Two Populations of p27 Use Differential Kinetics to Phosphorylate Ser-10 and Thr-187 via Phosphatidylinositol 3-Kinase in Response to Fibroblast Growth Factor-2 Stimulation*

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The cyclin-dependent kinase inhibitor p27 regulates cell cycle progression. We investigated whether FGF-2 uses PI 3-kinase to facilitate phosphorylation of p27 on serine 10 (Ser-10) and threonine 187 (Thr-187) and whether the two phosphorylation sites were differentially regulated. FGF-2 stimulation dramatically increased p27 phosphorylation at Ser-10 and Thr-187 using differential kinetics, and the FGF-2-induced p27 phosphorylation was completely blocked at both sites by LY294002. We determined the physical and biochemical interaction of p27 with the Cdk2-cyclin E complex in response to FGF-2 stimulation. Maximal p27 binding to Cdk2-cyclin E occurred at 12 h; the maximal level of p27 phosphorylation at Thr-187 in the ternary complex was observed at 16 h; ubiquitination of the Thr-187-phosphorylated p27 (pp27Thr-187) was observed starting at 12 h and continuing up to 24 h. However, maximum p27 phosphorylation at Ser-10 occurred in the nucleus 6 h after FGF-2 stimulation; maximal export of Ser-10-phosphorylated p27 (pp27Ser-10) occurred 8 h after FGF-2 treatment, and pp27Ser-10 was simultaneously ubiquitinated. We further investigated which of the two phosphorylated p27 was involved in G1/S progression. LY294002 blocked 64% of the cell proliferation stimulated by FGF-2. Use of leptomycin B to block nuclear export of pp27Ser-10 greatly decreased the FGF-2-stimulated cell proliferation (44%), suggesting that phosphorylation of p27 at Ser-10 is the major mechanism for G1/S transition. Our results suggest that differential kinetics are observed in p27 phosphorylation at Ser-10 and Thr-187 and that pp27Thr-187 and pp27Ser-10 may represent two populations of p27 observed in the G1 phase of the cell cycle.

Corneal endothelium is a monolayer of differentiated cells located in the posterior portion of the cornea. Recent work shows that human corneal endothelial cells (CECs) remain arrested in the G1 phase of the cell cycle throughout their life span (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. Alternatively, in a small fraction of wound healing processes, CECs are transformed into mesenchymal cells that subsequently produce 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 transmission. One clinical example of corneal fibrosis observed in corneal endothelium is the development of a retrocorneal fibrous membrane (RCFM) in Descemet’s membrane (the basement membrane of corneal endothelium) (3, 4). In RCFM, CECs are converted to fibroblast-like cells: the contact-inhibited monolayers of CECs are 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. Our in vitro model 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 CECs (7–9). We reported that, among the phenotypes altered during EMT, FGF-2 directly regulates cell cycle progression through the action of phosphatidylinositol (PI) 3-kinase and that this phenotype alteration leads to a marked stimulation of cell proliferation (7), as opposed to the G1-arrested CECs. We further reported that FGF-2 phosphorylates p27kip1 (p27) at Thr-187 via the PI 3-kinase pathway and that the Thr-187-phosphorylated p27 (pp27Thr-187) is degraded in the nuclei (7).

The PI 3-kinase pathway is centrally involved in cell proliferation and survival, and the importance of p27 as a regulator of PI 3-kinase-mediated cell cycle progression is well established (10–12). Protein kinase B (commonly known as Akt) is an important downstream effector of the PI 3-kinase pathway.

* The abbreviations used are: CECs, corneal endothelial cells; p27kip1, p27; PI 3-kinase, phosphatidylinositol 3-kinase; RCFM, retrocorneal fibrous membrane; FGF-2, fibroblast growth factor-2; Cdk, cyclin-dependent kinase; hKIS, human kinase-interacting stathmin; LMB, leptomycin B; EMT, endothelial mesenchymal transformation; FITC, fluorescein isothiocyanate; PCNA, proliferating cell nuclear antigen; BrdU, bromodeoxyuridine; DTT, dithiothreitol; DAPI, Dimeleuro’s modified Eagle’s medium; DAPI, 4′,6-diamidino-2-phenylindole; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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Recently Akt has been shown to directly phosphorylate p27 on Thr-157 in breast cancer cell lines; the Thr-157 site is present within the nuclear localization signal of p27. Thus, phosphorylation of Thr-157 causes retention of p27 in the cytoplasm (13–15). Akt also phosphorylates Thr-198, promoting 14-3-3 association and cytoplasmic retention; the Thr-198-phosphorylated p27 exists only in the cytoplasm (16). Among the multiple sites of phosphorylation of p27, the major phosphorylation site is Ser-10 (17), known to be phosphorylated by either human kinase-interacting stathmin (hKIS), the nuclear serine-threonine kinase (18) or Akt (16). It is likely that phosphorylation of p27 on the Ser-10 residue by either enzyme takes place in the nuclei, after which the Ser-10-phosphorylated p27 (pp27Ser-10) is exported out of the nucleus to the cytoplasm via the CRM1-dependent pathway (19) and is further involved in protein stability (20). Of great interest, all of the phosphorylated p27s, other than pp27Thr-187, are primarily localized in the cytoplasm. Such cytoplasmic localization of p27 prevents it from inhibiting the cell cycle progression in the nucleus.

Although phosphorylation as a major post-translational modification of p27 has been studied extensively, the means by which p27 phosphorylation is coordinated at multiple sites remains unclear. Does p27 undergo phosphorylation at all sites per molecule? If so, are the events of p27 phosphorylation sequential, with each one leading to another phosphorylation? Hara et al. (21) reported that pp27Ser-10 is exported out of the cytoplasm at the G0–G1 transition, whereas Cdk2-mediated p27 phosphorylation at Thr-187 results in complex formation with ubiquitin ligase SCFSkp2, leading to proteasome-mediated p27 degradation in the nucleus of proliferating cells during G1-S transition and S phase. Degradation of p27 at the G0–G1 transition is also known to be independent of Skp2 and to occur in the cytoplasm (22). This information, thus, suggests differential regulation of the two populations of the phosphorylated p27 (pp27Thr-187 and pp27Ser-10) at different phases of the cell cycle. These data further indicate that at least the two populations of p27 respectively regulate the different stage of the cell cycle: pp27Ser-10 regulates via the nuclear export of p27 during reentry of quiescent cells into the cell cycle, while pp27Thr-187 regulates the cell cycle during late G1-S transition. Most importantly, it has not been determined whether the different phosphorylation sites of p27 were sequentially phosphorylated or whether there were two populations of p27 that are respectively phosphorylated. Furthermore, the method by which p27 phosphorylation at respective sites is regulated at a single cell level has not been studied.

Most studies of the negative activity of p27 during cell cycle have employed the strategy of overexpressing exogenous mutant proteins in cultured cells. Such transfection conditions could not determine the kinetics of p27 phosphorylation. We, therefore, determined the kinetics of p27 phosphorylation at Thr-187 and Ser-10 sites in response to mitogenic signals at a single cell level. In the present study, we show that phosphorylation of Ser-10 occurred much earlier than phosphorylation of Thr-187 (maximum phosphorylation time: 6 h versus 16 h following FGF-2 stimulation). We also showed that pp27Ser-10 was no longer observed 12 h after FGF-2 stimulation, whereas pp27Thr-187 had only reached half its maximum level of phosphorylation, suggesting that the two phosphorylated p27s are two different populations. Thus, we have shown that at least two respective populations of p27 undergo phosphorylation and that each of these two populations of p27 functions at a different stage of the G1 phase of the cell cycle in response to mitogenic signals.

**EXPERIMENTAL PROCEDURES**

**Materials**—LY294002 and monoclonal antibodies against β-actin and proliferating cell nuclear antigen (PCNA) were obtained from Sigma-Aldrich. Anti-Cyclin E, anti-phospho-histone H1 antibody, and purified histone H1 protein were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-p27 antibody and anti-lamin B antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pp27Ser-10 and anti-pp27Thr-187 were obtained from Zymed Laboratories Inc. (South San Francisco, CA). Leptomycin B and anti-ubiquitin antibody were obtained from Biomol (Plymouth Meeting, PA). FGF-2 and anti-Cdk2 antibody were obtained from Calbiochem and BD Biosciences (San Jose, CA), respectively. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Chemicon (Temecula, CA). Mounting solution and peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories Inc. (Burlingame, CA).

**Cell Culture**—Rabbit eyes were purchased from Pel Freez Biologicals (Rogers, AR). Isolation and establishment of rabbit CECs were performed as previously described (23). Briefly, the corneal endothelium–Descemet’s membrane complex was treated with 0.2% collagenase and 0.05% hyaluronidase (Worthington Biochemical, Lakewood, NJ) for 90 min at 37 °C. Primary cultured cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 15% fetal bovine serum (Omega Scientific, Tarzana, CA) and 50 µg/ml of gentamicin (DMEM-15) in a 5% CO2 incubator. For subculture, confluent primary cultures were treated with 0.05% trypsin and 5 mM EDTA in phosphate-buffered saline for 5 min. First passage CECs maintained in DMEM-15 were used for all experiments. Heparin (10 µg/ml) was added to cell cultures treated with FGF-2 (10 ng/ml) since our previous study showed that CECs require the addition of supplemental heparin for FGF-2 activity to occur (5). In some experiments, pharmacologic inhibitors were used in the presence of FGF-2 stimulation: LY294002 (20 μM), or leptomycin B (10 ng/ml).

**Cell Proliferation Assays**—Cell proliferation was assayed by the bromodeoxyuridine (BrdU) cell proliferation assay kit (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer’s protocol. Cells were seeded in 6-well plates at a concentration of 1 × 10⁵ cells/well. On the following day, the medium was changed from DMEM-15 to DMEM, and the cells were incubated for 30 h. The serum-starved cells were then subjected to the respective experimental conditions for 24 h, with each culture containing BrdU (10 ng/ml). Cells were then fixed and permeabilized, and the DNA was denatured by treatment with fixative/denaturing solution. Detector peroxidase-conjugated anti-BrdU monoclonal antibody was then used, and the color reaction was developed using the chromogenic sub-
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strate tetramethylbenzidine. The product was quantified using a spectrophotometric plate reader at dual wavelengths of 370 and 492 nm.

Cell Cycle Analysis—CECs were synchronized by starvation in DMEM and released after 30 h by adding DMEM containing FGF-2. PCNA immunofluorescent staining was performed as described previously (24). We counted the number of PCNA-stained cells per ten 400 × viewing fields and determined the percentage of labeled nuclei in a total of over 300 nuclei using four coverslips per experimental condition.

Cytoplasmic and Nuclear Protein Extractions—CECs cultured in each culture condition on 100-mm tissue culture dishes were washed twice with sterile PBS, followed by incubation in an Enzyme-free Cell Dissociation Solution (Chemicon) for 3 min at room temperature. Cells were detached by scraping, transferred to microcentrifuge tubes, and pelleted at 5,000 × g for 1 min. Nuclear and cytoplasmic proteins were extracted using a Nuclear Extraction kit containing cytoplasmic lysis buffer and nuclear lysis buffer (Chemicon), according to the manufacturer’s instructions. Briefly, the harvested cells were resuspended in two cell pellet volumes of cold cytoplasmic lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 mM dithiothreitol (DTT), and allowed to swell on ice for 15 min. IGEPAL CA-630 (Sigma-Aldrich) was then added to a final concentration of 0.1%, and the swollen cells were incubated an additional 5 min on ice followed by homogenization with a 27-gauge needle. Nuclei were pelleted at 8000 × g for 20 min at 4 °C. The supernatant containing the cytosolic portion was transferred to a fresh tube and stored at −80 °C until further use. The remaining pellet containing the nuclear portion was washed by centrifugation with cold nuclear extraction buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 mM DTT and then resuspended in 2/3 of the original cell pellet volume of cold nuclear extraction buffer. Nuclei were disrupted by drawing and ejecting 10 times, using a syringe and a 27-gauge needle.

Nuclear proteins were extracted at 4 °C for 60 min, with gentle agitation using an orbital shaker. The nuclear protein extract was clarified by centrifugation at 16,000 × g for 5 min at 4 °C and then dialyzed with the Slide-A-Lyzer MINI Dialysis Unit, 7K MWCO (Pierce). Nuclear and cytoplasmic protein concentrations were determined using the Bradford reagent (Bio-Rad) and then dialyzed with the Slide-A-Lyzer MINI Dialysis Unit, 7K MWCO (Pierce). Nuclear and cytoplasmic protein concentrations were determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. To verify the purity of the fractions, 15 μg of nuclear or cytoplasmic proteins were immunoblotted with lamin B and α-tubulin antibodies. These nuclear and cytoplasmic proteins were used for immunoprecipitation and immunoblotting.

Protein Preparation, Protein Assay, SDS-PAGE, Immunoprecipitation, Western Blotting Analysis, Immunofluorescent Analysis, and Confocal Microscopy—All details of methods and procedures have been presented previously (24–26). The following concentrations of gel were used to separate proteins: a 12.5% gel for p27, Cdk2, and phosphohistone H1, and a 10% gel for cyclin E, actin, and lamin B.

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was carried out as described previously (8) with some modifications. CECs were lysed in a rehydration buffer containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.001% bromophenol blue, and 0.2% ampholyte (pH 3–10). For the one-dimensional isoelectric focusing gel, cell lysates containing 200 μg of protein were applied to an IPGStrip (11 cm, pH 4–7; Bio-Rad). The strips were covered with immersion oil to prevent samples from evaporation and rehydrated at room temperature overnight. The immersion oil was then removed from the surface. One-dimensional isoelectric focusing gel electrophoresis was carried out using a Protean I-Blotting Apparatus (Bio-Rad) for 20 min at 0 V, 3 h at 250 V, and 3 h at 8000 V. After focusing, the IPG-Strips were soaked for 15 min in equilibration buffer (0.375 M Tris-HCl, pH 8.8, 6.0 M urea, 2% SDS, and 2% DTT) at room temperature with gentle shaking, then for 10 min in the same buffer containing 2.5% iodoacetamide. The separated and equilibrated proteins were resolved in the second dimension by standard PAGE on a 4–16% gradient gel (Bio-Rad) and subjected to immunoblot analysis.

Protein Phosphorylation Assay in Vitro—Phosphorylation of histone H1 by activated Cdk2-associated kinase activity was carried out as described previously (27) with some modifications. Immune complexes precipitated with anti-p27, anti-pp27Ser-10, and anti-pp27Thr-187 antibody were washed two times with phosphate-buffered saline and resuspended in 50 μl of kinase buffer (50 mM Tris-HCl, pH 7.2, 10 mM MgCl₂, 1 mM DTT) containing 100 μM ATP. One microgram of commer-

FIGURE 1. Effect of FGF-2 on cell proliferation and cell cycle. A, first passage cells were plated in a 4-well chamber at a cell density of 1 × 10⁴ and maintained in DMEM-15. When cells reached ~70% confluence, they were starved of serum for 30 h. The serum-starved cells were treated with FGF-2 for 4, 16, 20, and 24 h and then maintained in DMEM-0 for up to 24 h. The cultured cells were fixed and labeled with anti-PCNA and DAPI, respectively. Total cell number and PCNA-positive cells were counted. S-phase entry is presented as the percentage of PCNA-positive cells to total cell number. Relative fold differences were then compared with the values of unstimulated CECs (0 h). B, to establish the serum starvation condition for maintaining cells in the G₀ phase of the cell cycle, Cdk2 activity was detected. Cells were maintained under serum-free conditions for up to 36 h, and cell lysates recovered from each time point were immunoprecipitated with anti-Cdk2 antibody followed by a protein phosphorylation assay using histone H1 and anti-phosphorylated histone H1 antibody as described under “Experimental Procedures.” Cdk2 activity was no longer observed in cells maintained under serum-free conditions for 30 h. The results represent data obtained in three independent experiments.
cally purified histone H1 (Upstate Biotechnology Inc.) was then added to the immunoprecipitates, and the mixtures were incubated for 30 min at 30 °C. The reaction mixtures were resolved by SDS-PAGE, and phosphorylated histone H1 was detected by immunoblotting with anti-phosphohistone H1-specific antibody. For positive control experiments, cell lysates were immunoprecipitated with anti-Cdk2 antibody, and the immune complex was assayed for Cdk2-associated kinase activity using histone H1 as a substrate as stated above.

RESULTS

Effect of FGF-2 on Cell Proliferation and Cell Cycle—In our previous study (7), we reported that CECs required prolonged and continuous treatment with FGF-2 for cell proliferation. Because one goal of this study is to determine the kinetics of p27 phosphorylation, it is important to confirm the experimental conditions previously used in which CECs were treated with FGF-2 for a designated time and were further maintained under serum-free conditions for a total incubation time of 24 h. We confirmed the characteristic cell proliferation pattern of CECs stimulated by FGF-2 using BrdU incorporation into DNA (data not shown). G1/S phase transition was determined with PCNA staining in CECs; Fig. 1A demonstrated that CEC progression to S phase in response to FGF-2 stimulation took 16 h. Another control experiment was performed to determine the time period for CECs to re-enter G0 phase following serum starvation. Fig. 1B demonstrated that CECs maintained in serum-free medium for 24 h still possess Cdk2 activity, confirming that our experimental conditions were adequate to pursue the kinetic study. On the other hand, in light of the Cdk2 activity observed in cells starved of serum for 24 h, we chose to starve the cells of serum for 30 h before stimulating the cells with FGF-2 in the following experiments.

Effect of FGF-2 on Phosphorylation of p27—Phosphorylation kinetics were determined in CECs treated with FGF-2 for 4, 8, 12, 16, or 24 h. The total protein level of p27 was gradually decreased in a time-dependent manner, reaching 80% of the p27 level observed in the serum-starved cells, confirming the findings of Fig. 2A. We, therefore, used the
4-h treatment time for the study of pp27 Ser-10 in the following experiments.

The differential phosphorylation kinetics were further confirmed using immunocytochemistry and two-dimensional gel electrophoresis followed by immunoblot analysis. When cells were stained with antibody specific to pp27 Thr-187, positive staining of pp27 Thr-187 was barely observed in the nuclei of cells treated with FGF-2 for 8 h, after which the staining potential was enhanced for up to 16 h treatment of cells with FGF-2 (Fig. 3A). Positive staining with anti-pp27 Thr-187 antibody was greatly decreased in cells treated with FGF-2 for 24 h. On the other hand, the specific antibody to pp27 Ser-10 could not be used for immunocytochemical studies. Two-dimensional gel electrophoresis followed by immunoblot analysis with antibodies to pp27 (pp27 Thr-187 or pp27 Ser-10) was, therefore, employed to distinguish the differential kinetics of phosphorylation patterns of pp27. When the spots separated by two-dimensional electrophoresis were stained with anti-pp27 antibody, cells maintained in the absence of serum showed only the p27 spot (Fig. 3B). When cells were stimulated with FGF-2 for up to 24 h, the intensity of the p27 spots was decreased in a time-dependent manner, while the intensity of the phosphorylated p27 spots was not greatly altered throughout the time studied. When these immunoreactive spots with anti-p27 antibody complex precipitated with anti-p27 antibody showed that it contained Cdk2 and cyclin E; association of p27 with both G1 proteins (Cdk2 and cyclin E) began 8 h after stimulation with FGF-2, and maximal association was observed in cells stimulated with FGF-2 for 12 h (Fig. 4A). Likewise, the immune complex precipitated with anti-Cdk2 antibody also contained cyclin E and p27 (Fig. 4B). The association of Cdk2 and cyclin E took place much earlier than the association of Cdk2 and p27. Both Fig. 4A and Fig. 4B showed that it took 8 h for p27 to bind to the Cdk2-cyclin E complex. Fig. 4B further demonstrated that p27 present in the ternary complex obtained from the cells stimulated with FGF-2 for 12 h was phosphorylated at the residue of Thr-187, reaching a maximum phosphorylation level during the next 4 h. Interestingly, p27 in the immune complex obtained from cells treated with FGF-2 for 8 h was barely phosphorylated at the Thr-187 residue. On the other hand, the immune complex precipitated with Cdk2 antibody did not contain pp27 Ser-10, even during the early stage of FGF-2-mediated stimulation (Fig. 4B). These findings clearly demonstrated that the events of physical and biochemical interaction among G1 proteins progress sequentially; first is the association of Cdk2 and cyclin E, followed by binding of p27 to the Cdk2-cyclin E complex, and finally phosphorylation of p27 at Thr-187 in the ternary complex.
Because association of p27 can turn off the Cdk2-cyclin E complex, we tested the activity of Cdk2 of the ternary complex formed with Cdk2-cyclin E and p27. When cells were stimulated with FGF-2 for up to 24 h, the immune complex was precipitated with either Cdk2 or p27 antibody. Histone H1 was then added to the immune complex for an in vitro kinase assay followed by immunoblot analysis with anti-phosphorylated histone H1 antibody. The presence of phosphorylated histone H1 confirmed the Cdk2 activity of the ternary complex (Fig. 5A). When anti-pp27Thr-187 antibody was used to precipitate the ternary complex, the complex containing Cdk2-cyclin E and pp27Thr-187 also demonstrated Cdk2 activity (Fig. 5B). Lack of a phosphorylated histone H1 band in the immune complex containing pp27Ser-10 confirms that Cdk2 activity is related only to the phosphorylation of p27 at Thr-187 (Fig. 5C). Of interest, when the immune complex was precipitated with either anti-p27 or anti-pp27Thr-187 antibody, the maximal Cdk2 activity was observed in cells stimulated for 16 h with FGF-2, at which time phosphorylation of Thr-187 was also maximum (Fig. 4B). We further confirmed these findings using nuclear fractions. The purity of the subcellular fractions was confirmed with respective nuclear (lamin B) and cytoplasmic (α-tubulin) proteins. When nuclear fractions were precipitated with either anti-Cdk2 or anti-p27 antibody, the ternary complex phosphorylated histone H1, suggestive of Cdk2 activity (Fig. 6). The immune complex precipitated with anti-pp27Thr-187 antibody also demonstrated Cdk2 activity from 4 h of stimulation, which is earlier than the Cdk2 activity observed in the total cell lysates (Fig. 5B). Maximal activity was reached at 16 h after FGF-2 stimulation. Unlike the Cdk2 activity observed in the total cell lysates, the band intensity of the phosphorylated histone H1 was gradually increased in the nuclear fractions in a time-dependent manner. Similar to the findings observed in Fig. 5C, the immune complex containing pp27Ser-10 was not able to phosphorylate histone H1 (Fig. 6). The protein levels of Cdk2, p27, pp27Thr-187, and pp27Ser-10 in the respective immune complexes were similar to those determined by immunoblot analysis.

**Involvement of PI 3-Kinase in Phosphorylation of p27 at Thr-187 and Ser-10 in Response to FGF-2 Stimulation**—We then determined whether FGF-2 used the PI 3-kinase pathway for phosphorylation of p27. Our previous study showed that FGF-2 phosphorylates p27 at Thr-187 via the PI 3-kinase pathway and

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**FIGURE 4.** Effect of FGF-2 on the physical association of p27 with the Cdk2-cyclin E complex. The serum-starved cells were stimulated with FGF-2 for the indicated time, as previously described in Fig. 2A, after which cell lysates were immunoprecipitated with anti-p27 (A) or anti-Cdk2 (B) antibodies. The immunoprecipitated complexes were then immunoblotted with the designated antibody. β-Actin was used to control the protein concentration on immunoprecipitation. The results represent data obtained in three independent experiments.

**FIGURE 5.** Phosphorylation of histone H1 by the Cdk2-cyclin E/pp27Thr-187 complex. The serum-starved cells were stimulated with FGF-2 for the indicated time as described in the legend for Fig. 2A, after which the cells were lysed. Lysates were immunoprecipitated with anti-Cdk2 or p27 (A), anti-pp27Thr-187 (B), or anti-pp27Ser-10 (C) antibodies. The phosphorylation of histone H1 was determined using the anti-phospho-histone H1 antibody as previously described in Fig. 1B. The reaction mixtures were resolved by SDS-PAGE, and then phosphorylated histone H1 was detected by immunoblotting with anti-phospho-histone H1-specific antibody. The immune complex was subjected to immunoblot analysis with respective antibodies to confirm the efficiency of immunoprecipitation. The data are representative of four experiments.
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![Image](https://example.com/image.png)

**FIGURE 6. Phosphorylation of histone H1 by nuclear complexes of Cdk2-cyclin E/pp27Thr187.** Serum-starved cells were stimulated with FGF-2 for the indicated time as described in the legend for Fig. 2A, after which the cells were lysed with cytoplasmic lysis buffer. The nuclei were harvested by centrifugation and then treated with nuclear extraction buffer to rupture the nuclei. Nuclear extracts were immunoprecipitated and then immunoblotted with designated antibodies. The phosphorylation of histone H1 was determined using the anti-phospho-histone antibody. The phosphorylation of histone H1 by nuclear complexes of Cdk2-cyclin E/pp27Thr187 was quantified by densitometry, and the data are representative of four experiments.

of the inhibitor (Fig. 4B). Formation of the ternary complex was greatly reduced in cells treated with FGF-2 for 12 h when cells were pretreated with LY294002 (Fig. 7C). The PI 3-kinase inhibitor further blocked the formation of the ternary complex in cells treated with FGF-2 for 16 h. On the other hand, association of Cdk2 and cyclin E was not disturbed by LY294002 (Fig. 7C). These findings suggest that association of p27 to the Cdk2-cyclin E complex is at least partly facilitated by PI 3-kinase. When the immune complex, precipitated with anti-Cdk2 antibody, was further tested with anti-pp27Thr187 antibody, the phosphorylated p27 at Thr-187 was no longer observed in the presence of LY294002 (Fig. 7C). These findings together confirm that PI 3-kinase is involved not only in phosphorylation of p27 at both Ser-10 and Thr-187 sites but in physical association of p27 to the Cdk2-cyclin E complex at least in part.

**Role of the Ser-10-phosphorylated p27—Ser-10 is reported as the major phosphorylation site of p27 (17) and is known to be involved in nuclear export of p27.** Our kinetic study of phosphorylated p27 at the Ser-10 residue showed that the molecule no longer exists 12 h after FGF-2 stimulation (Fig. 2). To confirm the findings, the kinetics of nuclear export and removal of pp27Ser-10 were further examined using nuclear and cytoplasmic fractions. The purities of the nuclear and cytoplasmic fractions were confirmed with lamin B and α-tubulin, respectively. Fig. 8 clearly demonstrated that phosphorylation of p27 at the Ser-10 residue took place in the nuclei in response to FGF-2 stimulation, and the amount of pp27Ser-10 was gradually increased in a time-dependent manner for up to 6 h. By 12 h after stimulation with FGF-2, pp27Ser-10 was no longer present in the nucleus (Fig. 8). On the other hand, nuclear export of the molecule was detected in the cells treated with FGF-2 for 2 h, and a high amount of pp27Ser-10 was observed in the cytoplasmic fractions until 12 h after FGF-2 stimulation (Fig. 8). The kinetic study suggests that the phosphorylated p27 at the Ser-10 site is transported out of the nucleus between 4 and 12 h after stimulation with FGF-2, and the molecule completely disappears between 12 and 16 h. This kinetic study further confirms that pp27Ser-10 and pp27Thr-187 are apparently two distinct populations of p27. Of great interest, no p27 is present in the cytoplasm of the cells stimulated with FGF-2 for 1 h; p27 is solely localized in the nucleus.

We further confirmed the notion of the two distinct populations of p27 with respect to phosphorylation sites by investigating the degradation kinetics. When the nuclear fraction was precipitated with anti-ubiquitin antibody followed by immunoblot analysis using anti-p27 antibody, polyubiquitinated p27 was accumulated in a time-dependent manner; the ubiquitinated p27 was substantially observed at 12 h following FGF-2 stimulation and reached the maximal level of ubiquitination at 24 h (Fig. 9). We further demonstrated that the polyubiquitinated p27 is phosphorylated at Thr-187 site, but not at Ser-10 site, using immunoprecipitation with either anti-p27Thr-187 or pp27Ser-10 antibody followed by immunoblotting with anti-ubiquitin antibody. On the other hand, the cytoplasmic fraction showed an early ubiquitination event of p27; within 4 h following FGF-2 stimulation, p27 was polyubiquitinated, reaching
degradation it in the nuclei (7). Cells were pretreated with LY294002 for 20 h followed by stimulation with FGF-2 for either 0 or 16 h, at which time maximum phosphorylation of the Ser-10 residue or the Thr-187 residue is respectively observed (Fig. 2A). Phosphorylation of p27 at Ser-10 after 4 h of stimulation with FGF-2 was completely blocked by LY294002, and phosphorylation of p27 at Thr-187 after 16 h of stimulation with FGF-2 was also completely blocked by LY294002 (Fig. 7A). Additional experiments were performed with the cells simultaneously treated with FGF-2 and LY294002; these experiments demonstrated the identical results (data not shown). The inhibitory activity of LY294002 on phosphorylation of p27 at the Thr-187 and Ser-10 sites was further confirmed with two-dimensional gel electrophoresis and immunoblot analysis using a specific antibody to pp27Thr187 or pp27Ser10 (Fig. 7B). The immunoreactive spots of both pp27Ser10 and pp27Thr187 were no longer observed in the presence of the inhibitor to PI 3-kinase. These data clearly indicate that phosphorylation of p27, at least at Thr-187 and Ser-10 sites, is mediated by the PI 3-kinase pathway in response to FGF-2 stimulation. We further investigated whether the association of p27 to the Cdk2-cyclin E complex was also facilitated by PI 3-kinase. Cells were pretreated with LY294002 for 20 h followed by stimulation with FGF-2 for up to 24 h; the binding pattern of p27 to the Cdk2-cyclin E was then compared with that observed in the absence of the inhibitor (Fig. 4B). Formation of the ternary complex was greatly reduced in cells treated with FGF-2 for 12 h when cells were pretreated with LY294002 (Fig. 7C). The PI 3-kinase inhibitor further blocked the formation of the ternary complex in cells treated with FGF-2 for 16 h. On the other hand, association of Cdk2 and cyclin E was not disturbed by LY294002 (Fig. 7C). These findings suggest that association of p27 to the Cdk2-cyclin E complex is at least partly facilitated by PI 3-kinase. When the immune complex, precipitated with anti-Cdk2 antibody, was further tested with anti-pp27Thr187 antibody, the phosphorylated p27 at Thr-187 was no longer observed in the presence of LY294002 (Fig. 7C). These findings together confirm that PI 3-kinase is involved not only in phosphorylation of p27 at both Ser-10 and Thr-187 sites but in physical association of p27 to the Cdk2-cyclin E complex at least in part.

**Role of the Ser-10-phosphorylated p27—Ser-10 is reported as the major phosphorylation site of p27 (17) and is known to be involved in nuclear export of p27.** Our kinetic study of phosphorylated p27 at the Ser-10 residue showed that the molecule no longer exists 12 h after FGF-2 stimulation (Fig. 2). To confirm the findings, the kinetics of nuclear export and removal of pp27Ser-10 were further examined using nuclear and cytoplasmic fractions. The purities of the nuclear and cytoplasmic fractions were confirmed with lamin B and α-tubulin, respectively. Fig. 8 clearly demonstrated that phosphorylation of p27 at the Ser-10 residue took place in the nuclei in response to FGF-2 stimulation, and the amount of pp27Ser-10 was gradually increased in a time-dependent manner for up to 6 h. By 12 h after stimulation with FGF-2, pp27Ser-10 was no longer present in the nucleus (Fig. 8). On the other hand, nuclear export of the molecule was detected in the cells treated with FGF-2 for 2 h, and a high amount of pp27Ser-10 was observed in the cytoplasmic fractions until 12 h after FGF-2 stimulation (Fig. 8). The kinetic study suggests that the phosphorylated p27 at the Ser-10 site is transported out of the nucleus between 4 and 12 h after stimulation with FGF-2, and the molecule completely disappears between 12 and 16 h. This kinetic study further confirms that pp27Ser-10 and pp27Thr-187 are apparently two distinct populations of p27. Of great interest, no p27 is present in the cytoplasm of the cells stimulated with FGF-2 for 1 h; p27 is solely localized in the nucleus.

We further confirmed the notion of the two distinct populations of p27 with respect to phosphorylation sites by investigating the degradation kinetics. When the nuclear fraction was precipitated with anti-ubiquitin antibody followed by immunoblot analysis using anti-p27 antibody, polyubiquitinated p27 was accumulated in a time-dependent manner; the ubiquitinated p27 was substantially observed at 12 h following FGF-2 stimulation and reached the maximal level of ubiquitination at 24 h (Fig. 9). We further demonstrated that the polyubiquitinated p27 is phosphorylated at Thr-187 site, but not at Ser-10 site, using immunoprecipitation with either anti-pp27Thr-187 or pp27Ser-10 antibody followed by immunoblotting with anti-ubiquitin antibody. On the other hand, the cytoplasmic fraction showed an early ubiquitination event of p27; within 4 h following FGF-2 stimulation, p27 was polyubiquitinated, reaching
maximal activity at 8 h, after which the ubiquitination event was gradually decreased. Unlike the nuclear fraction, the ubiquitinated p27 in the cytoplasmic fraction is phosphorylated at the Ser-10 site, but not at the Thr-187 site. Interestingly, ubiquitination kinetics of both pp27Thr-187 and pp27Ser-10 followed their characteristic time courses (early event for pp27Ser-10; late event for pp27Thr-187) but ubiquitination required slightly prolonged kinetics for both phosphorylated p27s. To confirm that the polyubiquitinated protein is p27 itself rather than an associated protein, we used the anti-p27 antibody to show the immunoblot analysis of total proteins obtained from either nuclear or cytoplasmic fractions. Fig. 9 demonstrates that these ladders are indeed p27 bands (Fig. 9).

We finally investigated whether phosphorylation of p27 at Ser-10 is involved in G1/S progression. Our results demonstrated that removal of pp27Ser-10 took place between 12 and 16 h after stimulation with FGF-2 (Figs. 2 and 8) and that CECs required 16 h for G1/S transition (Fig. 1). Thus, it is likely that phosphorylation of p27 at Ser-10 is involved in G1/S transition, as is phosphorylation of p27 at Thr-187. Because the phosphorylated p27 at Ser-10 needs to be exported out of the nuclei, leptomycin B (LMB) was used to block the CRM-1-mediated nuclear export of pp27Ser-10. Cell proliferation was measured using BrdU incorporation into DNA; cells maintained under a serum-free condition (control) demonstrated very little BrdU incorporation (Fig. 10). When cells were stimulated with FGF-2 for 4 h, a marked increase in BrdU incorporation occurred, whereas in cells that were simultaneously treated with FGF-2 and LMB, BrdU incorporation was inhibited by 50%. When cells were stimulated with FGF-2 for 24 h, a 3.7-fold increase in BrdU incorporation was seen compared with the value observed in cells stimulated for 4 h. Interestingly, LMB blocked incorporation of BrdU into DNA mediated by FGF-2 by 44%, whereas LY294002 blocked BrdU incorporation by 64%. Because phosphorylation of p27 at both Thr-187 and Ser-10 sites is mediated by the PI 3-kinase pathway in response to FGF-2 stimulation, the inhibitory level observed with LY294002 may be caused by the inhibited phosphorylation of both sites. On the other hand, the inhibitory level achieved by LMB (44%) is solely caused by the phosphorylation of p27 at the
Ser-10 site. Therefore, the difference between LY294002- and LMB-mediated inhibition on FGF-2-stimulated cell cycle progression (20%) may be caused by the phosphorylation of p27 at Thr-187. It is likely that phosphorylation of p27 at the Ser-10 site regulates the G1/S transition much more than phosphorylation at Thr-187 did.

DISCUSSION

Regulation of the G1/S transition of the cell cycle is considered important for control of cell proliferation. Among the mechanisms responsible for regulation of G1 cyclin-associated kinase activity, control of the p27 level mediated by mitogenic signals appears 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 post-translational modification. Phosphorylation of p27 on Thr-187 has been extensively investigated and is known to be prerequisite for binding to Skp2, the F-box component of an SCF (Skp1-Cul1-F-box protein) ubiquitin ligase complex. Such binding results in degradation of p27 through the ubiquitin-proteasomal machinery (32, 33). In addition to phosphorylation of p27 on Thr-187 site, the major phosphorylation site on p27 is Ser-10, phosphorylation of which leads to CRM1-mediated nuclear export and increased protein stability in the cytosol (34, 35). Moreover, pp27Ser-10 is exported out to the cytoplasm at the G0-G1 transition, while Cdk2-mediated phosphorylation of p27 at Thr-187 and subsequent degradation of pp27Thr-187 via the proteasome pathway occurs in the nucleus of proliferating cells during G1-S transition and S phase (21). Degradation of p27 at the G0-G1 transition is known to be independent of Skp2 and to occur in the cytoplasm. This information taken together suggests that the two populations of the phosphorylated p27 (pp27Thr-187 and pp27Ser-10) may be differentially regulated at different phases of the cell cycle. These data further indicate that at least two populations of p27, respectively, regulate the different stages of the cell cycle; pp27Ser-10 regulates the early stage of G1 of the cell cycle via the nuclear export of p27, while pp27Thr-187 regulates the cell cycle during the late G1-S transition.

Most studies that have evaluated the regulatory mechanism of p27 expression and cellular activity of the phosphorylated p27 have employed the strategy of overexpressing exogenous mutant proteins in cultured cells. Such transfection conditions could not determine the kinetics of p27 phosphorylation. Our attempt to determine whether different phosphorylation sites of p27, at least the Thr-187 and Ser-10 residues, were sequentially phosphorylated or whether there were two populations of p27 that were respectively phosphorylated led us to the first direct demonstration that there are at least two populations of p27 that undergo respective phosphorylation on the Thr-187 and Ser-10 residues at a single cell level. The kinetic studies demonstrated that phosphorylation of Ser-10 occurred much earlier than did phosphorylation of Thr-187. We also showed that pp27Ser-10 was no longer observed 12 h after FGF-2 stimulation, whereas pp27Thr-187 did not even reach the maximum phosphorylation state under the identical conditions, confirming that pp27Thr-187 and pp27Ser-10 are two distinct populations. This notion of two distinct populations of p27 for its phosphorylation was further confirmed with the ubiquitination patterns of pp27Ser-10 and pp27Thr-187: ubiquitination of pp27Ser-10 was observed as early as 4 h after FGF-2 stimulation in the cytoplasmic fraction; the maximal rate of the ubiquitination of this class p27 was maintained up to 12 h after FGF-2 stimulation, after which ubiquitinated pp27Ser-10 is barely present in the cytoplasmic fraction. On the other hand, ubiquitinated pp27Thr-187 was only observed in the nuclear fractions of the cells treated with FGF-2 for at least 12 h, and ubiquitination of pp27Thr-187 was strongly maintained in cells treated with FGF-2 for 24 h. The differential ubiquitination kinetics and distinct subcellular localization for ubiquitination events observed in pp27Thr-187 and pp27Ser-10 further confirmed the two distinct populations of p27 existing in response to mitogenic signal.
We also explored the role of FGF-2 on phosphorylation of p27 at Thr-187. The physical and biochemical interactions between the Cdk2-cyclin E complex and p27 are essential for phosphorylation of p27 at the Thr-187 residue (29). Association of p27 can turn the Cdk2-cyclin E complex on and off as a Cdk inhibitor, while the Cdk2-cyclin E complex is able to antagonize p27 by phosphorylating it and triggering the proteolysis of p27, subsequently leading to its own activation, rather than p27 acting as a Cdk inhibitor in the ternary complex. Our data also demonstrate that the whole event with respect to the phosphorylation of p27 at Thr-187 is facilitated by the PI 3-kinase pathway in response to FGF-2 stimulation in CECs; however, binding of p27 to the Cdk2-cyclin E complex is partially regulated, while phosphorylation of p27 at Thr-187 is completely regulated by the PI 3-kinase pathway. Although it is not clear how the PI 3-kinase pathway regulates the association of p27 to the Cdk2-cyclin E complex, it is likely that either the nuclear PI 3-kinase or the nuclear Akt may facilitate such a physical interaction among these G1 proteins. However, it remains to be determined if the interaction is directly downstream of Akt or if other molecules activated by PI 3-kinase, independent of Akt, directly facilitate the association of p27 to the Cdk2-cyclin E complex. Through the action of PI 3-kinase, FGF-2 also regulates phosphorylation of p27 on the Ser-10 residue; phosphorylation on Ser-10, nuclear export of pp27Ser-10, and subsequent degradation via the ubiquitin-proteasome machinery progress sequentially. Phosphorylation on the Ser-10 residue begins within 1–2 h of FGF-2 stimulation; nuclear export of pp27Ser-10 was greatly facilitated within 4 h of stimulation; most molecules were ubiquitinated by 12 h after FGF-2 stimulation, and pp27Ser-10 is barely detectable after 16 h of stimulation with FGF-2. A recent study indicated that Ser-10 phosphorylation is required for p27 localization in the cytosol and subsequent phosphorylation at the Thr-157 residue (36). This role of pp27Ser-10 relating to phosphorylation of p27 at Thr-157 has not been explored in the present study. Our attempt to determine which of the two phosphorylated p27s functions as a major G1 inhibitor led us to another new finding: that pp27Ser-10 controls two-thirds of the cell proliferation stimulated by FGF-2 via the PI 3-kinase pathway, while the remaining stimulatory activity of FGF-2 via PI 3-kinase is mediated by pp27Thr-187. Whether this differential regulatory mechanism for p27 phosphorylation is applied to other cell systems must yet be determined.

Of interest, CECs utilize the two pathways largely to remove p27 in response to FGF-2 stimulation. We have reported that IL-1β induces a rapid and high level of FGF-2 in CECs. The newly synthesized FGF-2, acting as an autocrine cytokine,
causes mesenchymal transformation of CECs, leading to corneal fibrosis (8). Thus, during the pathological wound repair process observed in corneal endothelium, FGF-2 facilitates phosphorylation of p27 to remove the potent G1 inhibitor of the cell cycle through the action of PI 3-kinase.

REFERENCES

1. Joyce, N. C., Navon, S. E., Roy, S., and Zieske, J. D. (1996) Invest. Ophthal. Vis. Sci. 37, 1566–1575
2. Senoo, T., and Joyce, N. C. (2000) Invest. Ophthal. Vis. Sci. 41, 660–667
3. Brown, S. I., and Kitano, S. (1966) Arch. Ophthal. 75, 518–525
4. Kay, E. P., Cheung, C. C., Jester, J. V., Nimni, M. E., and Smith, R. E. (1982) Invest. Ophthal. Vis. Sci. 22, 200–212
5. Kay, E. P., Gu, X., Ninomiya, Y., and Smith, R. E. (1993) Invest. Ophthal. Vis. Sci. 34, 663–672
6. Kay, E. P., Gu, X., and Smith, R. E. (1994) Invest. Ophthal. Vis. Sci. 35, 2427–2435
7. Lee, H. T., and Kay, E. P. (2003) Invest. Ophthal. Vis. Sci. 44, 1521–1528
8. Lee, H. T., Lee, J. G., Na, M., and Kay, E. P. (2004) J. Biol. Chem. 279, 32325–32332
9. Ko, M. K., and Kay, E. P. (2005) Invest. Ophthal. Vis. Sci. 46, 4495–4503
10. Roymans, D., and Slegers, H. (2001) Eur. J. Biochem. 268, 487–498
11. Liang, J., and Slingerland, J. M. (2003) Cell Cycle 2, 339–345
12. Cantley, L. C. (2002) Science 296, 1655–1657
13. Shin, I., Yakes, F. M., Rojo, F., Shin, N. Y., Bakin, A. V., Basenga, J., and Arteaga, C. L. (2002) Nat. Med. 8, 1145–1152
14. Vigniello, G., Motti, M. L., Bruni, P., Melillo, R. M., D’Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A., Fusco, A., and Santoro, M. (2002) Nat. Med. 8, 1136–1144
15. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M. K., Han, K., Lee, J. H., Ciarallo, S., Catzavelos, C., Beniston, R., Franssen, E., and Slingerland, J. M. (2002) Nat. Med. 8, 1153–1160
16. Fujita, N., Sato, S., Katayama, K., and Tsuru, T. (2002) J. Biol. Chem. 277, 28706–28713
17. Ishida, N., Kitagawa, M., Hatakeyama, S., and Nakayama, K. (2000) J. Biol. Chem. 275, 25146–25154
18. Boehm, M., Yoshimoto, T., Crook, M. F., Nallamshetty, S., True, A., Nabel, G. J., and Nabel, E. G. (2002) EMBO J. 21, 3390–3401
19. Connor, M. K., Kotchetkov, R., Cariou, S., Resch, A., Lupetti, R., Beniston, R. G., Melchior, F., Hengst, L., and Slingerland, J. M. (2003) Mol. Biol. Cell 14, 201–213
20. Kotake, Y., Nakayama, K., Ishida, N., and Nakayama, K. I. (2005) J. Biol. Chem. 280, 1095–1102
21. Hara, T., Kamura, T., Nakayama, K., Oshikawa, K., and Hatakeyama, S. (2001) J. Biol. Chem. 276, 48937–48943
22. Kamura, T., Hara, T., Matsumoto, M., Ishida, N., Okumura, F., Hatakeyama, S., Yoshida, M., Nakayama, K., and Nakayama, K. I. (2004) Nat. Cell Biol. 6, 1229–1235
23. Kay, E. P., Smith, R. E., and Nimni, M. E. (1982) J. Biol. Chem. 257, 7116–7121
24. Lee, J. G., and Kay, E. P. (2006) Invest. Ophthal. Vis. Sci. 47, 2358–2368
25. Ko, M. K., and Kay, E. P. (2001) Exp. Cell Res. 264, 363–371
26. Lee, J. G., and Kay, E. P. (2006) Invest. Ophthal. Vis. Sci. 47, 1376–1386
27. Bhattacharjee, R. N., Banks, G. C., Trotter, K. W., Lee, H. L., and Archer, T. K. (2001) Mol. Cell. Biol. 21, 5417–5425
28. Aikawa, T., Segre, G. V., and Lee, K. (2001) J. Biol. Chem. 276, 29347–29352
29. Xu, X., Nakano, T., Wick, S., Dubay, M., and Brizuela, L. (1999) Biochemistry (Moscow) 38, 8713–8722
30. Swanson, C., Ross, J., and Jackson, P. K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7796–7801
31. Nguyen, H., Gitig, D. M., and Koff, A. (1999) Mol. Cell. Biol. 19, 1190–1201
32. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) Nat. Cell Biol. 1, 193–199
33. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) Genes Dev. 13, 1181–1189
34. Ishida, N., Hara, T., Kamura, T., Yoshida, M., Nakayama, K., and Nakayama, K. I. (2002) J. Biol. Chem. 277, 14355–14358
35. Rodier, G., Montagnoli, A., Di Marcotullio, L., Coulombe, P., Draetta, G. F., Pagano, M., and Meloche, S. (2001) EMBO J. 20, 6672–6682
36. Shin, I., Rotty, J., Wu, F. Y., and Arteaga, C. L. (2005) J. Biol. Chem. 280, 6055–6063