Ubiquitin-Conjugating enzyme 3 Delays Human Lens Epithelial Cells in Metaphase

Qing Liu, Fu Shang, Elizabeth Whitcomb, Weimin Guo, Wei Li, and Allen Taylor

PURPOSE. Ubc3/Cdc34 is a ubiquitin-conjugating enzyme (Ubc) with well-established functions in the G1-to-S-phase transition. Expecting to find similar effects in human lens epithelial cells (HLECs), the authors explored roles for this ubiquitin-conjugating enzyme in regulation of the HLEC cycle.

METHODS. Catalytically incompetent Ubc3 (G88S, L97S), wild-type (wt)Ubc3, and mutant (mt)Ubc2 (C93A) were expressed in HLECs, by using an adenoviral vector, and cell cycle progression was assessed.

RESULTS. Expression of mt- and wtUbc3, but not empty virus or mtUbc2, delayed the cell cycle in metaphase, rather than the expected G1 phase. Expression of both Ubc3s also stabilized M-phase regulators, cyclin A, cyclin B, and securin. Thus, it appeared that the Ubc3 enzymes were playing roles different from canonical proteolytic functions in targeting G1/S regulators for degradation. We also directly investigated the effect of inhibiting the proteasome on the cell cycle of HLECs. When the proteasome inhibitor was added to S-phase cells, the M-phase regulators were stabilized, and the cells were arrested in the G2/M phase. In contrast, if the proteasome inhibitor was added before the cells entered the S phase, stabilization of the G1 kinase inhibitors p21WAF and p27KIP was observed and the cells were arrested in the G1 phase.

CONCLUSIONS. The ubiquitin-proteasome pathway is involved in regulation of transitions between all phases of the HLEC cycle. However, in contrast with previously described roles for Ubc3 in governing G1/S transitions, expression of Ubc3 delays the HLEC cycle in metaphase. The data suggest novel roles for Ubc3 that do not involve the transfer of ubiquitin in the M phase in the HLEC cell cycle. (Invest Ophtalmol Vis Sci. 2006; 47:1302-1309) DOI:10.1167/iovs.05-0935

Lens formation and clarity require a carefully choreographed program of epithelial cell proliferation, exit from the cell cycle, and differentiation of the epithelial cells into fibers. Proliferation involves the orderly transition of cells through the cell cycle, which can be roughly divided into four phases, G1, S, G2, and M. In most cell types studied, transitions from one stage of the cell cycle to the next depend on activities of cyclin-dependent kinases (Cdks). The mitotic Cdk is activated by cyclin A and cyclin B and is involved in the control of the G2/M transition. Specifically, elevated cyclin A is essential for progress through the S phase and passage into G2. Cyclin A levels decline precipitously on completion of the prometaphase. Cyclin B rises shortly after Cyclin A and its destruction occurs near the end of mitosis, continues into G1, and ceases around the time of the G1/S transition. Securin, which inhibits sister-chromatid separation during mitosis, rises along with or after cyclin B and is degraded on completion of mitosis. G1 regulators include inhibitors of Cdk2, such as p21WAF, p27KIP1, and p57KIP2, and the Cdk2 activators cyclins D and E. The balance between positive and negative regulators of Cdks controls progress from one phase to another of the cell cycle. Most of the cell cycle regulators, including cyclins D, E, A, B, Rb, p21WAF, p27KIP1, p57KIP2, securin, and early mitosis inhibitor (Em1) are often short lived, their levels being primarily regulated by the ubiquitin-proteasome pathway (UPP).

In the UPP, ubiquitin (Ub) is first activated by formation of a high-energy thiolester with Ub-activating enzyme (called E1). Next, the activated Ub is transferred to one of many Ub-conjugating enzymes (called E2s or Ubcs), also as a thiolester. Finally, the Ub is transferred to the protein substrate with the aid of Ub-isopeptide ligases (called E3s). There are at least 20 E2s and dozens of E3s. There is extensive homology within these groups of enzymes throughout the plant and animal kingdoms. The wide variety of E2s and E3s and combinations of them in different UPPs allow for substrate specificity and adaptation to changing cellular conditions.

Work in several model systems suggests that distinct UPPs play critical roles in regulating different parts of the cell cycle. It is generally thought that Ubc3, in concert with the E3, SCF (which comprises Skp1, Cul1/Cdc53, and an F box protein), regulates the transition from the G1 to the S phase by ubiquitin-dependent degradation of phosphorylated forms of the Cdk inhibitors p21WAF, p27KIP, and the activator cyclin D. Degradation of G1 substrates is regulated by phosphorylation and levels of enzymes, such as Ubc3 remain constant throughout the cell cycle. Another UPP, which is governed by a large multiprotein complex, E3, called the anaphase-promoting complex (APC), is thought to control mitosis and related events such as sister chromatid separation during anaphase and exit from mitosis. The APC ubiquitinates substrates along with the concerted action of one of two E2s, either UbcH10 or Ubc4.

In previous work, we showed that for HLECs, G1, S, and G2/M begin approximately 16, 28, and 38 hours after release from G0, respectively, and that the G1/S transition is coupled to degradation of p21WAF and p27KIP. The present study tested the hypothesis that Ubc3 is necessary for the G1/S transition of the lens cell cycle. The study produced the surprising finding that expression of inactive mutated (mt)Ubc3 and wild-type (wt)Ubc3 delayed the cell cycle in metaphase, rather than in G1 as anticipated. The metaphase delay was associated with stabilization of the usually labile M phase regulators, even when active Ubc3 was expressed.
Materials and Methods

Cell Culture, Synchronization, and Cell Cycle Analysis

Human lens epithelial cells (HLECs) SRA 01/04 were grown at 37°C in the presence of 5% CO2 and maintained in DMEM (Invitrogen-Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen-Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen-Life Technologies). HLECs were synchronized in G0 by contact inhibition. This was accomplished by allowing the cells to grow and remain at confluence for 72 hours. The cells arrested in G0 were induced to resume the cell cycle by replating at ~20% confluence. To synchronize cells in prometaphase, we added 100 ng/mL nocodazole (Sigma-Aldrich, St. Louis, MO) to the DMEM for 14 to 16 hours when the HLECs were arrested in prometaphase. Then the cells were washed with complete PBS, and blocking with PBS/0.3% BSA for 30 minutes at room temperature. Incubation with anti-tubulin primary antibody (Sigma-Aldrich) was performed as described. The titer of the adenovirus stock was 3.5 \times 10^9 plaque forming unit (pfu)/mL. Viral stock was diluted with PBS buffer, and added directly to the cultures. HLECs were arrested in G0 by contact infection and infected with wtUbc3, mtUbc3, mtUbc2, and empty adenovirus for 24 hours. The cells were replated at 20% confluence. Then samples were harvested at 0, 24, 28, 32, 40, and 44 hours, and the cell cycle profile was analyzed.

Immunofluorescence

To obtain sufficient amounts of cells in prometaphase, nocodazole was added to the culture medium for 14 to 16 hours when the HLECs were ~80% confluent. The addition resulted in 30% of the HLECs being arrested in prometaphase. Then the cells were washed with complete DMEM at various times, were rinsed in PBS, and fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature, followed by permeabilization in 0.1% Triton X-100 for 3 minutes, washing with PBS, and blocking with PBS/0.3% BSA for 30 minutes at room temperature. Incubation with anti-tubulin primary antibody (Sigma-Aldrich) was for 3 hours. After three washes in PBS, the cells were incubated for 30 minutes with Texas red-conjugated secondary antibody (Calbiochem, San Diego, CA) and the DNA counterstained with 4',6-diamidino-2-phenylindole (DAPI; Calbiochem) for 30 minutes at room temperature. Finally, the samples were sequentially washed 5 times with PBS buffer, once with dH2O and analyzed by fluorescence microscopy. Cells in prophase were indicated by the disassembly of the nuclear membrane. Metaphase was indicated by the alignment of chromosomes, and anaphase was indicated by the movement of sister chromosomes to the poles (see Fig. 3).

Generation of Recombinant Adenoviruses and Infection with Adenoviral Vectors

Adenoviruses expressing green fluorescent protein (GFP) and along with Ad empty, wild type human Ubc3 (wtUbc3), mutant Ubc3 (mtUbc3 C88S, L97S) or mutant Ubc2 (mtUbc2 C93A), a kind gift from Michele Pagano, New York University Medical School, were generated by an adenoviral vector GFP system (AdEasy; Stratagene). In brief, wild-type, and mutant Ubc3 cDNAs were subcloned into the pShuttle-CMV vector by using the EcoRV restriction site. The expression vector was linearized with PstI and cotransformed with supercoiled pAdEasy viral DNA into BJ5183 bacterial cells. Adenoviral amplification was performed as described. The titer of the adenovirus stock was 3.5 \times 10^9 plaque forming unit (pfu)/mL. Viral stock was diluted with PBS and added directly to the cultures. HLECs were arrested in G0 by contact infection and infected with wtUbc3, mtUbc3, mtUbc2, and empty adenovirus for 24 hours. The cells were replated at 20% confluence. Then samples were harvested at 0, 24, 28, 32, 40, and 44 hours, and the cell cycle profile was analyzed.

Ubc3 Activity Assay

The activity of Ubc3 was determined by monitoring thiol ester formation with ubiquitin. This assay is based on the known formation of thiol esters between ubiquitin and E2s; thus, the levels of thiol ester reflect the activities of E2s. HLECs infected with empty, wtUbc3, or mtUbc3 were homogenized in 50 mM Tris-HCl and 1 mM DTT (pH 7.6). This assay was performed in a final volume of 50 µL containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 106 cpm of [35S]Ubiquitin, and 25 µL (100 µg) of supernatant of lens cells. The reaction was started with the addition of 25 µL of HLEC supernatant. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 25 µL of 2 × Laemmli buffer or thiol ester assay buffer (50 mM Tris-HCl and 1 mM DTT, pH 7.6). This assay was performed in a final volume of 50 µL containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 106 cpm of [35S]Ubiquitin, and 25 µL (100 µg) of supernatant of lens cells. The reaction was started with the addition of 25 µL of HLEC supernatant. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 25 µL of 2 × Laemmli buffer or thiol ester assay buffer (50 mM Tris-HCl and 1 mM DTT, pH 7.6). This assay was performed in a final volume of 50 µL containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 106 cpm of [35S]Ubiquitin, and 25 µL (100 µg) of supernatant of lens cells. The reaction was started with the addition of 25 µL of HLEC supernatant. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 25 µL of 2 × Laemmli buffer or thiol ester assay buffer (50 mM Tris-HCl and 1 mM DTT, pH 7.6). This assay was performed in a final volume of 50 µL containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 106 cpm of [35S]Ubiquitin, and 25 µL (100 µg) of supernatant of lens cells. The reaction was started with the addition of 25 µL of HLEC supernatant. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 25 µL of 2 × Laemmli buffer or thiol ester assay buffer (50 mM Tris-HCl and 1 mM DTT, pH 7.6). This assay was performed in a final volume of 50 µL containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 106 cpm of [35S]Ubiquitin, and 25 µL (100 µg) of supernatant of lens cells. The reaction was started with the addition of 25 µL of HLEC supernatant. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 25 µL of 2 × Laemmli buffer or thiol ester assay buffer (50 mM Tris-HCl and 1 mM DTT, pH 7.6). This assay was performed in a final volume of 50 µL containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 106 cpm of [35S]Ubiquitin, and 25 µL (100 µg) of supernatant of lens cells. The reaction was started with the addition of 25 µL of HLEC supernatant. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 25 µL of 2 × Laemmli buffer or thiol ester assay buffer (50 mM Tris-HCl and 1 mM DTT, pH 7.6). This assay was performed in a final volume of 50 µL containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 106 cpm of [35S]Ubiquitin, and 25 µL (100 µg) of supernatant of lens cells. The reaction was started with the addition of 25 µL of HLEC supernatant. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 25 µL of 2 × Laemmli buffer or thiol ester assay buffer (50 mM Tris-HCl and 1 mM DTT, pH 7.6). This assay was performed in a final volume of 50 µL containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 1 mM DTT, 2 mM ATP, 106 cpm of [35S]Ubiquitin, and 25 µL (100 µg) of supernatant of lens cells. The reaction was started with the addition of 25 µL of HLEC supernatant. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 25 µL of 2 × Laemmli buffer or thiol ester assay buffer (50

FIGURE 1. Expression and activities of wild-type Ubc3 and mutant Ubc3 in HLECs. (A) HLECs were infected with wtUbc3, mtUbc3, and empty adenovirus and were harvested at 24, 48, and 72 hours after infection, respectively. Protein extracts (20 µg/well) were resolved on a 12% SDS-PAGE gel. Levels of Ubc3 were determined by Western blot analysis using polyclonal antibody to Ubc3. For normalization of protein loading, the nitrocellulose membrane was stripped and reprobed with a polyclonal antibody to E1. (B) HLECs were infected with wtUbc3, mtUbc3, and empty adenovirus for 48 hours. The activities of Ubc3 were determined by a thiol ester assay. Equal amounts of protein (100 µg) were used in each assay. Ubc3-Ub indicates the thiol ester of Ubc3. Thiol ester formation is confirmed by the absence of this moiety when β-mercaptoethanol (βME) is added.
mM Tris, 4% SDS, 8 M urea [pH 6.8]. After standing at room temperature for 20 minutes, 20 μL of the mixture was separated by 12% SDS-PAGE gel. The gels were exposed to film after drying. The activities of Ubc3 were quantified by determining the density of the bands that disappeared from samples that were treated with β-mercaptoethanol.

**Cell Extract Preparation**

Cell monolayers were washed with ice-cold PBS and harvested by scraping. The cells were homogenized with lysis buffer containing 10 mM Tris·HCl (pH 7.6), 50 mM EDTA, 1% NP-40, 0.1% SDS, 20 mM N-ethylmaleimide, and 2 mM 4-(2-aminoethyl)-benzene-sulfonylfluoride. After it was kept on ice for 20 minutes with occasional vortexing, the lysate was centrifuged at 13,000 g for 15 minutes at 4°C, and the supernatant was recovered. Protein concentrations in the supernatant were determined by the Coomassie plus protein assay kit (Pierce Biotechnology, Rockford, IL), with bovine serum albumin used as the standard.

**Western Blot Analysis**

The anti-E1 antibody was produced in this laboratory. Antibodies to cyclin A, cyclin B, and p27KIP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody to securin (PTTG) was purchased from Zymed Laboratories (San Francisco, CA). For each experimental condition, 20 μg of protein was separated with SDS-PAGE according to published techniques. Proteins were transferred to nitrocellulose membranes and were stained with 1% ponceau S (Sigma-Aldrich) to check loading and transfer efficiency. The membrane was incubated with primary antibodies overnight at 4°C in a solution containing TST (50 mM Tris·HCl [pH 7.6] 150 mM NaCl, and 0.02% Tween 20) and 2.5% milk proteins. After washing four times with TST, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour. Then the membrane was washed five times with TST and incubated with chemiluminescent detection reagents (Supersignal; Pierce Biotechnology) and exposed to a film (Eastman Kodak, Rochester, NY). Quantitative analysis of the

**Figure 2.** (A) Overexpression of mt- and wtUbc3 delays the cell cycle at the G2/M phase. HLECs were arrested at the G0 phase by maintaining cells at confluence for 48 hours and then infected with adenoviral vectors. After infection for 24 hours, the cells were induced to resume the cell cycle by replating at ~20% confluence. The cells were collected at the indicated times and fixed, and the DNA was stained with propidium iodide and quantified with a flow cytometry system. The population of cells in each phase of the cell cycle was analyzed on computer. Experiments were performed at least three times with reproducible results. *Significant difference (P<0.05) compared with cells infected with empty virus. Comparison was made with Student’s t-test. (B) HLECs were infected with the same amount of different adenovirus. HLECs infected with empty, mtUbc3, and mtUbc2 adenoviruses, which also express GFP, were harvested at 48 hours after infection. Protein extracts (20 μg/well) were resolved on a 12% SDS-PAGE gel. Levels of GFP were determined by Western blot analysis with a polyclonal antibody to GFP. For normalization of protein loading, the nitrocellulose membrane was stripped and reprobed with a polyclonal antibody to E1.

**Figure 3.** Immunofluorescence staining of HLECs with DAPI and anti-tubulin antibodies. Nocodazole 100 ng/mL was added to the culture medium for 14 to 16 hours to synchronize HLECs in prometaphase. The cells were then fixed and incubated with tubulin antibody to stain the microtubules and with DAPI to stain the nuclei. DNA staining of HLEC cells with DAPI during early prometaphase (a), late prometaphase (c), metaphase (e), and late anaphase (g). Immunofluorescence staining of HLECs with anti-tubulin antibodies during early prometaphase (b), late prometaphase (d), metaphase (f), and late anaphase (h).
immunoblots was accomplished by densitometry using an image analyzer (MD ImageQuant software ver. 3.3; GE Healthcare, Piscataway, NJ).

RESULTS

Effect of Ubc3 Expression on the HLEC Cycle

To investigate the roles of Ubc3 in regulation of the HLEC cycle, we constructed an inactive mtUbc3 and expressed it by using an adenoviral vector. As a control, wtUbc3 was also expressed in HLECs with the same vector. Western blot analysis indicated that in HLECs a low level of endogenous Ubc3 was noted in the absence of virus or in the presence of empty virus (Fig. 1A, lanes 1 and 2). Twenty-four hours after infection, an increase in expression of Ubc3 was noted (Fig. 1A compare lanes 3 and 4 vs. lanes 1 and 2). By 48 hours of infection with wt- and mtUbc3 adenovirus, there were >10-fold higher levels of wild-type and mutant Ubc3 (Fig. 1A, compare lanes 6 and 7 vs. 5). Further increases in expression levels with prolonged infection time were not observed (Fig. 1: compare lanes 8 and 9 with lanes 6 and 7).

To determine the activities of Ubc3 in HLECs that expressed wt- and mtUbc3, we explored the formation of 125I-ubiquitin thiol esters of Ubc3. A low level of endogenous Ubc3 thiol ester was observed in empty adenovirus-infected HLECs (Fig. 1B, lane 1). In comparison, thiol ester levels increased >10-fold when wtUbc3 was expressed (compare lane 2 vs. lane 1). Thiol ester levels did not change significantly when similar levels of mutant Ubc3 were expressed (Fig. 1: compare lane 3 with lane 1). As expected, in the presence of β-mercaptoethanol, the thiol ester was destroyed (Fig. 1: compare lane 5 with lane 2). These assays confirm that we expressed active wtUbc3 and an inactive variant, mtUbc3. E1-ubiquitin thiol ester was also observed in samples without β-mercaptoethanol, and the levels were not affected by expression of wt- or mtUbc3. This suggests that relaying ubiquitin from E1 to Ubc3 or other acceptors is not a rate-controlling step in Ubc3-dependent UPP processes.

To investigate the roles of Ubc3 in regulation of the HLEC cell cycle, the cells were infected with equivalent levels of viruses which expressed GFP along with nothing, mtUbc3, wtUbc3 (Fig. 2A, left), and mtUbc2 (Fig. 2A, right). HLECs infected with empty adenovirus showed indistinguishable patterns and timing of progress through the different phases of the
The G1/S transition in the initial cell cycle occurred at 16 hours after release from G0 and the M/next G1 transition occurred at 38 hours after release from G0 (data not shown). This is corroborated by the observation that at 40 hours more than 50% of the cells are in G1 phase, and this proportion increased at 44 hours as more cells move into the G1 phase of the next cell cycle. In comparison, expression of mtUbc3 resulted in lower proportions of cells in G1 and a greater proportion of the cells at G2/M of the first cell cycle (Fig. 2A, 40 hours). Arrest of the mtUbc3-infected HLEC in G2/M of the first cell cycle is corroborated by the observation that fewer of these cells progressed to the G1 phase of the next cell cycle at 44 hours. The G2/M arrest is curious, since most previous studies indicated roles for Ubc3 in the G1/S transition, and it might be anticipated that when mtUbc3 is expressed, the greatest proportion of cells would be expected to be in the G1 phase. The proportion of mtUbc3-expressing cells that are arrested at the G2/M phase and of the empty-virus–infected cells that progress to the next cell cycle are actually more dramatic, because approximately 25% of the confluence-synchronized cells do not reenter the cell cycle. Of interest, HLECs in which wtUbc3 was expressed also showed increases in the proportion of cells in the G2/M phase, along with proportional decreases in the fraction of cells at the G1 phase. These data are due to specific effects of Ubc3, as demonstrated in Figure 2A, right panel, since cells that were infected with the same amount of adenovirus that encodes mtUbc2 did not show a delay at G2/M. These results were not due to unequal viral loads, because GFP levels of all three samples are indistinguishable (Fig. 2B).

To more accurately determine the point of cell cycle arrest due to expression of the Ubc3s, we synchronized the cells at prometaphase with nocodazole. Then, at various times after nocodazole washout, we quantified specific mitotic forms of the cells. DNA (Figs. 3A, 3C, 3E, 3G) and tubulin (Figs. 3B, 3D, 3F, 3H) staining indicated cells at the expected stages of the cell cycle. Quantification of five fields of cells at the indicated times showed that most of the prophase synchronized cells moved from prophase to metaphase synchronously (Figs. 4A, 4B). By 10 minutes, there was no significant difference between empty and mtUbc3-infected or wtUbc3-infected HLECs. However, 20 minutes after nocodazole washout, differences between the groups began to appear: 68% of HLECs infected with empty adenovirus were in anaphase, whereas only 2.7% of mtUbc3-infected HLECs were in anaphase (Fig. 4C). Instead, at this time point, most mtUbc3-infected HLECs remained in metaphase. The difference became more apparent at 30 minutes, when 63% of HLECs infected with empty adenovirus moved into telophase, whereas none of mtUbc3-infected HLECs were in telophase (Fig. 4D). At this time point, most mtUbc3-infected HLECs remained in metaphase. The difference became more apparent at 30 minutes, when 63% of HLECs infected with empty adenovirus moved into telophase, whereas none of mtUbc3-infected HLECs were in telophase (Fig. 4D). At this time point, most mtUbc3-infected HLECs were still in metaphase. By 40 minutes, the differences were even more dramatic: virtually all the cells, which were infected with empty adenovirus, had exited mitosis and entered the next interphase. In contrast, 77% of mtUbc3-infected HLECs remained in telophasp. These data clearly indicate that the expression of mtUbc3 delays the cell cycle in metaphase. Expression of wtUbc3 in HLECs resulted in a similar metaphase arrest phenotype as did expression of mtUbc3.

Completion of the M-phase of the cell cycle requires timely ubiquitination-dependent destruction of mitotic cyclins and other mitotic regulators, such as cyclin A, cyclin B, and securin by the UPP. To determine the mechanism of cell cycle delay in metaphase by expression of Ubc3, we assessed the effects of
expression of Ubc3 on the stability of these mitotic regulators. In cells infected with the empty adenovirus, levels of cyclins A and B and securin fell as the cells exited the mitosis phase of the first cycle (Fig. 5, left side of each panel). This decrease is consistent with their being effective substrates for the UPP. In contrast, levels of these mitosis regulators remained elevated in cells that were infected with mtUbc3 and surprisingly with wtUbc3 (Fig. 5, middle and right side of each panel). These data show that expression of Ubc3, in either its active or inactive form, interfered with the timely destruction of mitotic regulators and delayed the cell cycle in metaphase. Because the same effects were obtained with active and inactive Ubc3, they also suggest that the effect observed with the mtUbc3 is not due to a dominant negative effect of the thiol-ester-incompetent Ubc3.

**Rules of the UPP in Regulating Different Transitions within the Cell Cycle**

The apparently paradoxical stabilization of mitotic regulators by an inactive variant and an active form of an enzyme that usually regulates G1/S transitions suggested that we test the role of UPP-dependent proteolysis in regulating transitions between different phases of the HLEC cycle. This test was performed in two ways. In the first, the cells were synchronized at the G0 phase, and the specific proteasome inhibitor, clastolactacystin β-lactone before they entered the cell cycle. As seen in Figure 7B, the cells were arrested in G1. In contrast, the control cells moved from the G0 to the S phase and then to the G2/M phase (Fig. 7A). We further examined the changes of protein levels of p21WAF and p27KIP, two known Cdk-inhibitory proteins. Figure 7C shows that the protein levels of p21WAF are highest in G0 phase-arrested cells. At 16 hours after replating, the cells that were not treated with lactacystin started entering the S phase and by 24 hours the p21WAF levels were markedly reduced. In contrast, in the presence of proteasome inhibitor, the levels of p21WAF were >8, 5, and >4-fold higher at 8, 16, and 24 hours, respectively. A similar
pattern of cell cycle-dependent changes in levels of p27kip1 was observed (Fig. 7D). Because degradation of these inhibitors is a prerequisite for progress through the cell cycle, these data explain the arrest at the G1 phase when the inhibitor was added as the cells are allowed to enter the cell cycle.

**DISCUSSION**

Ubiquitination-dependent proteolysis has been established as a requirement for progress through the cell cycle in various cell types, and here we confirm the importance of the UPP in cell cycle control in HLECs. It has been demonstrated that the proteolysis of p21waf1 and p27kip1 by a Ubc3-catalyzed UPP is a prerequisite for the transition from the G1 to the S phase.16 Because of this precedent, we anticipated that progression through the G1/S phase transition would be hastened by expression of additional wtUbc3 and delayed by expression of an enzymatically inactive form of Ubc3. Surprisingly, we found that expression of wt- and mtUbc3 in HLECs did not affect the transition through G1/S. Instead, expression of these Ubc3s resulted in a delay in metaphase during the cell cycle (Fig. 4). The M-phase delay that we observed on expression of mt- or wtUbc3 is specific, since infection with Ubc2-expressing or empty adenovirus was without effect. Moreover, expression of Ubc3 stabilized mitotic regulators, such as cyclin A, cyclin B, and securin (Fig. 5), indicating that expression of Ubc3 interferes with the timely destruction of mitotic regulators by the UPP. This was unexpected because these mitogenes are known to be ubiquitinated by UbcH10 and Ubc4, along with the mitotic E3, the APC.6 In addition, proteasome inhibitor also stabilized mitotic regulators and arrested the cells in G2/M of the cell cycle. Taken together, the data suggest the novel concept that the Ubc3-induced delay in mitosis in HLECs is due to effects that do not require binding of ubiquitin at the Ubc3 active site or direct transfer of ubiquitin from Ubc3 to the substrate (with or without the APC), and that this specific delay of the cell cycle involves domains that are present in Ubc3 but not present in Ubc2.

Albeit rare, effects of Ubc3 on mitosis have been reported in two other types of cells. Topper et al.19 found that microinjection of bacterially expressed active but not mutant Ubc3 into Ptk1 cells results in cell cycle arrest in prometaphase. They concluded that elevated levels of Ubc3 block centromere protein E (CENP-E, a mitotic kinesin) from associating with kinetochores and subsequently inhibit chromosome alignment in metaphase. Our data corroborate a role for wtUbc3 in the M phase and we further demonstrated that expression of inactive mtUbc3 also arrested the cell cycle in M phase. These results promote the secondary hypothesis that the Ubc3-induced metaphase delay does not require the activity of Ubc3. The observation also agrees with the findings of Topper et al., in that they also observed that Ubc3-mediated cell cycle arrest is proteasome independent. We demonstrated that, like overexpression of Ubc3, inhibition of the proteasome resulted in stabilization of mitotic regulators and G2/M arrest. Ubc3 was also reported to associate with the mitotic spindle in anaphase, suggesting that it may play a role in the events of late mitosis.31,32 In addition, Ubc3 is involved in the degradation of the budding yeast Cdk inhibitory kinase Swe1 and the Xenopus homologue Wee1.33 Both act to inhibit entry into mitosis.

How might Ubc3 interfere with progress through the M phase? It is possible that Ubc3, which shares 36% sequence identity and 50% homology with UbcH10, competes with UbcH10, the E2 that usually collaborates with the APC to allow progress through M phase. Levels of UbcH10 vary significantly during the cell cycle.6 In contrast, levels of Ubc3 are constant throughout the cell cycle in HLECs, as well as in yeast and other mammalian cells.18,30 We speculate that at elevated levels, Ubc3, but not the other E2s, may compete with UbcH10 for binding to the APC. Such competition would inhibit the APC-UbcH10-mediated degradation of the mitotic regulators and explain both the observed stabilization of cyclins A and B and securin, as well as the mitotic arrest in cells in which mutant Ubc3 is expressed. This would also explain why mt- and wtUbc3 have the same effect, since the wt- and mtUbc3 constructs can be expected to have the
same structure and compete similarly for UbcH10 binding to the APC.

Finally, we hypothesize that the constantly low level of Ubc3 meets the requirement for functions of Ubc3 in each phase of the cell cycle, while it also avoids the potential nonspecific interactions with other E3s. Elevation of this E2 induces interactions with E3s, such as the APC, thus interfering with their functions and inducing biological consequences. These hypotheses are being tested.

References