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The Proteasome Controls the Expression of a Proliferation-Associated Nuclear Antigen Ki-67

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Abstract The proteasome is a protease complex responsible for rapid, selective, and irreversible removal of regulatory proteins, as well as many other cellular proteins. In this study, we have demonstrated that a proliferation-associated nuclear protein Ki-67 depended on the proteasome for its rapid degradation. A proteasome-specific inhibitor lactacystin augmented Ki-67 protein levels in pancreatic cancer BxPC-3 cells while repressed the level of steady-state Ki-67 mRNA. Inhibition of the proteasome also led to accumulation of two CDK inhibitors $p27^{kip1}$ and $p21^{cip1}$ in the BxPC-3 cells. Failed reduction of Ki-67 protein and enhanced levels of the two CDK inhibitors are likely contributing factors for the suppressed BxPC-3 proliferation after proteasome inhibition. J. Cell. Biochem. 76:596–604, 2000. © 2000 Wiley-Liss, Inc.

Key words: proteasome; lactacystin; pancreatic tumor; Ki-67; $p27^{kip1}$; $p21^{cip1}$

Proteasomes are large protease complexes located in both cytoplasm and nuclei. These complexes degrade cellular proteins in a ubiquitination-dependent and ATP-dependent manner [Jentsch et al., 1995]. The proteasome has at least five distinct peptidase activities: chymotrypsin-like, trypsin-like, peptidylglutamyl peptide-hydrolyzing, branched-chain amino acid-preferring, and small neutral amino acid-preferring activities [Orlowski et al., 1993]. The first three are better characterized. The proteasome is the major intracellular machinery for protein degradation [Rock et al., 1994]; it was regarded as a housekeeping enzyme to dispose of spent proteins. However, it has become increasingly clear that the proteasome plays critical and active roles in regulating many different cellular functions. This is achieved by the ability of the proteasome to destroy regulatory protein factors in a timely, selective, and irreversible manner and to process precursors of regulatory factors into active ones. For example, the degradation of several important regulators of cell activation and proliferation, such as cyclin 2 [Deshaies et al., 1995], cyclin 3 [Yaglom et al., 1995], cyclin B [Seufert et al., 1995], p53 [Scheffner et al., 1993], $p27^{kip1}$ [Pagano et al., 1995], IκBα [Palombella et al., 1994], IκBβ [Cui et al., 1997], and c-Jun proteins [Treier et al., 1994] occurs by means of the proteasome pathway. The p50 component of a transacting nuclear factor NF-κB matures after cotranslational processing of its precursor peptide by the proteasome [Lin et al., 1998]. Selective ubiquitination of target proteins is an important mechanism in controlling the dis-

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criminative nature of protein degradation via the proteasome [Jentsch et al., 1995]. Other regulatory mechanism might also exist. For example, a CDK inhibitor $p27^{kip1}$ must associate with Jab-1 in order to translocate into cytoplasm, where it is degraded through the proteasome pathway [Tomoda et al., 1999].

A proliferation-associated nuclear antigen defined by monoclonal antibody (MAb) Ki-67 was discovered in 1983 by Gerdes et al. [1983]. As a marker for cell proliferation, it is a useful prognostic indicator for assessing clinical outcomes of cancers. The Ki-67 protein has two isoforms of apparent molecular weights of 345 kDa and 395 kDa as detected in immunoblotting, and these isoforms are likely derived from alternative splicing of exon 7 of the Ki-67 gene [reviewed in detail by Duchrow et al., 1996]. The biological function of Ki-67 has not been fully elucidated. However, blocking Ki-67 protein synthesis by antisense oligonucleotides to Ki-67 mRNA leads to inhibition of cell growth [Schluter et al., 1993], suggesting that Ki-67 plays an important role in regulating cell proliferation. Indeed, the Ki-67 protein is expressed in most active parts of the cell cycle, including the G1, S, and G2/M phases, but is not detectable in resting cells at the G0 phase [Gerdes et al., 1984; Braun et al., 1988; Tazzari et al., 1990]. In the M phase, Ki67 is rapidly degraded with a half-life of about 1 h at the anaphase and telophase after the metaphase [Bruno et al., 1992]; this finding suggests that removal of Ki67 might be necessary for cells to progress from the M phase.

A proteasome inhibitor lactacystin (LAC) can repress the three major peptidase activities of the proteasome (i.e., chymotrypsin-like, trypsin-like, and PGPH activity), but it does not affect other proteases, such as calpain, cathepsin B, chymotrypsin, trypsin, and papain [Fenteany et al., 1995]. Using this proteasome inhibitor, we investigated how the proteasome regulates the expression of Ki-67 and several other regulatory protein factors in a pancreatic tumor cell line, BxPC-3. In this study, we have found that degradation of Ki-67 and CDK inhibitors $p21^{cip1}$ and $p27^{kip1}$ is proteasome-dependent and that proteasome activity is also required for Ki-67 mRNA expression. The implications of these findings are discussed.

MATERIALS AND METHODS

Reagents

RPMI 1640, fetal calf serum (FCS), penicillin-streptomycin, and L-glutamine were purchased from Life Technologies (Burlington, Ont, Canada). Lactacystin was obtained from Dr. E. J. Corey [Fenteany et al., 1995]. Rabbit antibodies against $p27^{kip1}$ and $p21^{cip1}$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse MAb against Ki-67 was purchased from Boehringer-Mannheim (Montreal, Quebec), and a PE-conjugated goat anti-mouse IgG1 Ab was from Caltag (South San Francisco, CA). Enhanced chemiluminescence (ECL) kits were ordered from Amersham (Oakville, Ont). A human pancreatic tumor cell line BxPC-3 was purchased from American Type Culture Collection (Rockville, MD).

Cell Culture

BxPC-3 pancreatic tumor cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics [Luo et al., 1992].

^{3}H-Thymidine Uptake

Cell proliferation was measured according to ^{3}H-thymidine uptake as described by Luo et al. [1992]. Briefly, BxPC-3 cells were seeded in 96-well flat-bottomed plates at 2×10^4 cells/100 µl/well with LAC of different concentrations. ^{3}H-thymidine (0.5 µCi/well) was added 2 h later. The cells were harvested after 16-h further culture and ^{3}H-thymidine uptake by the cells was measured by a scintillation counter.

Immunofluorescent Microscopy

BxPC-3 cells were cultured in plain medium or in medium with 10 µM LAC for 10 h. The cells were washed three times with ice-cold phosphate-buffered saline (PBS) and then transferred onto glass slides by Cytospin. After air-drying at 56°C for 10 min, the slides were fixed in 4% paraformaldehyde at 4°C for 10 min, followed by three washes with cold PBS. The cells were permeabilized by a solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 7 min at room temperature. Mouse anti-Ki-67 MAb (1:25 dilution of the culture supernatant supplied by Boehringer-Mannheim) was reacted with the cells at 4°C overnight. After wash, PE-conjugated goat anti-mouse IgG1 (1:200) was reacted with the cells for 30 min at room temperature. The slides were washed in PBS 3 times and sealed with PBS containing 50% glycerol and 1 mg/ml phenyldiamine. The slides were examined under a Leitz fluorescent microscope.

Flow Cytometry

BxPC-3 cells were cultured, fixed, and permeabilized as for the immunofluorescent microscopy, except that the cells were always in suspension. The cells were stained with anti-Ki-67 MAb (1:25 dilution) on ice for 4 h, followed by PE-conjugated goat anti-mouse IgG1 (1:200) for 30 min at room temperature.

Northern Blot Analysis

The method was described in our previous publication [Shan et al., 1992]. Total cellular RNA from BxPC-3 cells with or without LAC treatment was extracted with the guanidine-CsCl method. After electrophoresis, the RNA was blotted onto a nylon membrane. Even loading of RNA in different samples was shown on the basis of the intensity of 18S and 28S bands of ribosomal RNA (rRNA). A 194-bp polymerase chain reaction (PCR) fragment corresponding to positions from 3671 to 3865 (transcribed from exon 13) of Ki-67 cDNA was excised from a pCRII construct (a generous gift from Dr. M. Duchrow, Surgical Clinic, Medical University of Luebeck, Germany), labeled with ^{32}P, using random primers, and used as a probe.

Immunoblotting

Immunoblotting was employed to evaluate the levels of $p21^{cip1}$ and $p27^{kip1}$. The general protocol was described in our previous publication [Chen et al., 1996]. Briefly, BxPC-3 cells were lysed in the presence of proteinase inhibitors. The cleared lysates were quantitated for protein concentrations. An equal amount of lysate proteins (40 μg) of each sample was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were then blocked with 5% milk, and hybridized with rabbit antisera against $p27^{kip1}$ and $p21^{cip1}$ at dilutions suggested by the manufacturer. The signals on the membranes were detected by ECL.

RESULTS

Effect of Proteasome Inhibition on Proliferation and Viability of BxPC-3 Cells

The role of the proteasome in growth of a cell line derived from a solid pancreatic tumor was first examined. Proliferation of BxPC-3 cells was inhibited dose-dependently by LAC in an overnight culture (Fig. 1A). LAC-induced cell death likely contributed moderately to the inhibition of cell proliferation. After overnight culture in 10 μM LAC, viability of the cells dropped to 73% according to trypan blue staining, compared with 93% for the cells cultured in plain medium (Fig. 1B). However, inhibition of the cell proliferation by 8 μM LAC overnight reached more than 50%, as shown in Figure 1A, and such a degree of inhibition cannot be totally attributed to the reduced cell viability. The results indicate that the proteasome is

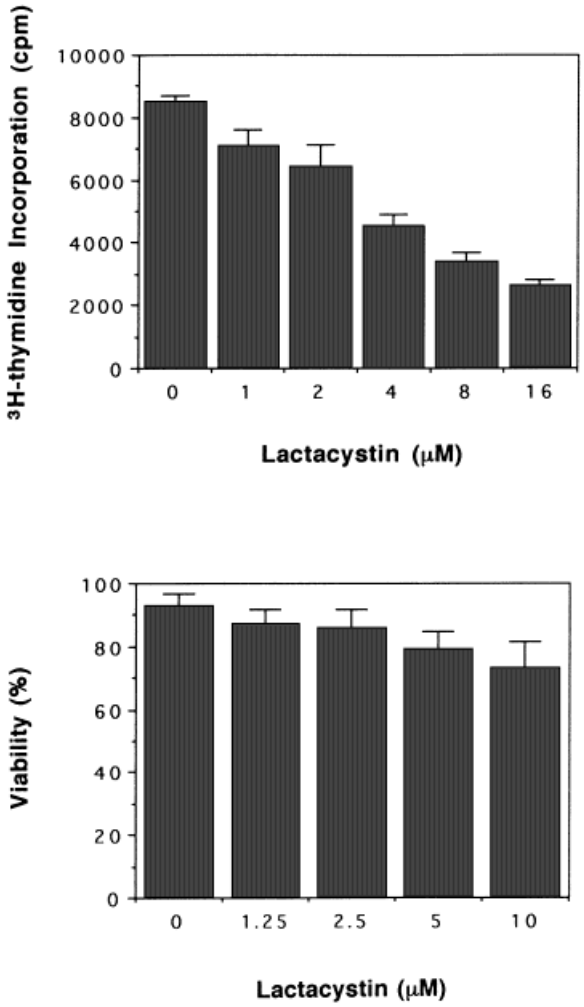

Fig. 1. Lactacystin (LAC) inhibits proliferation of pancreatic tumor BxPC-3 cells and moderately affects their viability. **A:** ^{3}H-thymidine uptake of BxPC-3 cells treated with LAC. BxPC-3 cells were cultured in a 96-well flat-bottomed plate with LAC of different concentrations. ^{3}H-thymidine was added 2 h later. ^{3}H-thymidine uptake of the cells was measured after 16 h further culture. **B:** Viability of BxPC-3 cells treated with LAC. BxPC-3 cells were cultured in a 24-well flat-bottomed plate in the presence or absence of LAC of different concentrations as indicated. After 24-h culture, the cell viability was determined by trypan blue exclusion.

critical for both cell proliferation and viability, as reported previously [Wang et al., 1998].

Role of the Proteasome in Regulation of Ki-67 Expression

Ki-67 is implicated in controlling cell proliferation. As a short-lived protein, it falls into a category of molecules whose removal might be important for its regulatory function and whose degradation might be controlled by the proteasome. To test this possibility, Ki-67 protein levels in BxPC-3 cells were assessed after proteasome inhibition. As shown in Figure 2, a small number of untreated BxPC-3 cells were Ki-67 positive, as observed by immunofluorescent microscopy; when the cells were treated with LAC (10 μM) for 10 h, the number of Ki-67 positive cells was greatly increased.

To assess the change more quantitatively, the level of Ki-67 protein was measured by flow cytometry (Fig. 3). BxPC-3 cells were treated with LAC of different concentrations for 16h and a biphasic expression pattern of Ki-67 was observed. With 4 μM of LAC, 26.8% of the cells became Ki-67 positive, compared with 1.1% Ki-67 positive for cells cultured in medium. When LAC was increased to 8 μM, the Ki-67-positive cells reached 70.3%. However, further increase in LAC concentration to 16 μM in this overnight culture repressed the Ki-67 expression, and only 13.1% cells remained positive.

The above findings suggest that there is a rapid turnover of Ki-67 protein in BxPC-3 cells and degradation of the protein is a proteasome-dependent process. Moreover, the biphasic expression of Ki-67 after proteasome inhibition also suggests that synthesis of Ki-67 is also regulated by the proteasome. We then examined the level of steady-state Ki-67 messenger RNA in LAC-treated BxPC-3 cells. After 10-h LAC treatment at 10 μM, the steady-state Ki-67 mRNA as detected by our probe derived from exon 13 of Ki-67 [Schluter et al., 1993] was significantly reduced, compared with that of untreated cells (Fig. 4, left panel). A prolonged LAC (8 μM) treatment for 16 h repressed the Ki-67 message to an almost undetectable level (Fig. 4, right). The 10-h LAC treatment and the 16-h LAC treatment were from two separate Northern hybridizations and two different exposures of ECL. Therefore, the absolute signals between Figure 4A and 4B are not comparable.

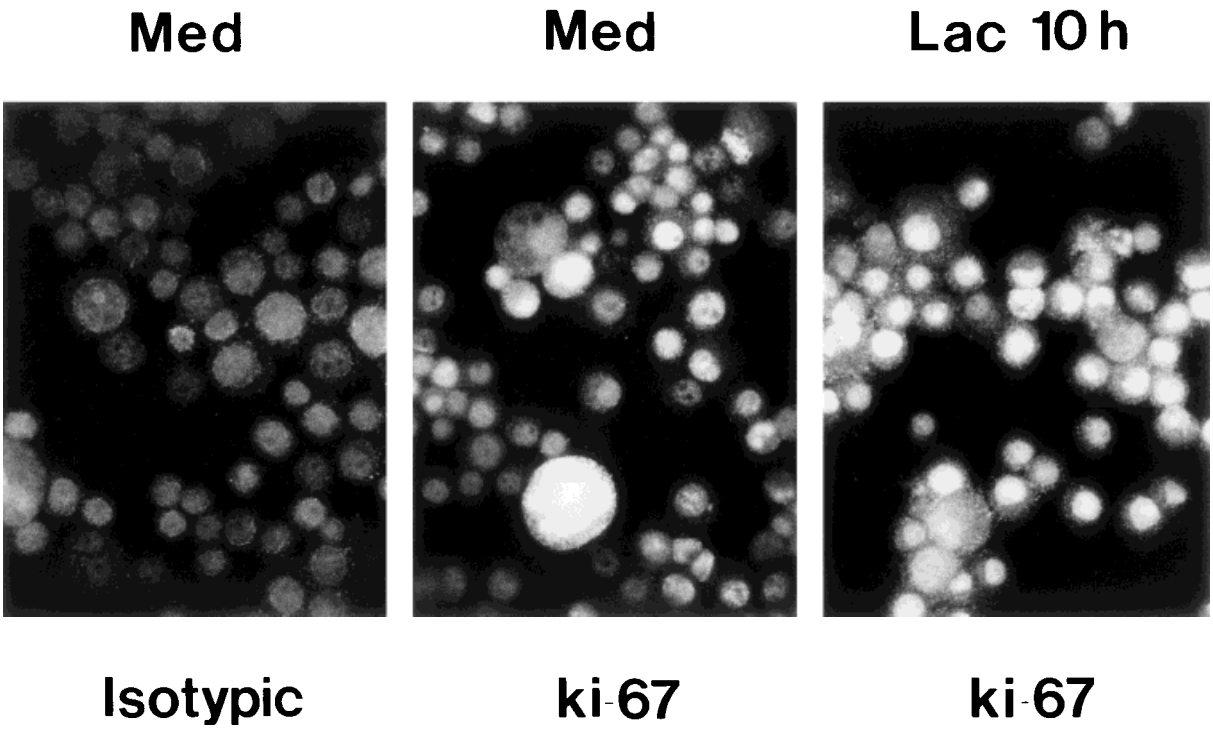

Fig. 2. Immunofluorescent microscopy of Ki-67 protein in BxPC-3 cells after lactacystin (LAC) treatment. BxPC-3 cells were cultured in medium or 10 μM LAC for 10 h. The cells were then permeabilized and stained with either Ki-67 MAb or isotypic control MAb as indicated. PE-conjugated goat anti-mouse IgG1 was used as a second antibody and the samples were examined under a fluorescent microscope.

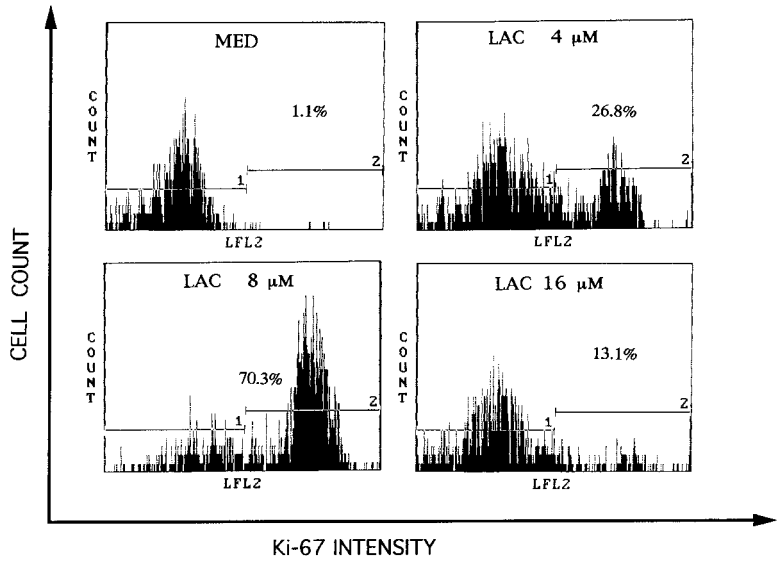

Fig. 3. Flow cytometry on expression of Ki-67 in lactacystin (LAC)-treated BxPC-3 cells. BxPC-3 cells were cultured in the absence or presence of LAC for 16 h as indicated. The cells were stained with Ki-67 MAb followed by PE-conjugated goat anti-mouse IgG1. Region 2 contains PE-positive cells; their percentages are indicated. Background staining by an isotypic control MAb has already been deducted from the percentages shown.

However, the medium-treated and LAC-treated samples in each case were from the same experiment, and LAC consistently and strongly suppressed the mRNA expression of Ki-67. This result indicates that the increase of Ki-67 protein seen in Figures 2 and 3 (4 μM and 8 μM) is not due to increased transcription of Ki-67 mRNA, and that the Ki-67 transcription is also a proteasome-dependent process.

The Proteasome Regulates Levels of CDK Inhibitors

Since the proteasome degrades of a large number of proteins, it is conceivable that other regulatory factors essential in cell proliferation might also be channeled to the proteasome for their demise. We therefore studied the protein levels of two CDK inhibitors—$p21^{cip1}$ and $p27^{kip1}$, which are critical for CDK activities and consequently pivotal for cell cycle progresses. As shown in Figure 5, $p21^{cip1}$ was hardly detectable in untreated BxPC-3 cells. When the cells were incubated with 10 μM LAC, accumulation of $p21^{cip1}$ was apparent as early as 6 h. Treating the cells with different concentrations of LAC for 16 h led to a dose-dependent increase in the protein. For $p27^{kip1}$, an obvious increase in its protein level occurred when the cells were treated with LAC (10 μM) for 10 h. These results suggest that, in BxPC-3 cells, these two CDK inhibitors are normally degraded in a proteasome-dependent fashion, and accumulation of these inhibitors after proteasome inhibition is a plausible contributing cause for the observed inhibition of proliferation of the LAC-treated BxPC-3 cells.

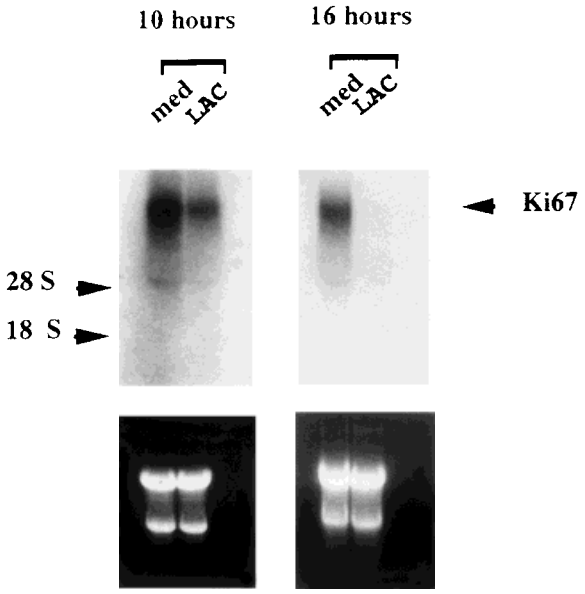

Fig. 4. Effect of lactacystin (LAC) on steady-state mRNA of Ki-67 according to Northern blot analysis. BxPC-3 cells were treated with 10 μM LAC for 10 h or with 8 μM for 16 h as indicated. Total cellular RNA of the cells was extracted and analyzed by Northern blotting. Bands of about 12 Knt in size, corresponding to the Ki-67 message, are indicated. Even loading of RNA is evidenced according to 18S and 28S ribosomal RNA (bottom).

DISCUSSION

In this study, we have reported that degradation of a proliferation-associated nuclear antigen Ki-67 in pancreatic tumor cells BxPC-3 was

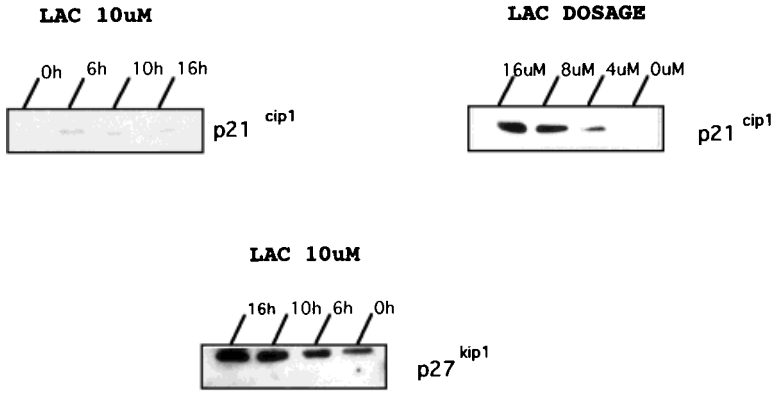

Fig. 5. Immunoblot of $p21^{cip1}$ and $p27^{kip1}$ in lactacystin (LAC)-treated BxPC-3 cells. BxPC-3 cells were treated with LAC (10 μM) for different periods **(top left and bottom)** or with LAC of different concentrations for 16 h **(top right).** The cellular proteins were analyzed by immunoblotting for $p21^{cip1}$ and $p27^{kip1}$ levels.

proteasome-dependent, as blocking the proteasome activity by LAC led to accumulation of Ki-67 protein in the cells, accompanied by a decrease in Ki-67 mRNA. We have also shown that protein levels of two CDK inhibitors were augmented after proteasome inhibition. Our results suggest that the changes in the levels of these regulatory proteins are likely contributing factors for the repressed proliferation when the proteasome is inhibited in the BxPC-3 cells.

LAC-treated BxPC-3 cells had significantly repressed proliferation and at the same time that their viability demonstrated moderate reduction. The difference between degrees of these two effects suggests that the former is not entirely caused by the latter. Considering that the proteasome degrades a large number of regulatory proteins, it would not be surprising if both proliferation and cell death can be shown to be affected independently by the proteasome. Our previous study in lymphocytes showed that this is indeed the case [Wang et al., 1998].

Inasmuch as the proteasome is essential for cell viability, different types of cells have different sensitivities to the proteasome inhibitors. In general, cells of hematopoietic origin are more sensitive to LAC than are cells derived from solid tumors (data not shown). Indeed, the viability of BxPC-3 pancreatic tumor cells was only moderately affected by LAC (10 μM) after 16-h treatment in this study. We previously demonstrated that LAC-induced death in T-leukemia Jurkat cells is due to apoptosis [Wang et al., 1998], a finding consistent with Imajoh-Ohmi's report on induction of apoptosis in U937 after proteasome inhibition [Imajoh-Ohmi et al., 1995]. However, we could not find evidence of apoptosis for the moderate degree of cell death seen in the BxPC-3 cells after LAC treatment, using either DNA laddering or a TUNEL assay (data not shown). Therefore, it appears that not all cells die in the same way after inhibition of their proteasome.

Ki-67 protein has a short half-life of less than 1 h, as Ki-67 immunostaining rapidly decreases during anaphase and telophase [Bruno et al., 1992]. However, no study has been conducted so far to elucidate pathways of Ki-67 degradation. Our study showed for the first time that degradation of Ki-67 protein is proteasome-dependent. The accumulation of Ki-67 protein after proteasome inhibition is not attributable to increased synthesis of the protein because (1) there is no augmentation of Ki-67 mRNA after 6 h LAC treatment, and (2) there is no plausible mechanism suggesting that proteasome inhibition could lead to increased macromolecule synthesis. It should be mentioned that an accumulation of a protein after proteasome inhibition does not automatically qualify the protein as a substrate of the proteasome, even when augmentation of synthesis of the protein is ruled out. A recent study by Tomoda et al. [1999] showed that $p27^{kip1}$ depends on a shuttle protein Jab1 to facilitate its translocation from the nucleus to the cytosol before it could be degraded by the proteasome in the cytosol. This finding implies that, theoretically, if the proteasome interferes with translocation of a protein during its journey to degradation, the protein could accumulate without it being the direct substrate of the proteasome. Also, Ki-67 could accumulate if LAC stabilizes a protein that stabilizes Ki-67, or LAC affects a post-translational process that prolongs the half-life of Ki-67. However, we would argue that Ki-67 is likely a direct substrate of the proteasome because it has 10 strong and 40 weak PEST (Pro,

Glu, Ser, Thr) motifs in its peptide sequence and the PEST motif is believed to be a signature for rapid degradation of the protein, mainly via the proteasome pathway [reviewed by Rechsteiner et al., 1996]. Many proteins (e.g., IκBα cyclins and phytochrome) known to be degraded by the proteasome contain such PEST motifs. Not much progress has been made since the identification of the PEST motif and its suspected connection with ubiquitination. This motif might be recognized by components of the ubiquitination cascade, or by a kinase because protein phosphorylation is reported to be a prerequisite for ubiquitination of certain proteins such as $p27^{kip1}$ [Vlach et al., 1997] and IκBα [DiDonatol et al., 1996]. Of course, the final prove that Ki-67 is the direct target of the proteasome will be obtained if its ubiquitination can be shown, and such a further experiment is warranted.

Overnight inhibition of the proteasome resulted in strong repression of Ki-67 mRNA expression, as shown by Northern blot analysis. This finding suggests that Ki-67 mRNA has a short half-life and that its transcription is proteasome-dependent. In the 5′ flanking sequence at position 234 of the Ki-67 gene, there is an inverted NF-κB binding site [Duchrow et al., 1996]. The proteasome is known to degrade NK-κB inhibitor IκB subunits IκBα [Palombella et al., 1996] and IκBβ [Cui et al., 1997]. Without being able to remove the inhibitor IκBs when the proteasome is inhibited, NK-κB is not able to translocate from cytosol into nuclei to play its role. This is a possible contributing mechanism for LAC-caused inhibition of Ki-67 transcription and is certainly worthwhile to confirm such speculation.

We have noted an interesting biphasic change in the Ki-67 level when BxPC-3 cells were treated with LAC. At low (4 μM) to medium (8 μM) concentrations, LAC induced accumulation of Ki-67 protein dose-dependently after 16 h (Fig. 3). Treatment of 10 μM of LAC for 10 h still led to accumulation of Ki-67 in the cells (Fig. 2). This finding suggests that, under such conditions, inhibition of the protein degradation has overwhelmed de novo synthesis of the Ki-67 protein. The Ki-67 protein could still be translated from the existing or inhibited de novo-transcribed mRNA, while its protein degradation is drastically reduced, hence the accumulation of the Ki-67 protein that we saw in the cells.

However, when the cells were treated with a higher dose of LAC (16 μM) for a longer time (16 h), the Ki-67 protein was drastically reduced. How do we explain the reduction? First, it is possible that when the proteasome is repressed for a longer time (i.e., by 16 μM of LAC for 16 h), there is no more de novo Ki-67 translation. Such a supposition is supported by the complete disappearance of the Ki-67 message in the Northern blot after LAC treatment at 10 μM for 16 h (Fig. 4). Then, where does the existing Ki-67 protein go after a prolonged LAC treatment? A simple explanation is that the inhibition of the proteasome is leaky and that the proteasome still degrades Ki-67 at a lower rate. Under such conditions, the initially accumulated Ki-67 protein eventually disappears, while there is no more de novo synthesis of the Ki-67 protein, since the Ki-67 mRNA is no longer available as shown in Figure 4.

The short half-life and the disappearance of Ki-67 protein at the anaphase and telophase suggest that fluctuation of its protein level might be necessary for cell cycle progress. If this is the case, the accumulation of Ki-67 after proteasome inhibition could well contribute to the inhibition of BxPC-3 cell proliferation, although the precise function of Ki-67 still needs to be understood. Since the proteasome controls degradation of a large number of regulatory proteins, the accumulation of Ki-67 is obviously not solely responsible for the inhibited proliferation. We have found that CDK inhibitors $p27^{kip1}$ and $p21^{cip1}$ levels were enhanced after proteasome inhibition in the BxPC-3 cells. Our previous study in lymphocytes has shown that the enhanced $p27^{kip1}$ by LAC is correlated with inhibition of cyclin E-associated CDK2 activity [Wang et al., 1998]. Such an observation is consistent with the role of $p27^{kip1}$ as a G1 CDK inhibitor. In lymphocytes, there is actually an induction of $p21^{cip1}$ when the cells progress from the G0 to S phase, and this induction is inhibited by LAC [Wang et al., 1998]. It is speculated that a low level of $p21^{cip1}$ is required for the cells to enter the cell cycle [LaBaer et al., 1997]. BxPC-3 cells are cycling cells and probably have sufficient basal level $p21^{cip1}$. Therefore, further accumulation of the protein could well be inhibitory to CDKs, since it is a pan-CDK inhibitor, and this is also a contributing factor to the LAC-caused inhibition of cell proliferation. We

would like to reiterate that the proteasome might control degradation of other regulatory factors that are essential in proliferation and death of the BxPC-3 cells.

A rapid advance has been made in understanding the biological roles of the proteasome. We are now no longer considering it a housekeeping enzyme and a garbage collector. Its role in regulating cells growth and cell death starts to be appreciated and this will obviously lead to application of proteasome inhibitors in treating various pathological conditions.

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