 activation was observed in cells stimulated with FGF-2 after 4 hours. When cells were pretreated with Akt inhibitor for 2 hours and then stimulated with FGF-2, the inhibitor blocked not only activation of Akt but also activation of Rac (Fig. 2A). On the other hand, Rac1 inhibitor (NSC23766) was able to block only Rac1 activation but not Akt activation (Fig. 2A). When the cytoplasmic fraction was tested, similar findings were observed (Fig. 2B); Akt inhibitor completely blocked the FGF-2-mediated activation of both Akt and Rac1, while Rac1 inhibitor did not block Akt activation. These findings indicate that Rac1 is downstream to the PI 3-kinase/Akt pathways triggered by FGF-2. Both nuclear and cytoplasmic Rac1 are regulated by Akt. Of interest, activation of Rac1 is crucial for nuclear import of the molecule: The amount of total cytoplasmic Rac1 was not altered regardless of the presence or absence of either Rac1 inhibitor or Akt inhibitor. On the other hand, nuclear Rac1 was not observed in cells pretreated with Rac1 inhibitor, and the amount of nuclear Rac1 was greatly reduced in cells pretreated with Akt inhibitor before FGF-2 stimulation. These findings suggest that activation of Rac1 is a prerequisite to the translocation of Rac1-GTP into the nucleus. Figure 2A further shows that the nuclear accumulation of Rac1 occurs from the early G₁ phase of the cell cycle in CECs.

Our kinetic studies demonstrated that phosphorylation of p27 at Ser10 occurred much earlier than phosphorylation of p27 at Thr187.^{11,12} The characteristic differential phosphorylation kinetics of p27 at Thr187 and Ser-10 were also tested in the presence of Rac1 inhibitor. Cells were pretreated with Rac1 inhibitor for 2 hours and then stimulated with FGF-2

FIGURE 5. Involvement of ERK1/2 on phosphorylation of p27 at both the Thr-187 and Ser-10 sites. The serum-starved cells were pretreated with U0126 or both U0126 and NSC23766 for 2 hours and then maintained in DMEM with FGF-2 for the designated time. The cell lysates were immunoblotted with (A) anti-pp27Ser10 and (B) anti-pp27Thr187 antibodies. Relative density of immunoblotting bands was determined using a gel documentation system, and actin was used to control protein concentration on immunoblot analysis. The relative fold differences were then compared with the values of unstimulated CECs (0 hours). The graphs represent the mean $\pm$ SD from three independent experiments.

for 4, 8, or 12 hours to determine phosphorylation of p27 at Ser10; longer times (12, 16, or 24 hours) were used to determine phosphorylation of p27 at Thr187. Figure 3A shows the early kinetic event for phosphorylation of p27 at Ser10: The maximum phosphorylation of p27 at Ser10 was observed in cells stimulated for 4 hours with FGF-2, and the pp27Ser10 had largely disappeared from the 12-hour treatment condition (Fig. 3A), confirming our previous findings.¹¹ Figure 3A further shows that the Rac1 inhibitor markedly reduced phosphorylation of p27 at Ser10. When the cells pretreated with Rac1 inhibitor were further tested for the phosphorylation event of p27 at Thr187, the maximum phosphorylation on this site took place 16 hours after FGF-2 stimulation, and the Rac1 inhibitor blocked the phosphorylation event (Fig. 3B). These findings suggest that Rac1 is involved in the phosphorylation of p27 at both the Ser10 and Thr187 sites.

Involvement of ERK1/2 in p27 Phosphorylation at Both Ser10 and Thr187

The ERK1/2 is regarded as the central component of the growth-promoting signaling pathways. It is, therefore, natural to address whether ERK1/2 is also involved in G₁/S transition, leading to cell proliferation in CECs stimulated with FGF-2. The simple question was tested using MEK1/2 inhibitor (U0126), because MEK1/2 is the upstream kinase to phosphorylate ERK1/2. When serum-starved cells were pretreated with U0126 for 2 hours and then stimulated with FGF-2 for 24 hours, FGF-2-mediated cell proliferation was greatly blocked by MEK1/2 inhibitor, and its inhibitory level was similar to the level achieved with Rac1 inhibitor (Fig. 4). Simultaneous treatment of cells with the two inhibitors completely blocked the FGF-2-stimulated cell proliferation. We then investigated whether the ERK1/2 pathway was also involved in phosphorylation of p27 at both the Ser10 and Thr187 sites. In the presence of U0126, phosphorylation of p27 at Ser10 was markedly reduced, and simultaneous treatment of cells with U0126 and NSC23766 completely blocked phosphorylation of p27 at Ser10 (Fig. 5A). When the late kinetics was tested, phosphorylation of p27 at Thr187 was blocked by the MEK1/2 inhibitor, and simultaneous treatment of cells with U0126 and NSC23766 completely blocked phosphorylation of p27 at Thr187 (Fig. 5B). We then determined whether Rac1, a downstream effector of Akt, was also involved in the ERK1/2 pathway. Cells were pretreated with either Rac1 inhibitor or MEK1/2 inhibitor for 2 hours and then stimulated with FGF-2 for 4, 8, or 16 hours. MEK1/2 inhibitor did not block Rac1 activation (Fig. 6A), and Rac1 inhibitor did not hamper phosphorylation of ERK1/2 (Fig. 6B), suggesting that the two signaling molecules do not regulate each other, at least in response to FGF-2 stimulation.

Involvement of PI 3-Kinase/Rac1 and ERK1/2 in Phosphorylation of p27 at Ser10 by KIS and at Thr187 by Cdc25A-Activated Cdk2

KIS phosphorylates p27 on Ser10 *in vitro* and *in vivo*²⁷ and Cdk2/cyclin E complex phosphorylates p27 at Thr187.^{35,36} Cdk2 is regulated through posttranslational modification; Cdc25A is one such regulatory protein.^{29,37} We, therefore, addressed a series of questions linking the early signaling event (activation of PI 3-kinase and ERK1/2) and the final outcome (phosphorylation of p27). We first determined whether FGF-2 induced the two key enzymes, KIS and Cdc25A, in CECs. On FGF-2 stimulation, the serum-starved cells promoted KIS production at the protein level in a time-dependent manner beginning at 1 hour; the maximum induction was observed at 4 hours, after which the protein

A

B

FIGURE 6. Independent activation of Rac1 and ERK1/2 in response to FGF-2 stimulation. (A) The serum-starved cells were pretreated with U0126 for 2 hours and then maintained in DMEM with FGF-2 for the designated time. At the end of incubation, a Rac1 pull-down assay was performed as described in Figure 1B. The relative fold differences were then compared with the values of unstimulated CECs, and total Rac1 was used to control protein concentration on immunoblot analysis. (B) The serum-starved cells were pretreated with NSC23766 for 2 hours and then maintained in DMEM with FGF-2 for the designated time. The cell lysates were immunoblotted with anti-p-ERK1/2 and ERK1/2 antibodies. Relative density of immunoblotting bands was determined using a gel documentation system, and data were normalized to nontreated cells (0 hours). Total ERK1/2 was used to control protein concentration on immunoblot analysis. The relative fold differences were compared with the values of unstimulated CECs. The graphs represent the mean $\pm$ SD from three independent experiments.

level gradually decreased (Fig. 7A). Such early induction kinetics of FGF-2 on KIS agree with the phosphorylation kinetics involved in Ser10 of p27.^{11,12} When cells were pretreated with either U0126 or NSC23766, both inhibitors were able to greatly reduce the KIS protein level. Likewise, FGF-2 upregulates expression of Cdc25A in a time-dependent manner according to the kinetics observed for p27 phosphorylation at Thr187 (Fig. 7B); Cdc25A was expressed 2 hours after FGF-2 stimulation, reached a maximum level at 12 hours, and gradually decreased. Both U0126 and NSC23766 blocked the FGF-2 action on Cdc25A production. With these findings, we further confirmed that KIS is involved in phosphorylation of p27 on Ser10, while Cdc25A is responsible for the Cdk2-mediated phosphorylation of p27 at Thr187; a blockade of Cdc25A was pursued using a specific inhibitor of Cdc25A (BN82002), and a siRNA strategy was used for gene knockdown for KIS. Since the KIS sequence in rabbit species was unknown, we cloned the gene, sequenced it, and uploaded it at GenBank (Accession No. GU815100). Specific siRNA was produced and tested for its action on KIS transcription. Figure 8 shows that KIS siRNA greatly reduced the FGF-2-induced KIS at the protein level (53% inhibition). The KIS-specific siRNA greatly

A

FIGURE 7. Induction of KIS and Cdc25A through two distinct Rac1 and ERK1/2 pathways in response to FGF-2 stimulation. The serum-starved cells were pretreated with or without U0126 and NSC23766 for 2 hours and then maintained in DMEM with FGF-2 for the designated time. The cell lysates were immunoblotted with (A) anti-KIS and (B) anti-Cdc25A antibodies. Relative density of immunoblotting bands was determined using a gel documentation system. Actin was used to control protein concentration on immunoblot analysis. The relative fold differences were then compared with the values of unstimulated CECs (0 hours). The graphs represent the mean $\pm$ SD from four independent experiments.

B

blocked phosphorylation of p27 at Ser10 but had no effect on the phosphorylation of p27 at Thr187. We further tested whether there was cross-talk and/or convergence between the phosphorylation site-specific enzymes. The KIS siRNA neither blocked the expression of Cdc25A nor hampered the kinase activity of Cdk2 that is directly involved in phosphorylation of p27 at Thr187 (Fig. 8). On the other hand, Cdc25A inhibitor (BN82002) blocked neither KIS expression nor phosphorylation of p27 at Ser10 (Fig. 9A). The Cdc25A inhibitor completely blocked the phosphorylation of p27 at Thr187 and kinase activity of Cdk2 (Fig. 9B). These findings indicate that there is neither cross-talk nor convergence between the phosphorylation site-specific enzymes. Figure 9B further confirms our previous data¹¹ in which phosphorylation of p27 at Thr187 was not observed within 4 hours after FGF-2 stimulation. Finally, these inhibitory reagents to KIS and Cdc25A were tested for their action on the cell proliferation of CECs. CECs were transfected with siRNA to KIS for 6 hours, and cells were maintained in the experimental medium for an additional 30 hours. When the transfected cells were maintained in the absence of FGF-2, there was no cell proliferation activity (Fig. 10A). When the transfected cells with siRNA to KIS were maintained in FGF-2-containing medium, there was a marked decrease of cell proliferation; the KIS siRNA was able to block the FGF-2 action on cell proliferation, while control siRNA had no effect on the FGF-2-stimulated cell proliferation. Cdc25A inhibitor also partially blocked the cell proliferation activity of FGF-2 (Fig. 10B).

DISCUSSION

Regulation of the G₁/S transition of the cell cycle is important for control of cell proliferation. Among the mechanisms responsible for regulation of G₁/S progression, control of

the p27 level mediated by mitogenic signals appears to be important; the amount of p27 is relatively high in quiescent cells and decreases as cells enter into the cell cycle. The abundance of p27 is predominantly regulated by posttranslational modification, namely, phosphorylation followed by ubiquitin-proteasome-mediated degradation. Phosphorylation of p27 at Thr187 has been extensively investigated and is known to be a prerequisite for binding to Skp2, the F-box component of an SCF (SCF^{Skp2}) ubiquitin ligase complex. Such binding results in degradation of p27 through the ubiquitin-proteasomal machinery.^{38,39} Aside from the phosphorylation of p27 on the Thr187 site, the major phosphorylation site of p27 is Ser10, phosphorylation of which leads to CRM1-mediated nuclear export into the cytosol.⁴⁰ The phosphorylation kinetics of p27 performed in our previous studies^{11,12} demonstrate that phosphorylation of p27 at Ser10 occurred much earlier than did phosphorylation of p27 at Thr187, both in the nuclei. We also showed that pp27Ser10 is exported into the cytoplasm immediately after its phosphorylation and that the molecule was no longer observed 16 hours after FGF-2 stimulation unless it remains in a polyubiquitinated state. On the other hand, the nuclear pp27Thr187 did not even reach the maximum phosphorylation state under identical conditions, confirming that pp27Thr187 and pp27Ser10 are two distinct populations. This notion of two distinct populations of p27 for its phosphorylation was further confirmed with the ubiquitination patterns of pp27Ser10 and pp27Thr187 using their characteristic kinetics; ubiquitination of pp27Ser10 takes place much earlier than that of pp27Thr187. We further suggest that these events with respect to the phosphorylation of p27 are facilitated by the PI 3-kinase pathway in response to FGF-2 stimulation in CECs. However, the eukaryotic cells have developed several layers of redundant regulatory control to access to the G₁/S transition and subsequent mitotic

FIGURE 8. Inhibitory effect of siRNA to KIS on phosphorylation of p27 at the Ser-10 site but not at the Thr-187 site. CECs were transfected with KIS siRNA (50 nM final) designed to target the rabbit KIS gene. After transfection, cells were maintained with DMEM or DMEM with FGF-2 for an additional 30 hours. Total protein extracts were prepared as described in Figure 1B and immunoblotted with designated antibodies. Relative density of immunoblotting bands was determined using a gel documentation system. Actin was used to control protein concentration on immunoblot analysis. The relative fold differences were then compared with the values of unstimulated CECs (D-0). The graphs represent the mean $\pm$ SD from three independent experiments. C, negative control cells, which were transfected with siRNA; IP, immunoprecipitation; IB, immunoblot.

activity. Both PI 3-kinase and ERK1/2 are regarded as the central components of the growth-promoting signaling pathways.

In the present study, we showed that both PI 3-kinase and ERK1/2 signal transduction are independently involved in phosphorylation of p27 at Ser10 and Thr187 sites using the characteristic kinetics involved in the two respective phosphorylation events (Fig. 11). Search for the downstream molecules led us to find that Rac1, as a downstream molecule of Akt, is involved in phosphorylation of p27 at both sites; Rac1 induces the expression of both KIS and Cdc25A. KIS phosphorylates p27 on Ser10, and Cdc25A activates Cdk2, which subsequently phosphorylates p27 on Thr187 residue. Likewise, ERK1/2 upregulates KIS and Cdc25A at the protein level, after which each enzyme en-

gages the pathway-specific phosphorylation of p27 using the characteristic kinetics: phosphorylation of p27 at Ser10 by KIS takes place during early G₁ phase of the cell cycle, and the Thr187 site phosphorylation by Cdc25A-activated Cdk2/cyclin E complex occurs much later. It has been reported that ERK1/2 is directly involved in activating Cdc25A, which in turn increases Cdk2 activity and subsequent G₁/S transition,²⁹ similar to our findings. Other studies using Cdc25A inhibitors showed that Cdc25A is an ERK phosphatase,^{41,42} suggesting that Cdc25A is an upstream regulator of ERK1/2, as opposed to our findings that clearly show ERK1/2 regulates the expression of Cdc25A. There is no explanation for the discrepancy. Nonetheless, we have shown a few new findings in the present study: (1) Rac1 induces expression of the two key proteins (KIS and Cdc25A) that are involved in phosphorylation of p27 in response to mitogenic stimulation; and (2) both PI 3-kinase/Rac1 and ERK1/2 are involved in induction of KIS expression in addition to Cdc25A. These findings, taken together, suggest that CECs employ the two major central pathways (PI 3-kinase and ERK1/2) to execute the identical cellular activity, removal of p27 through the mechanism of phosphorylation, subsequently leading to

FIGURE 9. Inhibitory effect of Cdc25A inhibitor on phosphorylation of p27 at the Thr-187 site, but not at the Ser-10 site. The serum-starved cells were pretreated with or without BN82002 for Cdc25A inhibition for 2 hours and then maintained in DMEM with FGF-2 for the designated time. After incubation, the phosphorylation of p27 at (A) Ser-10 residue and (B) Thr-187 residue was detected with immunoblotting. To detect Cdk2 activity, cell lysates were immunoprecipitated with anti-Cdk2 antibody followed by a protein phosphorylation assay using histone H1 and anti-phosphorylated histone H1 antibody. Actin was used to control protein concentration on immunoblot analysis. The relative fold differences were then compared with the values of unstimulated CECs (D-0). Graphs represent the mean $\pm$ SD from four independent experiments. BN, BN82002.

G₁/S transition and cell proliferation. This event is crucial when corneal endothelium cannot handle the high degree of pathologic insult. We have reported that injury-mediated inflammation induces a rapid and high level of FGF-2 through the action of IL-1 β in CECs.⁴³ The newly synthesized FGF-2, acting as an autocrine cytokine, causes mesenchymal transformation of CECs, leading to corneal fibrosis.⁸ Thus, during the pathologic wound repair process observed in corneal endothelium, G₁/S transition requires parallel signaling from PI 3-kinase and ERK1/2. This combined requirement for PI 3-kinase/Rac1 and ERK1/2 signaling is associated with an increased requirement for removal of p27 through phosphorylation-mediated degradation. Thus, the corneal endothelium has developed at least two layers of redundant regulatory control to easily access G₁/S transition and mitotic activity. Such a mechanism could explain the pathologic wound repair processes leading to fibrosis observed in other tissues in general.

FIGURE 10. Involvement of KIS and Cdc25A in cell proliferation stimulated by FGF-2. (A) CECs transfected with KIS siRNA designed to target the rabbit KIS gene were maintained with DMEM or DMEM with FGF-2 for 36 hours. Cell proliferation was determined as previously described in Figure 1A. Data were normalized to untransfected cells. The graphs represent the mean $\pm$ SEM from four independent experiments ($*P = 0.016$, paired t -test). (B) The serum-starved cells were pretreated with BN82002 for 2 hours and then maintained in DMEM with or without FGF-2 for 24 hours. Cell proliferation was determined and data were normalized to unstimulated cells. The graphs represent the mean $\pm$ SEM from four independent experiments ($**P = 0.03$, paired t -test).

FIGURE 11. Schematic presentation of cell proliferation stimulated by FGF-2 in CECs. Parallel ERK1/2 and PI 3-kinase/Rac1 pathways induced by FGF-2 increase cell proliferation through phosphorylation of p27 at both Ser-10 and Thr-187 residues.

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