Role of Proteasomes in T Cell Activation and Proliferation

Xin Wang, Hongyu Luo, Huifang Chen, William Duguid and Jiangping Wu

*J Immunol* 1998; 160:788-801;  
http://www.jimmunol.org/content/160/2/788

References  This article cites 64 articles, 19 of which you can access for free at:  
http://www.jimmunol.org/content/160/2/788.full#ref-list-1

Subscription  Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Role of Proteasomes in T Cell Activation and Proliferation

Xin Wang,* Hongyu Luo,* Huifang Chen,* William Duguid,‡ and Jiangping Wu‡†§

The role of proteasomes in T cell activation, proliferation, and apoptosis was investigated using a proteasome-specific inhibitor lactacystin (LAC). Inhibition of the proteasome activity by LAC repressed the mitogen-induced T cell proliferation. The proteasome activity was definitively required for the T cells to progress from the G0 to S phase. It was necessary to optimize the progress from the G2/S boundary to the G2/M phase, but not for the progress from the G2/M phase to the next G1 phase. Probably as a result of a blockage of cell cycle progress, the cycling, but not the resting, T cells underwent apoptosis when treated with LAC. Mechanistically, we have found that cyclin-dependent kinase-2 (CDK2) and the cyclin E-associated kinase (largely CDK2), but not CDK4, in the G1 phase were strongly inhibited by LAC. This could be an important mechanism for the proteasome to regulate the cell cycle. The degradation of cyclin E in the late G1 and early S phases was dependent on the proteasome, although it was unlikely that this accounted for the observed inhibition of T cell proliferation. There was a reduced decay of p27Kip1 in the late G1 phase when the proteasome activity was suppressed, and this might be a contributing mechanism for the observed inhibition of CDK2 activity. Interestingly, p27Kip1 was up-regulated during the G1 phase, and the up-regulation was inhibited by LAC. Our study shows that the proteasome plays pivotal roles in regulating T cell activation and proliferation, and its effect is probably exerted through multiple mechanisms. The Journal of Immunology, 1998, 160: 788–801.

The proteasome is a large protease complex. It is the main nonlysosomal proteolytic system in the cell and resides in the cytoplasm as well as in the nucleus (1). The proteasome possesses up to five different peptidase activities in different catalytic domains (2), and the best characterized ones are chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide-hydrolyzing (PGPH) activities (3, 4). The proteasome is responsible for the degradation of 70 to 90% of cellular proteins (5). Yet its activity is well controlled, and only those destined to be destroyed are timely digested by the proteasome. It therefore plays a critical role in irreversibly removing short-lived regulatory proteins and other types of proteins. Indeed, the degradation of some important regulators of cell proliferation, such as cyclin 2, cyclin 3, cyclin B, p53, and p27Kip1, is mediated by the proteasome (6–11). The activities of several important regulators involved in cell activation are also controlled by the proteasome. For example, the transacting nuclear factor NF-κB is degraded after the enzymatic cleavage of its precursor by the proteasome (12); IkBα, the inhibitor of NF-κB, and c-Jun protein are degraded via the proteasome pathway (12, 13).

According to sedimentation rates, the proteasome could be purified as 26S and 20S complexes. The 20S proteasome is a cylindrical proteolytic core composed of multiple α and β subunits. Each subunit is coded by a different gene in high eukaryotic cells, and the total number of subunits varies among different species (14). In vitro, the purified 20S proteasomes can digest small peptides in an ATP-independent fashion, but they are inactive on intact folded proteins (15). The 20S proteasome can bind at its ends a 19S regulator and forms the 26S proteasome, which degrades ubiquitinated protein in an ATP-dependent fashion (1). The 20S proteasome can also complex with an 11S activator called PA28 (14). PA28 is a ring-like hexamer or heptamer composed of α and β subunits (PA28a and PA28b), both of which are about 29 kDa in size (16, 17). It is not clear whether the 20S proteasome can associate with both 19S and 11S regulators at the same time. There are two better characterized mechanisms regulating the protein degradation via the proteasome pathway. The first is that of substrate selection. This process is controlled by a cascade of enzymes called the ubiquitin-activating enzyme, the ubiquitin-conjugating enzyme, and the ubiquitin ligase (1). In addition, the 19S regulator controls the entry of the ubiquitinated protein into the 20S catalytic core. The second mechanism is the activity of the 20S proteasome, which is enhanced by the 11S PA28 (16). It is not clear whether and how the 11S PA28 exerts its effect on the 26S proteasome, since it and the 19S regulator do not seem to associate with the 20S at the same time. Moreover, whether the 20S complex exists in parallel to the 26S complex in vivo is still an open question. Nevertheless, it has been shown that overexpression of PA28α could indeed significantly augment Ag processing by the proteasome in vivo (18).

Our recent work has revealed that PA28 expression is up-regulated during T cell activation, and probably as a result, the ex vivo proteasome activity is fourfold higher in the activated T cells than that in the resting T cells (19). Such an augmented activity probably reflects the increased need to destroy short-lived regulatory proteins and other types of proteins during T cell activation and

---

*Laboratory of Transplantation Immunology, Louis-Charles Simard Research Center, and ‡Nephrology Service, Notre-Dame Hospital, University of Montreal; and †Montreal General Hospital and §Department of Surgery, McGill University, Canada

Received for publication May 13, 1997. Accepted for publication October 7, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grant MT-11543 from the Medical Research Council of Canada and grants from the Kidney Foundation of Canada and the Heart and Stroke Foundation of Quebec (to J.W.), by the Nephrology Service of Notre-Dame Hospital, and a scholarship from the Fonds de la Recherche en Santé du Québec (to J.W.).

2 Address correspondence and reprint requests to Dr. Jiangping Wu, Laboratory of Transplantation Immunology, Pavilion A. de Sève, Louis-Charles Simard Research Center, Room Y-5616, Notre-Dame Hospital, 1560 Sherbrooke St. East, Montreal, Quebec, Canada H2L 4M1.

3 Abbreviations used in this paper: PGPH, peptidylglutamyl peptide-hydrolyzing; NF-κB, nuclear factor-κB; LLnL, N-acetyl-L-leucinyl-L-leucinal-L-norleucinal; LAC, lactacystin; CDK2, cyclin-dependent kinase-2; SAC, Staphylococcus aureus Cowan I; PE, phytohemagglutinin; HU, hydroxyurea; PVDF, polyvinylidene difluoride.
proliferation. Consequently, it is logical to hypothesize that blocking the proteasome activity will interfere with the activation and proliferation of T cells.

Certain peptide aldehydes, such as N-acetyl-L-leucinyl-L-leucinal-N-norleucinal (LNNL) and N-carbobenzoxy-L-leucinyl-L-leucinyl-L-norvalinal (MG115) are competitive inhibitors of chymotrypsin (20, 21). These agents could effectively block the chymotrypsin-like activity and, to a lesser extent, the trypsin-like and PGPH activities of the proteasome (5). They have been employed to study the function of the proteasome in various cellular processes. A caveat of such studies is that these peptide aldehydes are not specific to the proteasome peptides, and other cellular cytosine protease, such as calpain and cathepsin B (5, 22), are also potentially inhibited. This makes some interpretations less assuring.

In 1991, Omura et al. reported the discovery of lactacystin (LAC), which could induce neurite outgrowth (23, 24). Fenteaney et al. subsequently found that LAC is a proteasome-specific protease inhibitor (25). It inhibits the three major peptidase activities (i.e., chymotrypsin-like, trypsin-like, and PGPH activities) of the proteasome, and the inhibition of the first two is irreversible in vitro assays. LAC does not affect other proteases, such as calpain, cathepsin B, chymotrypsin, trypsin, and papain. Currently, LAC is the only proteasome-specific protease inhibitor available. Taking advantage of LAC’s specificity and potency, in this study we used this compound to investigate the roles of proteasomes in T lymphocyte activation and proliferation. We have demonstrated that the proteasome is essential for progression of T cells from the G1 to S phase. Probably as a result of blockage of cycling, the activated, but not the resting, T cells underwent apoptosis when treated with LAC. We have also shown that the proteasome controls the protein levels of p21Cip1 and p27Kip1 as well as CDK2 activity in the G1 phase, and such a control mechanism might be essential in cell cycle progression.

Materials and Methods

Reagents

RPMMI 1640, FCS, penicillin/streptomycin, and l-glutamine were purchased from Life Technologies (Burlington, Canada). Lymphoprep was purchased from Nycomed (Oslo, Norway). PHA, hydroxyurea, nocodazole, and histone H1 were obtained from Sigma Chemical Co. (St. Louis, MO). Staphylococcus aureus Cowan I (SAC) were obtained from Calbiochem (La Jolla, CA), and lactacystin was obtained from Dr. E. J. Corey (25). Human rIL-2 was obtained from Hoffmann-La Roche (Nutley, NJ), and anti-CD3 mAb OKT3 was obtained from American Type Culture Collection (Rockville, MD). FITC-conjugated anti-CD3 mAb (clone SFC18W-8C8) and PE-conjugated anti-CD25 mAb (clone HIT44H3) were purchased from Coulter (Miami, FL). Anti-CD28 mAb (clone 9.3) was a gift from Dr. P. Linsley (26). A fluorogenic chymotrypsin substrate, sLLVY-MCA, was obtained from Peninsula Laboratories (Belmont, CA). Rabbit antisera against cyclin A, cyclin E, p21Cip1, p27Kip1, CDK2, and CDK4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-32P]ATP (3000 Ci/mmol) and [125I]protein A (30 Ci/mg protein) were obtained from Amersham (Oakville, Canada), and [methyl-3H]thymidine (2 Ci/mmol) was obtained from ICN (Irvine, CA).

Cell culture

PBMC and tonsillar T cells were prepared as described previously (27, 28). The cells were cultured in RPMI 1640 supplemented with 10% FCS, l-glutamine, and antibiotics. [3H]thymidine uptake was conducted as described previously (27, 28).

DNA fragmentation assay

The assay was performed according to a protocol described by Liu et al. (29) with some modifications. Briefly, 2 to 6 million cells were resuspended in 50 μl of PBS followed by 300 μl of lysis buffer (100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.2 M NaCl, 0.2% (w/v) SDS, and 0.2 mg/ml protease K). After overnight incubation at 37°C, 350 μl of 3 M NaCl was added to the mixture, and cell debris was removed by centrifugation at 13,000 × g for 20 min at room temperature. DNA in the supernatant was precipitated with an equal volume of 100% ethanol. The pellet was washed with cold 70% ethanol and then dissolved in 20 μl of Tris-EDTA buffer containing 0.2 mg/ml RNase A. After incubation at 37°C for 2 h, the DNA was resolved on 2% agarose gel and visualized with ethidium bromide staining.

Electron microscopy

T cells and Jurkat cells were examined by electron microscopy as described by Tsao and Duguid (30).

Flow cytometry for IL-2Ra

Two-color staining with FITC-anti-CD3 and PE-anti-CD25 was performed on tonsillar T cells. The method was described previously (28).

Proteinase assay

Jurkat cells were cultured with various treatments and were harvested and sonicated in 300 μl of PBS on ice for 40 s. Twenty micrograms of protein per sample from the cleared lysates was supplemented to 100 μl with 0.1 M Tris buffer (pH 8.2). The fluorogenic chymotrypsin substrate sLLVY-MCA was added at a final concentration of 10 nM. The samples were incubated at 37°C for 15 min, and the reaction was terminated by adding 4 μl of 2 M HCl. The samples were then diluted to 2 ml with 0.1 M Tris, pH 8.2, and their fluorescence intensity was measured using a PFI fluorometer (Photo Technology International, South Brunswick, NJ). The excitation wavelength was 380 nm, and the emission wavelength was 440 nm.

Cell cycle synchronization of T cells and Jurkat cells

Tonsillar T cells were cultured in the presence of 2 μg/ml PHA and 1 mM hydroxyurea for 40 h. The cells thus treated were synchronized at the G1/S phase. The synchronization was released by washing out hydroxyurea, and the cells were cultured in medium for additional 6 to 22 h according to the requirements of the individual experiments. The synchronization of Jurkat cells was described in our previous publication (31). Briefly, the Jurkat cells were starved in isoleucine-deficient medium for 24 h, followed by 16-h treatment with 2 mM hydroxyurea (HU). Cells thus treated were synchronized at the G1/S boundary. For synchronization at the G2/M boundary, the G2/S synchronized cells were released from hydroxyurea and cultured in regular medium for 6 h, and then treated with 0.1 μg/ml nocodazole for 16 h. The cells were thus synchronized at the G2/M boundary.

Cell cycle analysis

Flow cytometry was employed for cell cycle analysis for T cells and Jurkat cells as described previously (31), using propidium iodide staining.

Immunoblotting

Immunoblotting was employed to evaluate the levels of cyclin E, cyclin A, p21Cip1, and p27Kip1. The general protocol was described in our previous publication (32). Briefly, lymphocytes were lysed in the presence of protease inhibitors. The cleared lysates were electrophoresed on 10% SDS-PAGE gels, blotted onto nitrocellulose filters, and probed with specific antibodies. An equal amount of lysates (40 μg) of each sample was resolved by 10% SDS-PAGE and was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). The membranes were then blocked with 5% milk and hybridized with rabbit antisera against cyclin E, cyclin A, p21Cip1, and p27Kip1 at the dilutions suggested by the manufacturer. The signals on the membrane were detected by [125I]protein A followed by autoradiography.

Immunoprecipitation and the kinase assay

Lymphocytes were lysed by the lysis buffer used for immunoblotting (32), and cleared lysates were quantitated for their protein content. For immunoprecipitation, 50 μl of rabbit antibody against CDK2, CDK4, or cyclin E was added to the lyastes equivalent to 20 or 40 μg of protein depending on the experiment. After 2-h incubation at 4°C, the immune complexes were recovered by protein A-conjugated Sepharose (Pharmacia Biotech, Montreal, Canada). The immune complexes bound to protein A-Sepharose were extensively washed in lysis buffer without detergents or EDTA and resuspended in 50 μl of kinase reaction buffer (100 mM NaCl, 20 mM HEPES (pH 7.5), 5 mM MnCl2, 5 mM MgCl2, 25 μM cold ATP, 2.5 μCi of [γ-32P]ATP, and 3 μg of histone H1 as a substrate). The reaction was conducted for 10 min at room temperature and was stopped by adding SDS-PAGE loading buffer. After boiling for 3 min, the samples were subjected to 10% SDS-PAGE. The proteins were then transferred to PVDF membranes, and the signals were detected by autoradiography.
Results
Proteasome activity is obligatory for activation and proliferation of T and B cells

The role of proteasome in T cell activation and proliferation was first examined in PBMC, using the proteasome-specific inhibitor LAC. PBMC were activated with various stimulants. LAC was added to the cells at the beginning of culture (0 h) along with the stimulants. $^{3}H$thymidine uptake between 48 and 64 h of 64-h cultures was used as a parameter for cell proliferation. As shown in Figure 1, LAC strongly and dose-dependently inhibited the T cell proliferation induced by the T cell mitogen PHA (Fig. 1A), by cross-linking TCR with anti-CD3e (Fig. 1B), or by $Ca^{2+}$ ionophore plus cross-linking of the T cell costimulating molecule CD28 (Fig. 1C). The T cell-independent B cell proliferation induced with SAC plus IL-2 in tonsillar B cells was also potently inhibited by LAC (Fig. 1D). In all four systems employed, LAC at $5 \mu M$ exerted near-maximal inhibition. The results suggest that LAC’s effect is not lymphocyte type (T or B cells) or stimulant specific. Rather, it probably affects certain downstream events governing a more general process(es) in lymphocyte activation and proliferation.

LAC causes apoptosis in activated, but not resting, T cells

Since LAC has been reported to induce apoptosis in U937 cells (32), it is crucial to examine whether the LAC-induced inhibition of cell proliferation is due to cell death, be it apoptosis or necrosis.

The viability of T cells and Jurkat cells after LAC treatment was first evaluated with trypan blue exclusion. Resting T cells (T cells in medium) or PHA-stimulated T cells were cultured with 10 $\mu M$ LAC (LAC added at the beginning of the culture). As shown in Figure 2A, after 16-h culture the viability of the cells showed only a minor decrease (~12%) in LAC-treated cells compared with that in cells without LAC (97 vs 92% for cells in medium, 94 vs 83% for cells with PHA). After a prolonged culture for 64 h, the decrease was more prominent, although it was still less than 27% (97 vs 79% for cells in medium, 90 vs 63% for cells with PHA).

There was a tendency for the activated T cells to be more susceptible to LAC than were the resting T cells. This became more evident when LAC was added to T cells 40 h after PHA activation (Fig. 2B). The viability of the activated T cells dropped from 94 to 46% after an additional 24-h culture, although 9-h culture did not change the viability significantly according to trypan blue exclusion. On the other hand, the viability of the resting T cells in
medium had only a small decrease (from 98% of the control to 87% of the LAC-treated cells) after 24 h in the presence of LAC. Why did LAC added at 0 h along with PHA cause less cell death than LAC added 40 h after PHA stimulation (Fig. 2, A vs B)? We will demonstrate later in this paper that LAC is rapidly degraded in the cell culture. After 24 h in culture medium, LAC lost its activity, and at 40 h, when the T cells were fully activated and became more susceptible, there was no biologically active LAC in the culture. This could explain the observed difference in terms of viability between the 0 and 40 h addition of LAC to PHA-activated T cells.

The effect of LAC on Jurkat cells was quite similar to that on activated T cells. Less than 8-h exposure to 6 mM LAC did not induce apparent Jurkat cell death, while about 60% of the Jurkat cells were trypan blue positive after 24-h culture with LAC (Fig. 2 C).

We next employed DNA laddering to study the mode of cell death caused by LAC, and the result of this experiment also reflected the degree of cell death after different treatments. As shown in Figure 2D, resting T cells treated with 10 μM LAC for 24 h showed no apparent DNA breakdown (lanes 1 and 2). This correlated to the good cell viability, as shown in Figure 2B. On the other hand, clear DNA ladders could be observed in activated T cells (40 h post-PHA stimulation) treated with LAC for additional 9 h (lanes 3 and 4). After 24 h of LAC treatment, the ladders became less discrete, and this probably reflected further DNA fragmentation. For Jurkat cells, DNA fragmentation could be detected as early as 6 h after LAC treatment, and after 16 h, the fragmentation became more prominent (Fig. 2E).

Electron microscopy was also employed to examine the mode of cell death induced by LAC. The resting T cells (cells cultured in medium; Fig. 3A), activated T cells (40 h after PHA activation;
Fig. 3C), and Jurkat cells (Fig. 3E) were all healthy looking. Occasional condensed nuclei were observed in medium-cultured T cells (Fig. 3A), and this is not unusual. The resting T cells treated with LAC (10 μM) for 24 h were still healthy (Fig. 3B). However, nuclear condensation, which is a hallmark of apoptosis, was frequently observed in activated T cells and Jurkat cells after they were exposed to LAC (10 and 6 μM, respectively) for 24 h (Fig. 3, D and F).

The following conclusions are drawn from the results of this section. 1) Resting T cells or T cells in their early activation phase (24 h after PHA stimulation) are not sensitive to LAC in terms of cell viability. Consequently, there is still a significant percentage of live cells after 64-h culture if LAC is added once, at the beginning of culture. 2) Less than 8 to 9 h of LAC treatment does not significantly affect the viability of activated T cells (40 h post-PHA activation) or Jurkat cells, as determined by trypan blue exclusion. 3) Prolonged treatment (24 h) of activated T cells or Jurkat cells with LAC causes cell death in the form of apoptosis, although signs of apoptosis could be detected as early as 9 h, in T cells, and 6 h, in Jurkat cells, after LAC treatment.

The data in this section indicate the following. 1) LAC’s differential effect on the viability of resting vs cycling cells suggests that it is not simply nonspecific cytotoxicity, but relates to the status of the cell cycle. 2) Cell death, without a doubt, contributes to but cannot solely account for the observed inhibition of proliferation by LAC, since there is still a significant percentage (~60%) of live cells at the end of the culture, as determined by trypan blue exclusion. Moreover, we will elaborate later that the cell death is a consequence of blockage of cell cycle progress. 3) Admittedly the trypan blue-negative cells include some early apoptotic cells, as evidenced by the fact that DNA laddering could be detected in a largely trypan blue-negative population. However, it does not necessarily mean that the whole population is dead. We will later demonstrate that most Jurkat cells treated with LAC for 6 to 8 h could still progress normally in the cell cycle despite the fact that a certain degree of apoptosis could be detected in these cells. 4) LAC can be used to study the role of proteasomes in lymphocyte activation and proliferation as long as the compound is applied only once at the beginning of activation of resting T cells, and the experiment is conducted in 24 to 40 h, or LAC is present for <8 h in the case of cycling cells, since such treatments do not drastically affect the viability of the cells.

**Effect of LAC was rapid and reversible**

We next investigated how fast and how long LAC could exert its effects on the lymphocytes, since this information is necessary to assess the requirement for the proteasome activity in events related to cell activation and proliferation. PBMC were pretreated with LAC (10 μM) or medium for the period indicated in Figure 4A. The cells were then washed and recultured in the presence of PHA. Fig. 4C), and Jurkat cells (Fig. 3E) were all healthy looking. Occasional condensed nuclei were observed in medium-cultured T cells (Fig. 3A), and this is not unusual. The resting T cells treated with LAC (10 μM) for 24 h were still healthy (Fig. 3B). However, nuclear condensation, which is a hallmark of apoptosis, was frequently observed in activated T cells and Jurkat cells after they were exposed to LAC (10 and 6 μM, respectively) for 24 h (Fig. 3, D and F).

The following conclusions are drawn from the results of this section. 1) Resting T cells or T cells in their early activation phase (24 h after PHA stimulation) are not sensitive to LAC in terms of cell viability. Consequently, there is still a significant percentage of live cells after 64-h culture if LAC is added once, at the beginning of culture. 2) Less than 8 to 9 h of LAC treatment does not significantly affect the viability of activated T cells (40 h post-PHA activation) or Jurkat cells, as determined by trypan blue exclusion. 3) Prolonged treatment (24 h) of activated T cells or Jurkat cells with LAC causes cell death in the form of apoptosis, although signs of apoptosis could be detected as early as 9 h, in T cells, and 6 h, in Jurkat cells, after LAC treatment.

The data in this section indicate the following. 1) LAC’s differential effect on the viability of resting vs cycling cells suggests that it is not simply nonspecific cytotoxicity, but relates to the status of the cell cycle. 2) Cell death, without a doubt, contributes to but cannot solely account for the observed inhibition of proliferation by LAC, since there is still a significant percentage (~60%) of live cells at the end of the culture, as determined by trypan blue exclusion. Moreover, we will elaborate later that the cell death is a consequence of blockage of cell cycle progress. 3) Admittedly the trypan blue-negative cells include some early apoptotic cells, as evidenced by the fact that DNA laddering could be detected in a largely trypan blue-negative population. However, it does not necessarily mean that the whole population is dead. We will later demonstrate that most Jurkat cells treated with LAC for 6 to 8 h could still progress normally in the cell cycle despite the fact that a certain degree of apoptosis could be detected in these cells. 4) LAC can be used to study the role of proteasomes in lymphocyte activation and proliferation as long as the compound is applied only once at the beginning of activation of resting T cells, and the experiment is conducted in 24 to 40 h, or LAC is present for <8 h in the case of cycling cells, since such treatments do not drastically affect the viability of the cells.

**Effect of LAC was rapid and reversible**

We next investigated how fast and how long LAC could exert its effects on the lymphocytes, since this information is necessary to assess the requirement for the proteasome activity in events related to cell activation and proliferation. PBMC were pretreated with LAC (10 μM) or medium for the period indicated in Figure 4A. The cells were then washed and recultured in the presence of PHA.
Thymidine uptake was measured 3 days later. It was clearly demonstrated that 3-h preincubation with LAC was sufficient to cause significant inhibition of the subsequent mitogen-stimulated proliferation in T cells, although 16-h preincubation with LAC was more effective. This result indicates that LAC can enter the cells rapidly within 3 h.

We used Jurkat cells that have high constitutive proteasome activity to evaluate the duration of LAC’s effect once the drug entered the cells. Jurkat cells were treated with LAC (6 μM) for 3 h, which was sufficiently long for the compound to enter the cells as shown above. The cells were then thoroughly washed and reconstituted, and they were harvested 0, 5, and 21 h after the wash. The proteasome activity in the cell lysates was measured using a chymotrypsinogen substrate. We have previously established that the enzyme activity measured by this assay was predominantly (>90%) derived from the proteasome (19). As shown in Figure 4B, the proteasome activity in Jurkat cells was almost completely inhibited by 3-h preincubation with LAC at 6 μM. Five hours after the LAC was washed out, the proteasome activity in the cells was still significantly inhibited, but the inhibition was reduced compared with that at 0 h. By 21 h, the proteasome activity returned to a near-normal level. It should be noted that the short 3-h treatment with LAC did not affect the viability of the Jurkat cells, and this is also reflected by the normal proteasome activity of the treated cells at 21 h. The result shows that LAC is not stable and loses its activity within 21 h in the cells.

We also investigated whether LAC was stable in the culture supernatant. LAC (6 μM) was added to Jurkat cells culture for 4, 6, 16, or 24 h. The conditioned medium was harvested and used to treat fresh Jurkat cells for 3 h, then the proteasome activity in the lysates of the fresh Jurkat cells was assayed. As shown in Figure 4C, 4- to 24-h conditioned medium without LAC did not affect the proteasome activity of the fresh Jurkat cells. The medium conditioned with LAC up to 6 h could still actively inhibit the enzymatic activity, but after 16 h, the LAC-conditioned medium lost its inhibitory effect. The loss of LAC activity in the 16- and 24-h conditioned medium is unlikely to be due to trapping of LAC by proteasomes released by dead Jurkat cells, because LAC could rapidly enter the live cells, and the equilibrium of the LAC concentration between both sides of the cytoplasmic membrane should be established very fast. Thus, the proteasomes, whether released or not, should not make a difference in terms of trapping LAC. Besides, we have also noticed that LAC kept in cell-free culture medium at 4°C lost its activity within 24 h (data not shown). These results indicate that LAC is not only unstable within the cells, but is also unstable in the supernatant.

LAC’s capability to enter the cells to inhibit the proteasome activity rapidly (<3 h) and its short active duration within the cell and in the culture medium (~16 h) make the compound a useful reagent to evaluate the requirement for proteasome activity in various events during cell activation and proliferation, since we could pinpoint the period when proteasome activity is critical.

**Proteasome activity is required for IL-2Ra up-regulation**

In the four systems of T and B cell activation and proliferation studied in the first section, the growth-promoting activity of IL-2 is indirectly (for stimulation by PHA, anti-CD3, and anti-CD28 plus ionomycin) or directly (for SAC plus IL-2) involved. We then investigated the role of proteasome in IL-2Ra expression and IL-2 production. As shown in Figure 5, CD25 was up-regulated in CD3+ T cells 40 h after stimulation with PHA. When LAC (10 μM) was added at the beginning of the culture, the up-regulation was significantly inhibited. On the other hand, IL-2 production by PBMC 2 to 4 days after PHA stimulation in the absence or the presence of LAC (10 μM, added at the beginning of the culture) was also examined, but no consistent difference was found (data not shown). Under the experimental condition used, the viability of the LAC-treated cell was reasonable (>80% at 40 h), as described in the previous section, because LAC was added only once initially. Moreover, the absence of a consistent change in IL-2 production in LAC-treated cells was a functional indication that the cell viability was reasonable and is not of concern in interpreting the data. The results from this section indicate that IL-2Ra up-regulation, but not IL-2 production, is proteasome dependent, and the suppressed IL-2Ra expression probably contributes to LAC’s inhibitory effect on T cell activation and proliferation.

**Proteasome activity is critically required between the Go and G1/S boundary in T cells**

Like that of normal T cells, the proliferation of Jurkat cells was potently inhibited by LAC (data not shown). We used synchronized Jurkat cells to identify the LAC-sensitive phase(s) of the cell cycle.

Jurkat cells were first synchronized at the G2/M boundary by nocodazole (Fig. 6A). The cells were released from the blockage by washing out nocodazole. In the control sample, more than half the cells traversed through the M phase and arrived at the G1 phase within 4 h. In the test sample, LAC (6 μM) was added to the culture 3 h before the release, so the compound could have enough time to enter the cells. LAC was also added to the culture after the release. However, the Jurkat cells treated with LAC traversed through the M phase to the G1 phase at a similar pace as the control cells. Since the total duration of the assay was around 7 h (3-h preincubation plus 4 h after the release), LAC was certainly active during this period. The fact that most of the synchronized Jurkat cells could traverse through G2/M to G1 in the presence of LAC for 7 h again suggests that the viability of the cells thus treated is not a matter of concern. This result shows that the G2 to G1 progression is not proteasome dependent.

We next studied the requirement for proteasome activity of the progression from the G1/S boundary to the G2/M phase. Jurkat cells were synchronized at the G1/S boundary by HU blockage. The cells were then released by washing out HU. Within 9 to 12 h, the majority of the cells progressed to the S and G2/M phases (Fig. 6B). When LAC was added to the culture immediately after cell release, it slowed, but did not block, cell cycle progression from the G1/S boundary to the G2/M phase, as evidenced by the histograms 6 and 9 h after the release. It should be noted that although the percentage of cells in the S/G2/M phase in the LAC-treated sample was similar to that in controls (the inset table of Fig. 6B), the peak of fluorescence lagged behind (histogram array). Beyond 9 h, the cells gradually lost their synchronization, the viability of the cells started to decline, and LAC gradually lost its activity, so the data became difficult to interpret. The results from this part of the study suggest that proteasome activity is required for optimal progression from the G1/S boundary to the G2/M phase, because the progression could still proceed, albeit at a slower pace, when proteasome activity is inhibited. These results also imply that the absolutely proteasome-dependent window during the cell cycle, as evidenced by the near-total inhibition of S phase entry in LAC-treated mitogen-stimulated lymphocytes according to the proliferation data, must be in the G1/S boundary (33).

The cycling Jurkat cells are obviously not the best model to study the events in the G2 phase, since the G2/M synchronization becomes desynchronized by the time the cells re-enter the S phase, and there is no appropriate method to synchronize the Jurkat cells.
at the early G1 phase. We therefore decided to use mitogen-stimulated normal T cells to study the role of the proteasome in the G1 phase.

T cells from PBMC were at G0 when isolated. After 16-h stimulation with PHA, they remained at the pre-S phase (Fig. 6C). At 40 h, about 20% of the cells were in the S and G2/M phases. The peak of [3H]thymidine uptake according to a 16-h pulse was between 48 and 64 h (data not shown), although at 64 h, the percentage of cells in the S and G2/M phases was still about 20% (Fig. 6C). The lack of an increase in the percentage of cells in the S and G2/M phases at 64 h compared with that at 40 h was probably due to the exit of the cells from the S and G2/M phases. It should be noted that the cycling T cells in this system never reached 100%, because about 15% of the cells were non-T cells, and an additional 20% were nonresponsive T cells. Taking the cell proliferation and cell cycle analysis together, the G1/S boundary of the cycling T cells should be between about 35 and 48 h after PHA stimulation. The boundary was broad because the synchronization was not ideal.

In this model the role of the proteasome in the S phase entry was examined. As shown in Figure 6C, LAC added once at 16 h could totally block S phase entry when examined at 40 h. We have noticed that when the cell viability was evaluated at 40 h, there was an increase in cell death, comparing the 16 h addition of LAC with the 0 h addition (25 vs ~17%; data not shown). The increased cell death was also reflected in the cells with <2 N DNA in the 40-h histogram. However, such a viability was still reasonable and would not invalidate our conclusion. According to [3H]thymidine uptake, LAC was strongly inhibitory even when added as late as 40 h (Fig. 6D). However, no difference in the percentage of the population in the S and G2/M phases was observed at 64 h regardless of whether LAC was added at 40 h according to flow cytometry (Fig. 6C). The discrepancy could be explained by the fact that 20% of the cells were already in the S and G2/M phases at 40 h when LAC was added. LAC prevented additional cells from entering the S phase, thus explaining the lack of [3H]thymidine uptake. At the same time, the drug slowed cell cycle progression from the G1/S boundary to the G2/M phase, hence the finding of a lingering population in the S and G2/M phases according to flow cytometry.

It is worth mentioning the inhibition of proliferation by LAC was probably a combined effect of blocking cell cycle progress and cell death, with the latter possibly being the consequence of the former. The later the compound was added when more T cells are activated, the larger a proportion of the effect should be attributed to cell death caused by LAC. The extensive cell death for the sample treated with LAC at 40 h was not fully reflected in the flow cytometry (Fig. 6C) in the form of cells with <2 N DNA. This was due to the fact that the histogram was gated on a region of largely viable cells.
The results from this section indicate that the proteasome activity is not required from the G2/M to the G1 phase. It optimizes the progression from the G1/S boundary (as defined by the HU target point) to the G2/M phases, and it is absolutely required for the progression from the G1 to the S phase.

**Proteasome activity is essential for CDK2, but not for CDK4, function**

CDKs are critical for cell proliferation. CDK4 is essential in the early to mid-G1 phase to facilitate S phase entry (34, 35), and CDK2 is critical in the late G1 as well as throughout the S phase for cell cycle progression (36). We therefore examined the role of the proteasome in CDK4 and CDK2 activities in mitogen-stimulated T cells. In all the models used in this section, LAC was added only once at the beginning of culture. Consequently, the viability of the LAC-treated cells was good for the first 16 h and was reasonable at 40 h, but was not a factor that might interfere with the interpretation of the results.

As shown in Figure 7A, the resting T cells had some CDK4 activity, and the activity reached a plateau within 16 h of activation. This was in agreement with the critical role of CDK4 in the early G1 phase. Inhibition of the proteasome activity by LAC from 0 to 16 h (LAC added once at 0 h) did not affect CDK4 activity when examined at 16 and 40 h (Fig. 7A). This indicates that the induction and maintenance of CDK4 activity during the G1 phase are not proteasome dependent.

In contrast to CDK4, CDK2 activity was augmented at 16 h, but the augmentation was more prominent at a later stage close to 40 h after mitogen stimulation (Fig. 7B), and this reflected its essential role starting from the late G1 phase and extending to the early S phase. The presence of LAC from 0 to 16 h (LAC added once at 0 h) significantly inhibited CDK2 activity at 16 h and more so at 40 h. Therefore, proteasome activity during the early activation stage (0–16 h) is essential for activation of the kinase at the G1 phase and early S phase. The unchanged CDK4 activity in the LAC-treated cells at 40 h served as an internal control for the repressed CDK2 activity and indicated that the latter was not due to the viability problem.

Since at the late G1 phase cyclin E is the predominant partner of CDK2 (37), we next examined the cyclin E-associated CDK activity. As shown in Figure 7C, despite the fact that the level of cyclin E protein was increased after LAC treatment (LAC added once at 0 h), cyclin E-associated kinase activity was almost completely inhibited by LAC. These results indicate that CDK2 activity and most likely cyclin E-associated CDK2 activity in the late G1 phase are proteasome dependent. The results also suggest that the inhibition of CDK2 activity is probably an important mechanism accountable for LAC’s effect in blocking S phase entry.

**Degradation of cyclin E, but not cyclin A, is proteasome dependent**

Oscillation of cyclins during the cell cycle is a mode of regulation for CDK activities. Since the CDK2 activity is proteasome dependent, and CDK2 associates predominantly with cyclin E and cyclin A at the G1/S boundary and during the S phase, respectively (38, 39), we studied the role of the proteasome in degradation of these two cyclins. As shown in Figure 8A, the cyclin E level was apparently increased around 40 h after PHA stimulation of T cells, which were then at the G1/S boundary. If the activated cells were treated with HU, the cyclin E level was significantly enhanced compared with that in cells treated with PHA alone (Fig. 8A). This reflects a better synchronization at the G1/S boundary by HU and was consistent with our knowledge that the cyclin E level peaked at the boundary. After the boundary, the cyclin E level started to decline, and the decline was prevented by LAC (Fig. 8A). This clearly demonstrates that the degradation of cyclin E is a proteasome-dependent process, although whether the increased cyclin E level contributes to LAC’s effect on the cell cycle is a matter of debate (to be elaborated in Discussion).

For cyclin A, the level was increased around the late G1 phase after the mitogen stimulation as shown in Figure 8B. The blockage of the cycle at the G1/S boundary with HU did not further increase the cyclin A level. However, when the cycle passed the boundary and entered the S phase, the cyclin A level was significantly augmented (Fig. 8B), consistent with the idea that cyclin A is mainly an S phase cyclin. Unlike that of cyclin E, the level of cyclin A did not decline during the S phase, and LAC did not affect its level during this period. This suggests that the proteasome is not involved in cyclin A degradation, at least in the G1 and S phases, and that LAC’s effect on inhibiting cell proliferation is unlikely to be mediated via cyclin A levels.

The G1/S phase synchronized T cells represented activated cells, and prolonged exposure to LAC would cause significant cell death. However, 6-h treatment of LAC did not apparently affect cell viability, while the blockage of cyclin E degradation, but not cyclin A degradation, was obvious at that time point. Moreover, cyclin A could be considered an internal control for cyclin E, indicating that the LAC-induced cell death should not affect the conclusion in this section.

**Role of the proteasome in regulating levels of CDK inhibitors p27kip1 and p21Cip1**

In addition to the cyclin levels, the CDK activities were controlled by several low m.w. inhibitors. We have examined in this study the effect of the proteasome on the CDK inhibitors p27kip1 (39) and p21Cip1 (40). As shown in Figure 9A, the resting T cells had a high level of p27kip1, and the level decreased gradually when the cells moved to the G1/S boundary 40 h after mitogen stimulation. This is in agreement with previous reports (41, 42). The presence of LAC (added once at 0 h) significantly blocked the decrease when assayed at 16 h, showing that the degradation is a proteasome-dependent process. The blockage was less obvious when assayed at 40 h, probably because of the gradual loss of LAC activity during the 40-h culture. The result suggests that the blocking of p27kip1 degradation is a mechanism that contributes to the inhibitory effect of LAC on CDK2 activity.

Unlike p27kip1, p21Cip1 had a low level of expression in resting T cells. The level was rapidly augmented after 16-h PHA activation, and the high level was maintained at the G1/S boundary at 40 h (Fig. 9B). Such an induction suggests that p21Cip1 might be required in the G1 phase for roles other than a CDK inhibitor (to be elaborated in Discussion). Interestingly, LAC strongly suppressed the up-regulation of p21Cip1 in the G1 phase, indicating that the expression of p21Cip1 is proteasome dependent and suggesting that the proteasome might facilitate cell proliferation via its role in p21Cip1 up-regulation during the G1 phase. In this experiment, LAC was only added once at the beginning of the culture, and the viability of the treated cells at 16 h was good (83%) and should not be a concern in drawing conclusions.

**Discussion**

The proteasome is a large protease complex. More and more we realize that the proteasome does not simply perform a housekeeping duty to degrade spent proteins in the cell. It actually plays an active role in regulating various cell processes by selectively destroying regulatory proteins. In this study we investigated the role
FIGURE 6.

A

Medium

LAC

8h

4h

FL3

FL3

G2/M

Count

FL3

Percentage of cells in S/G2/M and G1 phases

<table>
<thead>
<tr>
<th></th>
<th>Med</th>
<th>LAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2/M (0h)</td>
<td>82.6</td>
<td>17.4</td>
</tr>
<tr>
<td>4h</td>
<td>33.0</td>
<td>67.0</td>
</tr>
<tr>
<td>8h</td>
<td>41.9</td>
<td>58.1</td>
</tr>
</tbody>
</table>

B

Medium

LAC

24h

15h

12h

9h

6h

3h

N0N.SYN

G1/S

FL3

FL3

Percentage of cells in G1 and S/G2/M phases

<table>
<thead>
<tr>
<th></th>
<th>Med</th>
<th>LAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Sync.</td>
<td>45.5</td>
<td>54.5</td>
</tr>
<tr>
<td>G1/S (0h)</td>
<td>75.0</td>
<td>25.0</td>
</tr>
<tr>
<td>3h</td>
<td>48.9</td>
<td>53.1</td>
</tr>
<tr>
<td>6h</td>
<td>34.4</td>
<td>65.6</td>
</tr>
<tr>
<td>9h</td>
<td>30.3</td>
<td>69.7</td>
</tr>
<tr>
<td>12h</td>
<td>42.3</td>
<td>57.7</td>
</tr>
<tr>
<td>15h</td>
<td>45.9</td>
<td>53.1</td>
</tr>
<tr>
<td>20h</td>
<td>58.0</td>
<td>44.0</td>
</tr>
</tbody>
</table>
of the proteasome in T cell cycle progress and the underlying mechanisms, using a proteasome-specific inhibitor LAC. There are several major findings from this study. 1) The entry into the S phase of mitogen-stimulated T cells depends on the proteasome activity. The proteasome activity is not only required in the early but also in the late G1 phase for a successful S phase entry of the lymphocytes. 2) Mechanistically, the activation of CDK2 and most likely the cyclin E-associated CDK2 during the G1 phase, which is pivotal for S phase entry, is proteasome dependent, while the CDK4 activity is not. 3) Probably as consequence of cell cycle
blockage, the cycling T cells, but not the resting T cells, undergo apoptosis when treated with LAC. 4) Further mechanistic study has revealed that the decline of a CDK inhibitor, p27Kip1, from the G0 phase to the S phase is blocked by LAC.

Some other interesting findings are also reported in this study. IL-2Rα CD25 up-regulation, but not IL-2 production, is proteasome dependent; this was previously undocumented. The degradation of cyclin E, but not that of cyclin A, depends on the proteasome. The induction of CDK inhibitor p21Cip1 relies on the proteasome; this is also a new finding.

LAC is the most selective proteasome inhibitor presently known. It has been reported that LAC irreversibly inhibits the chymotrypsin-like and trypsin-like activities of the proteasome when tested in vitro using the purified 20S complex (25). Interestingly, using the chymotrypsin-like activity of the proteasome as an indicator, we found that the inhibition of proteasome activity in the cells was rapidly reversible within <16 h, and the activity of LAC in the culture supernatants disappeared equally fast. This suggests that LAC is quickly degraded by certain enzymes present in the cells and culture supernatants. Alternatively, its spontaneous hydrolysis at high pH, as reported by Dick et al. (43), might be accelerated by certain enzymes from the cells at physiologic pH.

We have shown that LAC did not cause significant death in resting T cells or T cells in their early stage of activation. A short period of exposure (<8 h) of cycling T cells or Jurkat cells to LAC did not significantly affect the viability of these cells. This is consistent with the report by Grimm et al. (44). We, therefore, restricted analysis of our results within these limits to avoid possible complications in data interpretation due to extensive cell death.

We, however, have found that prolonged exposure (overnight culture) of cycling T cells to LAC, be they T cells 40 h after PHA stimulation or Jurkat cells to LAC did not significantly affect the viability of these cells. This is consistent with the report by Grimm et al. (44). We, therefore, restricted analysis of our results within these limits to avoid possible complications in data interpretation due to extensive cell death.

We, however, have found that prolonged exposure (overnight culture) of cycling T cells to LAC, be they T cells 40 h after PHA stimulation or Jurkat cells to LAC did not significantly affect the viability of these cells. This is consistent with the report by Grimm et al. (44). We, therefore, restricted analysis of our results within these limits to avoid possible complications in data interpretation due to extensive cell death.
inhibitor LAC could increase the cyclin E level in the G₁ phase and block the decrease in cyclin E in the S phase. This has confirmed and extended a previous observation that the degradation of cyclin E is ubiquitin dependent and can be inhibited by a less specific proteasome inhibitor, LLnL (51). However, it is unlikely that the inhibition of S phase entry by LAC is due to the block of cyclin E degradation for the following reasons. 1) The major inhibition of cyclin E degradation occurs in the S phase, and it is already irrelevant to S phase entry. 2) Guadagno et al. have reported that overexpression of cyclin E does not affect cell cycle progression (52). Thus, the moderate increase in the cyclin E level during the G₁ phase in LAC-treated cells is unlikely to be a cause of the inhibition of CDK2 activity. The level of cyclin A in the G₁ and S phase was not affected by LAC. This shows that the degradation of cyclin A in the G₁ and S phases, if it occurs, is not via the proteasome pathway, and that the inhibited CDK2 activity in the G₁ and S phases is not due to the altered cyclin A level in LAC-treated T cells.

Six low molecular weight (m.w.) CDK inhibitors have been recently identified in mammalian cells, and they play critical roles in regulating CDK activity. These inhibitors can be classified into two groups. The first group comprises four members of Ink4 family inhibitors (p16Ink4a, p15Ink4b, p18Ink4c, and p19Ink4d) (53–55). They specifically inhibit the activities of CDK4 and CDK6 by directly binding to these CDKs, and they are not known to affect other CDKs. Using polyclonal rabbit Abs (Santa Cruz Biotech) against these inhibitors, we could marginally detect the p16Ink4a signal in immunoblots of the T cell and Jurkat samples, but LAC treatment did not cause noticeable changes in the p16Ink4a level (data not shown). On the other hand, signals of p15Ink4b, p18Ink4c, and p19Ink4d in these cells were not detectable (data not shown). We have also shown that the CDK4 activity was not modulated by LAC. Together, these results suggest that the regulation of the CDK4 activity is not a proteasome-dependent process, and the observed antiproliferative effect of LAC is unlikely to be mediated through the protein levels of the Ink4 family CDK inhibitors.

The second group of inhibitors consists of p27Kip1 and p21Cip1 (56). They only interact with cyclin-CDK complexes, not with CDKs alone. p27Kip1 inhibits the activities of cyclin D-CDK2 and cyclin E-CDK2 (39), p27Kip1 is present at the highest level in the quiescent (G₀) and early G₁ phases, and the level decreases as the cells enter the S phase (42, 57, 58). The degradation of p27Kip1 is ubiquitin dependent and can be blocked by the chymotrypsin inhibitor LLnL (47). We have confirmed and extended these observations using the proteasome-specific inhibitor LAC. We have shown that in lymphocytes the decline of p27Kip1 from the G₀ phase to the S phase could be suppressed by LAC, and it was thus proteasome dependent. The lingering high level of p27Kip1 at the end of the G₁ phase in LAC-treated cells could well be a contributing mechanism for the LAC-caused CDK2 inhibition, which could, in turn, contribute to the block of S phase entry. In this regard, it is worth mentioning that rapamycin inhibits p27Kip1 degradation (42) and CDK2 activity (59), and that our study shows that rapamycin inhibits the mitogen-induced proteasome activity (19). Therefore, it is plausible that the proteasome/p27Kip1/CDK2 pathway might mediate to some extent the antiproliferative effect of rapamycin in lymphocytes.

The finding that LAC inhibited p21Cip1 induction in the G₁ phase was quite intriguing. p21Cip1 is reportedly a pan-CDK inhibitor, and it inhibits the activities of cyclin D-CDK4/6, cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDK1 (40). However, we have found that the p21Cip1 level was significantly augmented from the G₀ to the late G₁ phase, and that inhibition of p21Cip1 by LAC correlated with the blocking of S phase entry. These results
suggest that p21Cip1 might have positive functions in cell cycle progression during the G1 phase in addition to its inhibitory effect on CDK2 activity, which was actually enhanced in the late G1 phase despite the increase in p21Cip1 protein. Indeed, p21Cip1 protein has a domain that binds to the proliferation cell nuclear Ag, and a different domain that binds to the cyclin E-CDK2 (60). Such a structure suggests that it might serve as an adaptor to connect CDK2 with proliferation cell nuclear Ag, which, in turn, associates with a group of other proteins involved in DNA replication control (61). The adaptor function might explain the required induction of p21Cip1 in the G1 phase and might be responsible for its putative positive role. There are several recent articles describing the positive role of p21Cip1 in cell proliferation. Zhang et al. have reported that p21Cip1–containing cyclin kinases exist in both active and inactive states (62). Mentel et al. showed that mice lacking p21Cip1 have decreased cycling and absolute number of marrow and spleen hematopoietic progenitors (63). Most interestingly, Larber et al. have shown that at a low concentration, p21Cip1 promotes the assembly of the active CDK4/cyclin D complex, whereas at higher concentrations, it inhibits this activity (64). Of course, in our model, CDK4 activity is probably not important for LAC’s effect on cell proliferation, but the positive role of p21Cip1 has been supported by this finding in principle.

Inasmuch as the proteasome degrades a broad spectrum of proteins, its regulatory role in T cell proliferation probably involves multiple mechanisms and is not restricted to our findings on the CDKs and CDK inhibitors, as alluded to above. As an example, inhibition of CD25α up-regulation, as shown in this study, could be one of many other mechanisms. It should be noted that there is an NF-κB binding site in the 5′ flanking region of the CD25α gene (65), and NF-κB’s activation is achieved by the proteasome-dependent process of NF-κB precursors and degradation of the NF-κB inhibitor IκBα (12). However, we believe that factors other than NF-κB are also responsible for the inhibition of CD25α up-regulation, because the IL-2 promoter also contains an NF-κB promoter in the 5′ flanking region in the 5′-flanking region. However, the IL-2 promoter also contains an NF-κB binding site in the 5′ flanking region of the CD25α gene (65), and NF-κB’s activation is achieved by the proteasome-dependent process of NF-κB precursors and degradation of the NF-κB inhibitor IκBα (12). However, we believe that factors other than NF-κB are also responsible for the inhibition of CD25α up-regulation, because the IL-2 promoter also contains an NF-κB binding site in the 5′ flanking region. However, the IL-2 promoter also contains an NF-κB binding site in the 5′ flanking region. However, the IL-2 promoter also contains an NF-κB binding site in the 5′ flanking region. However, the IL-2 promoter also contains an NF-κB binding site in the 5′ flanking region. However, the IL-2 promoter also contains an NF-κB binding site in the 5′ flanking region.

Our study has revealed that the proteasome has a pivotal function in immune responses, and its importance in the immune system has been underestimated in areas other than Ag processing. Much attention has been devoted to regulation of the anabolic process or protein modification to achieve immune regulation. Our study has proved that the other side of the coin, i.e., protein degradation via the proteasome, is an equally important process in this regard.

Acknowledgments

The authors thank Dr. Julius Gordon for critical reading of the manuscript, and Ms. Carmen Rodriguez for excellent secretarial assistance.

References

50. Tsai, L. H., T. Takahashi, V. S. Caviness, Jr., and E. Harlow. 1993. Activity and
63. Tsai, L. H., T. Takahashi, V. S. Caviness, Jr., and E. Harlow. 1993. Activity and