Regulation of Gene Expression of Proteasomes (Multi-protease Complexes) during Growth and Differentiation of Human Hematopoietic Cells*

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We have reported that proteasomes are expressed at abnormally high levels in various hematopoietic tumor cells (Kumatori, A., Tanaka, R., Inamura, N., Sone, S., Ogura, T., Matsumoto, T., Tachikawa, T., Shin, S., and Ichihara, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 87, 7071-7075). In the present study, we examined changes in the expressions of proteasomes during growth of peripheral T-lymphocytes from healthy adults and differentiation of human leukemic cell lines. Up-regulation of mRNAs encoding multiple proteasome subunits was observed during proliferation of resting T-cells induced by mitogens such as phytohemagglutinin and interleukin-2. In contrast, in vitro terminal differentiation into monocyctic, granulocytic, and erythroid cells of various immature leukemic cell lines, such as HL-60 promyelocytic leukemia cells and K562 erythroleukemia cells, by various inducing agents caused rapid and marked down-regulation of proteasome expression, independently of the cell type, direction of differentiation, or type of signal. The syntheses of proteasome subunits of 21-31 kDa and their associated components of 35-110 kDa, measured by [35S]methionine incorporation, were much higher in mitogen-activated T-cells and unstimulated HL-60 cells, which grow rapidly, than in resting and differentiated cells, indicating apparent correlations of the mRNA levels of proteasomes with their translational activities. However, immunocytochemically, no detectable difference in the cellular contents of proteasomes was found in these cells in induced and uninduced states for proliferation and differentiation, suggesting accelerated turnover of proteasomes in rapidly proliferating cells. Inhibition of proteasome expression by an antisense oligodeoxynucleotide for the largest proteasome subunit, C2, caused partial arrest of cell cycle progression of T-lymphocytes, suggesting that up-regulation of proteasomes is dispensable for proliferation of the cells. We also observed that the nuclear fraction of proteasomes increased in proliferating T-cells and that proteasomes moved rapidly between the nucleus and cytoplasm during differentiation of HL-60 cells.

Proteasomes are unusually large polysubunit complexes with a sedimentation coefficient of 20 S and consist of approximately 15 distinct polypeptides of 21–31 kDa (Arrigo et al., 1988; Tanaka et al., 1988a). All the genes for these subunits determined so far encode similar but novel proteins that differ from all other known proteins and seem to have been highly conserved during evolution in organisms ranging from yeast to man (Tanaka et al., 1992). These findings suggest that proteasomes are involved in fundamental biological processes.

Proteasomes are multi-protease complexes with at least three distinct catalytic functions (Orlowksi, 1990; Rivett, 1988) and are involved in an extralysosomal energy-dependent proteolytic pathway (Goldberg, 1992). Recently they were demonstrated to be an essential component of an ATP/ubiquitin-dependent proteolytic complex of 26 S, which consists of proteasomal subunits and multiple protein components of 35-110 kDa (Hershko, 1991). Thus proteasomes are thought to be an important soluble proteolytic complex responsible for various biological events, although their exact physiological function(s) is still unknown.

Recently, we (Fujiwara et al., 1990) and others (Emori et al., 1991; Heinemeyer et al., 1991) demonstrated independently that proteasomes are essential for proliferation of yeast cells by showing that inactivations of chromosomal genes encoding several proteasomal subunits had lethal effects. We also found that the levels of mRNAs encoding proteasome subunits are high in rapidly proliferating human cells, such as various types of leukemic cells (Kumatori et al., 1990) and malignant tumor cells from renal and hepatic cell carcinomas (Kanayama et al., 1991). The latter findings suggest that proteasomes play an important role in abnormal proliferation of mammalian cells.

To clarify the cellular functions of proteasomes, we used an in vitro system of cultured human hematopoietic cells, which have been used as a model to study growth and differentiation of mammalian cells, because cell cycle progression of human peripheral T-lymphocytes can be induced by stimulation of the resting cells with various mitogens, such as phytohemagglutinin (PHA) or interleukin-2 (Furukawa et al., 1990), and because terminal differentiation of immature leukemic cell

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1 N. Shimbara, K. Tanaka, and A. Ichihara, unpublished data.
2 The abbreviations used are: PHA, phytohemagglutinin; MøSO₄, dimethyl sulfoxide; Suc, succinyl; MCA, 4-methylcoumaryl-7-amide; TPA, (12-O-tetradecanoylphorbol-13-acetate; DAPI, 4',6-diamino-2-phenylindole; sAb, monoclonal antibody; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NBT, nitro blue tetrazolium; MHC, major histocompatibility complex.
lines including HL-60 and K562 cells into various cell types such as monocytic, granulocytic, and erythroid cells is known to be induced by several factors including phorbol esters (Rovera et al., 1979), 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) (McCarthy et al., 1983), retinoic acid (Breitman et al., 1980), dimethyl sulfoxide (MeSO) (Collins et al., 1978), hemin (Cioe et al., 1981) and butyric acid (Cioe et al., 1981).

We examined the expressions of proteasomes at the mRNA and protein levels during proliferation and differentiation of normal and immature hematopoietic cells and found that the levels of mRNAs for proteasomes are high in rapidly proliferating cells and appear to be correlated with the activities of these cells for proteasome synthesis but that the cellular contents of proteasomes remain unchanged, regardless of the cell state, suggesting that intracellular proteasome turnover is in unusually dynamic states in these cells. From these findings, we propose a model of two pool sizes of proteasomes, a large pool with a slow turnover present in all cells and a small pool with a rapid turnover present only in rapidly growing cells (see “Discussion”). We also report immunocytochemical studies on hematopoietic cells showing that the distributions of the proteasomes between the nucleus and the cytoplasm changes with the inductions of proliferation and differentiation of these cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The materials used were as follows: [α-³²P]ATP (110 Tbsp/μmol), [³²P]Met (37 Tbsp/μmol), and Hybond-N nylon membrane (Amersham Corp.); the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-MCA, as described previously (Tanaka et al., 1988b); the protein content of cell extracts was determined by the method of Bradford (1976) with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in 10–20% gradient gel were transferred electrophoretically to Hybond-C (Amersham) with a semidry Electroblotter (Sartorius). The membranes were pretreated with Block Ace (Yukijirushi Co., Sapporo) and then treated with various antibodies, proteasome and anti-mouse or anti-rabbit IgGs conjugated with alkaline phosphatase of a ProteoBlot immunoblotting system (Promega Biotec). They were then washed extensively with buffer containing 0.05% Tween 20 and stained by the alkaline phosphatase reaction. In some experiments, [α-³²P]Met (3.7 MBq/ml) for 3 h in RPMI 1640 medium modified by addition of 0.10 the standard concentration of unlabeled Met. The cells were resuspended in 2–5 μl of buffer A (25 mM Tris-HCl buffer, 5 mM MgCl₂, and 1 mM dithiothreitol) or buffer B (buffer A without ATP) and lysed by sonication at 30 s in a Sonifier Cell Disruptor 250 (Branson). The sonicate was centrifuged for 30 min at 15,000 × g, and the resulting supernatant was incubated for 60 min at 57 °C in buffer A with an ATP-regenerant (200 μM MgCl₂, 200 μM ATP, 500 μM orthophosphate and alkaline phosphatase) or buffer B with an ATP-depleting system (10 mM glucose and 1 μg of hexokinase). In immunoprecipitation experiments, samples of cell extracts containing approximately 5 × 10⁶ cpm of radioactivity incorporated in acid-insoluble materials were first treated with nonimmunized mouse IgG and protein A-Sepharose CL4B to remove proteins with nonspecific absorption. The supernatant obtained by centrifugation was mixed at room temperature for 20 min with 5 μg of anti-proteasome mAb 2-17 and then for 30 min with protein A-Sepharose CL4B at a concentration corresponding to 2-fold the binding capacity of the added IgG. The resulting precipitates were washed extensively with 50 mM Tris-HCl (pH 7.5) buffer containing 0.15 M NaCl and 0.05% Tween 20 and then solubilized in 400 μl of 20 mM glycine-HCl buffer (pH 3.0) containing 0.15 M NaCl. The solubilized proteins were precipitated with cold acetone and subjected to SDS-PAGE (10–20% gradient gel). After electrophoresis, the gels were treated with Entilign (New England Nuclear Research Products), dried, and subjected to fluorography.

**Immunological Methods**—Antihuman proteasome antibodies were raised in rabbits (Tanaka et al., 1988b) and monoclonal antibody (mAb) 2-17, which reacted specifically with the largest component (named C9; molecular mass = 31 kDa) of human proteasomes, was produced in mouse hybridoma cells by a standard method as described (Fujitani, 1983) and determined quantitatively by fluorescence microscopy. 1,25-(OH)₂D₃ was detected by the avidin-biotin-peroxidase complex (ABC) method (Rovera, 1983) and quantitated by densitometry. The cellular DNA was counter-stained with methyl green solution.

**Biochemical Methods**—The protease activity of proteasomes was assayed by measuring the fluorescence liberated from a fluorogenic peptide, Suc-Leu-Leu-Val-Tyr-MCA, as described previously (Tanaka et al., 1988b). The protein content in cellular extracts was measured by the method of Bradford (1976) with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was carried out by the method of Laemmli (1970). For measurements of the synthesis of proteasomes and their associated components (Orino et al., 1991), cells were labeled with [³²P]Met (3.7 MBq/ml) for 3 h in RPMI 1640 medium modified by addition of 0.10 the standard concentration of unlabeled Met. The cells were resuspended in 2–5 μl of buffer A (25 mM Tris- HCl buffer (pH 7.5) containing 2 mM ATP, 5 mM MgCl₂, and 1 mM dithiothreitol) or buffer B (buffer A without ATP) and lysed by sonication at 30 s in a Sonifier Cell Disruptor 250 (Branson). The sonicate was centrifuged for 30 min at 15,000 × g, and the resulting supernatant was incubated for 60 min at 57 °C in buffer A with an ATP-regenerant (200 μM MgCl₂, 200 μM ATP, 500 μM orthophosphate and alkaline phosphatase) or buffer B with an ATP-depleting system (10 mM glucose and 1 μg of hexokinase). In immunoprecipitation experiments, samples of cell extracts containing approximately 5 × 10⁶ cpm of radioactivity incorporated in acid-insoluble materials were first treated with nonimmunized mouse IgG and protein A-Sepharose CL4B to remove proteins with nonspecific absorption. The supernatant obtained by centrifugation was mixed at room temperature for 20 min with 5 μg of anti-proteasome mAb 2-17 and then for 30 min with protein A-Sepharose CL4B at a concentration corresponding to 2-fold the binding capacity of the added IgG. The resulting precipitates were washed extensively with 50 mM Tris-HCl (pH 7.5) buffer containing 0.15 M NaCl and 0.05% Tween 20 and then solubilized in 400 μl of 20 mM glycine-HCl buffer (pH 3.0) containing 0.15 M NaCl. The solubilized proteins were precipitated with cold acetone and subjected to SDS-PAGE (10–20% gradient gel). After electrophoresis, the gels were treated with Entilign (New England Nuclear Research Products), dried, and subjected to fluorography.

**RESULTS**

Reciprocal Changes in Levels of mRNAs Encoding Proteasomal Subunits during Inductions of Proliferation and Differ-
entiation of Various Blood Cells—Previously we found that the level of mRNA encoding subunit C2 of proteasomes in human mononuclear cells increased greatly during culture with PHA, which is a potent T-cell mitogen inducing blastogenic transformation (Kumatori et al., 1990). Here, we examined whether induction of proliferation of T-cells is associated with increased expressions of other subunits of proteasomes. For this purpose, we measured the levels of mRNAs for subunits C3 and C5 as well as C2 of proteasomes in purified human peripheral lymphocytes during proliferation induced by PHA. On Northern blot analysis under the present experimental conditions, proteasomal mRNAs were hardly detected in freshly isolated resting lymphocytes or during culture without mitogen.

In contrast, the mRNAs encoding proteasome subunits C2, C3, and C5 were identified at significant levels after 6-h stimulation with PHA, and thereafter their levels increased dramatically to maxima on day 2–3 (Fig. 1A). Similar enhanced expression of proteasomal genes was induced by treatment with interleukin-2, although in this case, the levels were maximal on day 6–8 (Fig. 1B). The time difference in the effects of these two mitogens might be due to a difference in their abilities to promote cell proliferation, as PHA is more effective than interleukin-2 in inducing growth of T-cells. As shown in Fig. 1, the three mRNA levels changed similarly during proliferation of T-cells induced by these two mitogens, suggesting that multiple proteasome genes are regulated coordinately. These results also suggest that up-regulation of multiple proteasome genes is closely related to cell cycle progression of T-lymphocytes.

To clarify the role of elevated expressions of proteasomes in rapidly proliferating cells, we examined the changes in the mRNA levels of proteasomes during in vitro induction of terminal differentiation of various types of immature human leukemic cell lines. Treatments of the promyelocytic leukemia cell line HL-60 with phorbol ester, TPA, and an activated form of vitamin D₃ (1,25-(OH)₂D₃) resulted in the expression of a monocytic-macrophagic phenotype (McCarthy et al., 1983; Rovera et al., 1979), whereas treatments with Me.SO and retinoic acid lead to expression of a granulocytic phenotype (Breitman et al., 1980; Collins et al., 1978). Previously, we reported that the levels of mRNAs of proteasomes were abnormally high in a variety of malignant human hematopoietic cell lines, such as HL-60 and K562 cells (Kumatori et al., 1990). As shown in Fig. 2A (left panel), treatment of HL-60 cells with TPA caused marked down-regulation of the mRNA levels of proteasomes. This down-regulation was rapid, indicating half-lives of within 3 h during stimulation, suggesting rapid turnovers of the mRNAs. Similar down-regulations of the mRNA levels of proteasomes were observed during monocytic differentiation induced by 1,25-(OH)₂D₃, although the disappearances of the mRNAs were slower than on treatment with TPA (Fig. 2A, right panel). Thus down-regulation of proteasome expression seems to be independent of the type of signal. As rapid down-regulation of the mRNA level of the proto-oncogene c-myc is known as a differentiation marker in a blood cell culture system (Siebenlist et al., 1988), we compared its mRNA level with those of proteasome mRNAs and found that these two types of mRNA decreased similarly on treatments with TPA and 1,25-(OH)₂D₃, whereas the level of actin mRNA did not changed significantly during these treatments (Fig. 2).

Intracellular transduction of the signal of TPA is known to be mediated by protein kinase C, so we examined whether down-regulation of proteasomes by 1,25-(OH)₂D₃ was mediated through a similar protein kinase C pathway. For this, we tested the effects of protein kinase C inhibitors, such as H7 (Hidaka et al., 1984) and sphinganine (Merrill et al., 1986) on the down-regulation of proteasome expression during treatments of HL-60 cells with these inducing agents. As shown in Fig. 2B, down-regulation of proteasomes in HL-60 cells induced by TPA was markedly blocked by the co-existence of H7 or sphinganine, but these two inhibitors had no effect on decrease of the mRNA levels of proteasomes during 1,25-(OH)₂D₃-induced monocytic differentiation of these cells. These findings suggest that protein kinase C is involved in the TPA-mediated pathway of down-regulation of proteasomes, but not in the 1,25-(OH)₂D₃-dependent pathway. Interestingly, these protein kinase C inhibitors did not prevent down-regulation of the mRNA level of c-myc, suggesting that TPA down-regulates the c-myc gene by another pathway not involving protein kinase C. However, these inhibitors of protein kinase C resulted in partial inhibition of down-regulation of c-myc induced by 1,25(OH)₂D₃, consistent with a report by others (Simpson et al., 1989). Thus, the mechanisms of down-regulations of c-myc and proteasome genes induced by TPA and 1,25-(OH)₂D₃ seem to be different. These two inhibitors alone had no effect on the mRNA levels of proteasomes and c-myc, suggesting that down-regulation of proteasome expression is not dependent on the intracellular pathway for signal transduction evoked by TPA and 1,25-(OH)₂D₃ and that differentiation of the cells itself is important for their down-regulating effects.

It was of interest to determine whether TPA-induced down-regulation of proteasomes was specific for HL-60 cells. To examine this, we measured alterations of the mRNA levels during TPA-induced monocytic differentiation of other types of cells. As shown in Fig. 3A, TPA caused marked decreases in the levels of proteasomal mRNAs in U937, THP1, and K562 cells, suggesting that down-regulation of proteasomes induced by TPA is independent of the cell type. We also examined whether down-regulation of proteasomes was specific to monocytic differentiation of cells by studies on HL-60 cells treated with Me.SO and retinoic acid to induce their differentiation into granulocytic cells and K562 cells treated with butyric acid and hemin to induce their erythroid differentiation (Breitman et al., 1980; Cioe et al., 1981; Collins et al., 1978). Results showed marked down-regulation of proteasomes during both granulocytic differentiation of HL-60 cells.
Promyelocytic leukemic HL-60 cells.

Fig. 2. Down-expression of mRNAs for proteasome subunits during monocytic differentiation of promyelocytic leukemic HL-60 cells. A, time course: HL-60 cells (3 × 10^9 cells/ml) were treated with 5 × 10^-8 M TPA (left panel) or 5 × 10^-7 M 1,25-(OH)2D3 (right panel) for the indicated times. B, effects of protein kinase C inhibitors: HL-60 cells were treated for 48 h with TPA or 1,25-(OH)2D3 (1,25-D3) in the presence or absence of 18 μM H7 or 6 μM sphinganine (Sa). Total RNA (10 μg) extracted from these cells was used for Northern blot analysis with cDNAs for proteasomal subunits C2, C3, and C9, c-myc, and actin as probes.

Fig. 3. Effects of cell type and direction of differentiation on down-regulation of proteasome expression. A, U937, THP1, and K562 cells (3 × 10^7 cells/ml) were treated with TPA for 72 h. B, HL-60 cells were treated for 72 h with 1.25% Me2SO or 1.0 × 10^-5 M retinoic acid (RA). C, K562 cells were treated for 120 h with 1.4 × 10^-2 M n-butyric acid (BuAc) or 5 × 10^-5 M hemin. Northern blot analysis was performed as for Fig. 2. The direction of differentiation of the cells examined is shown at the bottom.

With previous reports (Siebenlist et al., 1988; Simpson et al., 1989). Monocytic and granulocytic differentiations were detected by measuring change in activity for reduction of nitro blue tetrazolium (NBT), and erythroid differentiation was monitored as hemoglobin production stained with benzidine. After 3 days of treatment, almost all the HL-60 cells were NBT-positive in cultures with TPA, and 55–95% were NBT-positive in cultures with 1,25-(OH)2D3 retinoic acid, or Me2SO (data not shown). Cultures of unstimulated control HL-60 cells contained less than 5% NBT-positive cells. Differentiation of these cells is known always to be associated with withdrawal from the cell cycle (Breitman et al., 1980; Cioe et al., 1981; Collins et al., 1979; McCarthy et al., 1983; Rovera et al., 1979). In fact, TPA almost completely suppressed increase in the cell number of HL-60 cells, but inhibitions of cell proliferation by other inducing agents, such as 1,25-(OH)2D3, Me2SO, and retinoic acid were only partial on day 3–4, although long-term treatments with these compounds resulted in marked inhibition of cell proliferation (data not shown). Similarly, TPA and hemin almost completely inhibited proliferation of K562 cells, but butyric acid caused only partial inhibition, although hemin and butyric acid caused similar induction of differentiation of these cells, as judged by measurements of benzidine-positive cells (data not shown). These findings and those on changes in the mRNA levels of proteasomes (Figs. 2 and 3) suggest that down-regulation of proteasomes precedes inhibition of cell proliferation and is parallel with induction of differentiation of leukemic cells. Conversely, down-regulation of proteasomes may be necessary for suppression of cell growth. To clarify this possibility, we examined whether down-regulation of proteasomes is serum-dependent, because removal of serum from cultures caused almost complete inhibition of cell growth, but had no effect on cell differentiation.

As shown in Fig. 4, serum-free cultures of all cells examined, such as HL-60, U937, THP1, and K562 cells, resulted in marked decreases in the mRNA levels of proteasomes irrespective of the cell type, suggesting that a high level expression of proteasomes is closely related with cell proliferation and...
not directly correlated with differentiation of these leukemic cells.

**Immunoblot Analyses of Cellular Proteasome Levels**—To determine whether changes in the levels of mRNAs for proteasomes in different states of growth and differentiation of these hematopoietic cells are correlated with those of cellular proteasome contents, we first compared the activities for proteasomes in different states of growth and differentiation. We then evaluated the absolute contents of proteasomes in cells by measuring these enzymatic activities, because proteasomes are present in cells in a latent state (Orlowski, 1990; Rivett, 1988), so their full activity was not determined. Moreover, cells may contain natural substrates that compete with synthetic peptide in the assay and whose cellular contents change in different states of these cells.

Therefore, we measured the cellular contents of proteasomes during blastogenic transformation of T-cells induced by PHA at the protein level by immunoelectrophoretic blot analyses with anti-proteasomal monoclonal and polyclonal antibodies. As shown in Fig. 5, mAb 2–17 reacted with only a single component corresponding to the proteasome subunit C2 of 31 kDa, while polyclonal antibodies reacted strongly with three species of proteasomal subunits and weakly with several other components of 21–30 kDa. Unfortunately, the proteasome content of T-cells was found not to change significantly during culture with PHA (Fig. 5, A and B). This finding was not due to unresponsiveness of the T-cells to PHA, because the PHA-treated T-cells showed marked morphological change, as judged by formation of a number of large aggregates on stimulation with PHA. To confirm cell cycle progression of T-cells treated with PHA, using the same cell extracts as for proteasomal analyses, we measured the levels of three proteins that are known to change during cell cycle progression, cdc2, cyclin-A, and cyclin-B, composing the maturation/M-phase promoting factor. Immunoblotting with anti-human polyclonal antibodies against these three proteins showed that these three proteins changed markedly in a cell cycle-dependent fashion (Fig. 5, C–E), the change being in accord with that described in a recent report (Furukawa et al., 1990). This observation is also consistent with our previous finding that the cell cycle progressed time-dependently with increase in DNA synthesis and cell number on day 4 of stimulation with PHA (Kumatori et al., 1990). Thus, it is clear that the proteasomal level in T-lymphocytes does not change during cell cycle progression. This was also clear from studies on subunit C2, which showed that the level of mRNA for C2 increased greatly during cell cycle progression of T-lymphocytes, but the amount of immunoreactivity of subunit C2 stained with mAb 2–17 remained unchanged (compare top rows of Figs. 1 and 5). These findings are of outstanding interest, because they indicate that dramatic changes of mRNAs are not correlated with their protein levels.

We also examined alterations of the cellular contents of proteasomes during differentiation of leukemic cells. On induction of monocytic and granulocytic differentiations of HL-60 cells by treatments with TPA and Me2S0, respectively, the relative contents of immunoreactive components did not change significantly, as shown by analyses with monoclonal and polyclonal antibodies (Fig. 6A). Similarly, inductions of monocytic and erythroid differentiations of K562 cells by TPA and butyric acid, respectively, did not affect the protein levels of proteasomes measured by similar immunoblot analyses (Fig. 6B). These results clearly indicate that differentiation-dependent down-regulations of the levels of mRNAs of proteasomes are not parallel to alterations of their protein levels in either the HL-60 or K562 cell line.
Enzyme Syntheses and Levels of 20 and 26 S Proteasomes in Cells with Different Proliferating Activities—It was of interest to know whether the increased levels of mRNAs were responsible for increased translational activities. For testing this possibility, we measured the rates of incorporation of \([^{35}S]\)Met into immunoprecipitable proteasomes of resting and growing T-cells.

As shown in Fig. 7A (lanes without ATP), the rate of synthesis of 20 S proteasomes of 21–31 kDa was very high in rapidly proliferating T-cells. Similar high rates of syntheses of proteasome components were found in rapidly growing HL-60 cells (Fig. 7B, lanes without TPA and ATP), but little incorporation of \([^{35}S]\)Met into proteasomes was detected in differentiated monocytic HL-60 cells induced by TPA (Fig. 7B, lanes without ATP). Thus, high translational activities of proteasomes in proliferating cells appeared to be correlated with increased levels of mRNAs as shown in Figs. 1 and 2. Subsequently, we examined whether proteasomes synthesized at high rates in rapidly proliferating cells are secreted into the culture media. In order to compare the activities for syntheses of intra- and extracellular proteasomes exactly, we measured the radioactivities of \([^{35}S]\)Met incorporated into immunoprecipitated proteasomes quantitatively with an image analyzer.

As shown in Fig. 8, treatment of T-cells with PHA resulted in increased syntheses of intracellular proteasomes, and treatment of HL-60 cells with TPA caused their dramatic decrease, as in Fig. 7. However, the amounts of newly synthesized proteasomes found in the cultured media were very low in all cases, suggesting that secretion of proteasomes into the extracellular space by these cells is low in all conditions.

Recently, we reported that in HL-60 cells the 20 S proteasome is associated ATP-dependently and reversibly with multiple protein components of 35–110 kDa to form the 26 S complex that catalyzes ATP-dependent breakdown of proteins ligated with ubiquitin (Orino et al., 1991, Kanayama et al., 1992). Thus, it was also important to examine whether the rates of syntheses of these other associated components are correlated with those of 20 S proteasomes in rapidly growing cells. As reported previously (Orino et al., 1991), 26 S proteasomes complexes in cell lysates can be immunoprecipitated with anti-proteasomal antibodies in the presence of ATP, which prevents their dissociation. Therefore, we immunoprecipitated 26 S proteasomes from the lysates of cells labeled with \([^{35}S]\)Met with mAb 2–17 in the presence of ATP. As shown in Fig. 7, A and B (lanes with ATP), the syntheses of these associated components of 35–110 kDa co-immunoprecipitated with proteasome subunits were also high in proliferating T-cells induced by PHA and in uninduced immature HL60 cells that grow rapidly, suggesting apparent coordination on the expressions of proteasome subunits and their multiple associated components. These findings strongly suggest accelerated turnover of proteasomes in rapidly proliferating T-lymphocytes and leukemic cells (for details, see “Discussion”).

To determine whether the level of the 26 S complex changes with alteration of the cell state, we analyzed cellular lysates directly by glycerol density gradient centrifugation in the presence of a high concentration of ATP to stabilize the 26 S complex as reported (Orino et al., 1991).

On fractionation of crude extracts prepared with ATP, active enzyme-catalyzing Suc-Leu-Leu-Val-Tyr-MCA breakdown was eluted in fractions with a sedimentation coefficient of about 26 S (Fig. 9, fractions 8–9), but no detectable activity was found in fractions corresponding to the elution position of purified 20 S proteasomes (Fig. 9, fractions 10–11). However, addition of 0.05% SDS, which is a potent artificial activator of purified latent proteasomes (Orlowski, 1990; Rivett, 1988), caused marked activation of the enzyme eluted in the fraction of 20 S, indicating the presence of 20 S proteasomes in a latent form. This concentration of SDS had little effect on the peptidase activity of 26 S fractions. As shown in Fig. 9A, the peak of the 26 S complex was much smaller than that of the latent 20 S proteasomes in lysates of resting T-cells. In contrast, similar analyses of the lysates of PHA-activated T-cells showed an increased level of the 26 S fraction with decrease of the 20 S peak (Fig. 9B). We examined the relationship between the 26 and 20 S enzymes directly by immunoblot analyses of the two fractions. Anti-proteasome mAb 2–17 reacted specifically with the 31-kDa component in both the 26 and 20 S fractions (Fig. 9, upper panel), and we found that the proteasome subunit C2 in the 26 S fractions was significantly enriched in the lysate of proliferating T-cells induced by PHA (Fig. 9B) over that in the lysate from the resting T-cells (Fig. 9A). Conversely, shifts of proteasomal activity and the immunoreactive C2 component from the 26 S fraction to the 20 S fraction were observed in response to monocytic differentiation of HL60 cells (data not shown). These findings suggest an important role of the 26 S complex of proteasomes in rapid cell proliferation.
Effect of Antisense Oligodeoxynucleotide for Proteasome Subunit C2 on Cell Cycle Progression of Mitogen-induced T-cells—For clarifying the role of enhanced expression of proteasome genes, we examined the effect of an antisense oligodeoxynucleotide on cell cycle progression of T-cells. The cell cycle was analyzed by measurement of the DNA contents of individual cells by fluorescence microscopy after staining with DAPI, a DNA-binding dye (Hamada and Fujita, 1983). Cells in the S- and M-phases of the cell cycle were stained strongly with DAPI. We determined the DNA contents of resting T-lymphocytes and those activated with PHA in samples of approximately 100 cells.

As shown in Fig. 10, unstimulated resting T-cells at G0 in the cell cycle consisted of a single population of cells with a low cellular DNA content (top panels), whereas in cultures with PHA cell populations with high contents of DNA were observed (second panels). Treatment with TN16, a metaphase-arresting reagent (Hashimoto et al., 1972), resulted in increase in the cell population with high DNA content (B, second panel). Culture with an antisense oligodeoxynucleotide corresponding to the ATG region of subunit C2 significantly blocked cell cycle progression of PHA-induced T-cells, whereas culture with a sense oligodeoxynucleotide had less effect (A, third and bottom panels). Similar growth arrest by antisense DNA against proteasome C2 was observed more clearly by cell cycle analysis of T-lymphocytes cultured with TN16 (B, bottom panel). Thus, inhibition of synthesis of proteasomal subunit C2 inhibits cell cycle progression of T-lymphocytes induced by mitogen, suggesting that proteasome expression is essential for T-cell proliferation.

Immunocytochemical Location of Proteasomes in HL-60 Cells and T-lymphocytes—We have reported the predominant location of proteasomes in the nucleus of various types of leukemic cells from patients with acute lymphocytic leukemia, adult T-cell leukemia, and acute myelocytic leukemia (Kumatori et al., 1990). We examined the intracellular distribution of proteasomes during differentiation of promyelocytic leukemia cells HL-60. As shown in Fig. 11A, proteasomes are present in the irregular-shaped nucleus of proliferating HL-60 cells as well as diffusely in their cytoplasm. Interestingly, induction of monocyte differentiation of cells
by TPA resulted in predominant location of proteasomes in the nucleus of the differentiated cells (Fig. 11B), suggesting that nuclear translocation of proteasomes occurs in a signal-dependent fashion. On differentiation of HL-60 cells into granulocytes induced by retinoic acid, high nuclear staining of proteasomes was also observed, but less than on treatment of TPA (Fig. 11C). However, there was no detectable proteasomal staining in the nucleus of human peripheral blood monocytes separated from healthy adults (data not shown). Thus, the TPA- or retinoic acid-induced nuclear translocation of proteasomes may be related with an intermediate state of monocytes during terminal differentiation. On the other hand, hardly any staining of proteasomes was observed in resting peripheral T-lymphocytes (Fig. 11D), but induction of proliferation of these cells with PHA or interleukin-2 caused nuclear enrichment of proteasomes (Fig. 11E), suggesting that nuclear translocation of proteasomes is an important step for cell cycle progression of mitogen-stimulated T-cells. Interestingly, considerable amounts of proteasomes appear to be associated with plasma membrane-like structures in T-lymphocytes activated with either PHA or interleukin-2 (Fig. 11E). Immunostaining of proteasomes in isolated T-cells was consistently very slight in repeated experiments (Fig. 11D), possibly due to low transportation of antibody into fresh cells. The exact role of proteasomes in the nucleus requires further study.

**DISCUSSION**

**A Model of Two Proteasome Pools, One of Which Is Closely Related to Cell Proliferation**—In the present study, we examined the expression of proteasomes at the mRNA and protein levels in normal and malignant hematopoietic cells, which proliferate and differentiate in response to several inducing factors. Our findings were as follows. 1) The expression levels of mRNAs of proteasomes were low in resting T-lymphocytes, but increased 10-20-fold during mitogen-stimulated blastogenic transformation (Fig. 1). 2) Proteasome expressions were abnormally high in human leukemia cell lines, but decreased rapidly and markedly on induction of differentiation of the cells by exposure of various inducers. This rapid down-regulation may be due to repression of transcription of the genes, because it was so rapid; for instance, the mRNAs were not detectable after a 6-h treatment with TPA. This down-regulation seemed to be independent of the cell type, direction of differentiation, or type of signal (Figs. 2 and 3). 3) The mRNA levels of proteasomes apparently correlated with their translational activities, because high incorporation of [35S]Met into proteasomes was found during proliferation of both T-lymphocytes and HL-60 cells (Fig. 7). 4) Proteasomes were not secreted appreciably into cultured media by either T-lymphocytes or HL-60 cells in the nonproliferating or rapidly growing state (Fig. 8). 5) Immunohistochemical studies with anti-proteasomal antibodies clearly showed that the absolute contents of proteasomes remained unchanged in nondonorizing and growth-stimulated T-lymphocytes and during differentiation of leukemic cells such as HL-60 and K562 cells regardless of their direction of differentiation (Figs. 5 and 6). These results are surprising, because they indicate no relationship between the levels of the mRNAs and proteins, despite the apparent correlation of the levels of the mRNAs of proteasomes with their translational activities. One possible explanation for these findings is that the intracellular proteasome pool may be in a dynamic state responsible for change in cellular activities. Marked increase in turnover of total cellular proteasomes may occur in proliferating cells. However, this is unlikely, because proteasomes are expressed constitutively and are abundant in cells, constituting about 1.0% of the total cellular proteins (Tanaka et al., 1988b), and turn over slowly with half-lives of 5-10 days (Hendil, 1988; Tanaka and Ichihara, 1989).

To explain the present unusual data, we propose that there may be two distinct proteasome pools in cells. We propose that these two putative pools differ in size and in turnover rate. One is a large pool with a slow turnover, is widely distributed in all cell types, and does not change regardless of alterations in the states of the cells during proliferation and differentiation. The other is a small pool with a rapid turnover which appears to be specifically expressed in rapidly growing cells, such as activated T-cells and immature leukemic cells. This small pool is in a dynamic state changing in parallel with rapid change of the mRNA levels of the proteasomes, which is presumably responsible for adaptation of cellular metabolic activities. This model of the two proteasome pools is shown schematically in Fig. 12.

This model could explain our surprising findings in the present work, but further substantial studies are required for direct demonstration of the presence of these two pools in cells.

Why are proteasome genes expressed at such high level in proliferating cells irrespective of the cell type? Accelerated turnover of proteasomes in the small proteasome pool shown in Fig. 12 is probably essential for proliferation of T-lymphocytes, because prevention of synthesis of one proteasome subunit, C2, by an antisense oligodeoxynucleotide, which inhibits gene expression by trapping and causing degradation of a specific mRNA, resulted in significant suppression of cell cycle progression (Fig. 10). Antisense oligonucleotides against several oncogenes and cell cycle-related proteins, such as c-myc, c-myb, and cdc2 were demonstrated to suppress cell cycle progression at the G1 to S transition in human T-lymphocytes (Furukawa et al., 1990). Interestingly, inhibition of only one subunit of the multisubunit proteasome complex causes growth arrest. However, this finding is not surprising, because recently we (Fujikawa et al., 1990) and others (Emori et al., 1991; Heinemeyer et al., 1991) reported that in yeast cells, inactivations of single genes encoding various subunits had lethal effects. A simple explanation of why cells in which a single subunit is inactivated cannot proliferate normally is that each subunit functions in some process necessary for cell cycle progression. Alternatively, each subunit may be important for assembly of the proteasome complex that is essential for proliferation of cells. Since proteasomes are involved in the intracellular protein turnover essential for maintaining cellular homeostasis and cell cycle progression, inactivation of a single subunit of proteasomes could result in loss of proteasomal activity, which could induce abnormal accumulation of various cellular proteins and might result in inhibition of cell proliferation.

**Roles of Proteasomes during Development and Differentiation**—Interestingly, we found that the synthesis of the multiple components that associate with the 26 S proteolytic complex were also enhanced in proliferating T-cells and undifferentiated HL-60 cells (Fig. 7) and that the 26 S form was also increased in extracts of proliferating T-cells (Fig. 9) and leukemic cells. As the 26 S protease complex catalyzes selectively ATP-dependent breakdown of ubiquitinated proteins (Hershko, 1991), judging from these findings this proteasome- and energy-dependent proteolytic system may be responsible for rapid elimination of unnecessary proteins, such as oncogene products, tumor suppressive proteins, and cyclin (Rechsteiner, 1991), which are expressed in a specific phase of the cell cycle and whose rapid...
turnover is necessary for progression through the cell cycle. These observations are consistent with a report suggesting that differentiation of murine erythroleukemia cells resulted in loss of the associated components of the 26 S complex, but not of proteasomes themselves (Tsukahara et al., 1991). Moreover, the levels of 20 S proteasomes and 26 S ubiquitin/ATP-stimulated protease were found to decrease by one-third during maturation of rabbit reticulocytes to erythrocytes (Cola et al., 1991), which may be correlated with reduction of energy-dependent proteolysis during reticulocyte maturation (Speiser and Etlinger, 1982) and marked loss of certain enzymes catalyzing protein ubiquitination during erythroid cell differentiation (Pickart and Vella, 1988). The compositions of cellular proteins probably differ in different cellular states, particularly in quiescent-differentiated and immature-proliferating cells. Large parts of intracellular proteins must be degraded, and syntheses of new protein are required for acquisition of cellular functions during blood cell development and differentiation. The functions of 26 S proteasome complexes may also be related with these degradation processes. 

Immunohistochemical studies showed that proteasomes are localized predominantly in the nuclei of leukemic cells (Kumatori et al., 1990) and of human primary cancer cells of the kidney (Kanayama et al., 1991). Consistent with these previous findings, we observed that proteasomes are concentrated in the nuclei of proliferating T-cells (Fig. 11E). Moreover, during induction of differentiation of HL-60 cells, proteasomes appeared to move rapidly between the nucleus and the cytoplasm (Fig. 11, A–C). Increased levels of proteasome mRNAs were also found in the early stage of development of various rat embryonic tissues such as the liver and kidney, and their levels gradually decreased during development.1 Moreover, during development of Drosophila, proteasomes were found to be highly expressed in the central nervous system and to be abundant in highly proliferating cells, indicating that their accumulation is differentially regulated during embryogenesis (Klein et al., 1990). Furthermore, proteasomes apparently move rapidly between the nucleus and cytoplasm during oogenesis and embryogenesis in lower organisms (for review, see Scherrer, 1990). These changes in intracellular distribution may be important for the physiological function(s) of proteasomes. Recently, several subunits of proteasomes were found to contain consensus signal sequences for nuclear translocation (Tanaka et al., 1990). Proteasomes in the nucleus are indistinguishable from those in the cytosol in proteolytic properties, size, and immunological reactivity (Tanaka et al., 1989), but further studies are necessary to determine whether proteasomes in the two compartments have exactly the same subunit compositions. It is also of importance to examine whether an ATP/ubiquitin-dependent 26 S proteasome form is present in the nucleus as well as the cytoplasm. Interestingly, Haass and Kloetzel (1989) reported that proteasomes undergo changes in subunit pattern during development of Drosophila and that these developmental changes in subunit multiplicity are due to phosphorylation of some components. Similarly, in chick embryonic muscle, the subunit pattern of proteasomes was found to change during development, suggesting that development-dependent diversification of subunit composition may be related to change in proteolytic events (Ahn et al., 1991). It is, however, unknown whether the subunit structure of proteasomes changes during proliferation and differentiation of hematopoietic cells. 

**Regulation of Gene Expression of Proteasomes**—All proteasome subunits seem to be present as components of multisubunit complexes in cells, because no free subunits have yet been found (Kumatori et al., 1990). Moreover, in this work, we showed that the up- and the down-regulation of the mRNAs encoding multiple proteasomal subunits occur simultaneously. These findings suggest the existence of some mechanism(s) regulating the coordinated expressions of the genes for proteasome subunits. Recently we found that the PRS1, PRS2, and PRS3 genes of yeast proteasomes are located on chromosomes XV, VII, and II, respectively; that is, in yeast these three genes are located on different chromosomes (Lee et al., 1992). Thus, in higher animals also the multiple proteasome genes are probably located on different chromosomes, although chromosomal mapping experiments are required to determine their exact locations. To obtain information on the coordinated expressions of the proteasome subunit genes in mammalian cells, it is essential to identify a specific consensus region common to the sequences of the upstream regions of...
the multiple genes for proteasome subunits. Studies on this problem are underway. Recently, two genes encoding putative proteasome subunits were reported to be located in the MHC class II region, the genetic locus which is essential for class I MHC-mediated presentation of intracellular antigens (Robertson, 1991; DeMars and Spies, 1992). The facts that these proteasomal subunit genes are polymorphic, tightly linked to two peptide transporter genes, and induced by interferon-γ suggest the possible involvement of proteasomes in the antigen processing pathway (Driscoll and Finely, 1992; Goldberg and Rock, 1992). It is interesting that some proteasome subunits are related with the immune response. It is important to determine whether the expressions of these putative proteasome subunit genes located in the class II MHC region are coordinated with those of other subunits of proteasomes.

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