Recent studies have increasingly implicated the proteasome in the regulation of cell surface receptors. In the present study, we investigated the role of the proteasome for ligand-dependent endocytosis and degradation of the interleukin-2 (IL-2)-interleukin-2 receptor (IL-2R) complex. Proteasome inhibitors impaired internalization of IL-2-IL-2R and prevented the lysosomal degradation of this cytokine. Based on time-course studies, proteasome activity is primarily required after initial endocytosis of the IL-2-IL-2R. Proteasome function was also necessary for the lysosomal degradation of IL-2 internalized by IL-2R that were comprised of cytoplasmic tailless β- or γc-subunits, suggesting that the target protein for the proteasome is independent of either the cytoplasmic tail of the IL-2R β- or γc-subunits and their associated signaling components. Therefore, a functional proteasome is required for optimal endocytosis of the IL-2R/ligand complex and is essential for the subsequent lysosomal degradation of IL-2, possibly by regulating trafficking to the lysosome.

Within the immune system, the proteasome is essential for production of peptides for MHC class I antigen presentation. The proteasome also has a well-described role in the degradation of cytosolic and nuclear proteins, including the elimination of misfolded proteins as well as controlling the levels of certain transcription factors or cell cycle regulatory proteins (8). Most proteins destined for degradation are targeted to the proteasome by ubiquitination (9). More recent studies have implicated ubiquitination in the internalization of a growing list of cells surface receptors (10, 11), suggesting a possible role for the proteasome in regulating the levels of cell surface receptors. In the present study we have used specific inhibitors to explore the potential role of the proteasome for endocytosis and degradation of the IL-2-IL-2R complex.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**1F1γcWT (6), 1F1γc284 (12), and CXβ (13) have been described previously. Activated T cells were prepared by the culture of C57BL/6 spleen cells with anti-CD3 for 48 h as described previously (14). E-64, MG132, PSI, and antipain were obtained from Peptide International, Inc. (Louisville, KY), lactacystin from Calbiochem-Novabiochem Corp. (San Diego, CA), and chloroquine from Sigma (St. Louis, MO).

**Internalization Assay—**Cells (10 *10⁶/ml) were incubated in RPMI 1640 containing 5% fetal calf serum (14) (complete medium) at 4 °C for 30 min in the presence of 50,000 cpm/ml human IL-2 (specific activity ~20-40 μCi/μg) that was radiolabeled with Na¹²⁵I using Iodo-gen precoated tubes (Pierce Chemical Co., Rockford, IL) according to the manufacturer’s instruction. The cells were then washed three times with cold Hank’s balanced salt solution. Unless otherwise indicated, all inhibitors were added at 50 μM final concentration to the cells just after these washes. In the case of lactacystin, the cells were also cultured with this proteasome inhibitor for 2 h in complete medium and cooled on ice for 15 min prior to treatment with ¹²⁵I-labeled IL-2. Dimethyl sulfoxide (DMSO), which was the solvent for all the proteasome inhibitors, was added to the control cells at a concentration of ≤1%. The IL-2-pulsed cells (4 *10⁶/ml) were then cultured in complete medium at 37 °C using a water bath in 1 ml of medium/tube using 1.5-ml microcentrifuge tubes. At the indicated times, the cells were pelleted by centrifugation in a microcentrifuge at 14,000 × g for 15 s. Protein released in the supernatant was precipitated by addition of one-fourth volume of 50% trichloroacetic acid. The trichloroacetic acid soluble counts represented internalized IL-2 that was degraded. The cell pellets were then resuspended in 0.5 ml of 0.1 M sodium citrate, 0.14 M NaCl, pH 2 buffer for 2 min at room temperature and centrifuged for 15 s at 14,000 × g in a microcentrifuge. The radioactivity that remained associated with the cells after this low pH buffer wash was represented the internalized (I) IL-2 whereas the portion of radioactivity in the pH 2 buffer supernatant represented cell surface-associated (S) IL-2. Before shifting the cells to 37 °C, usually 75–85% of the cpm was cell-surface associated. The I/S ratio was calculated at the indicated time points. The pH 2-resistant material at time 0 (I₀) was considered nonspecific material and was subtracted from I.

**Western Blot Analysis—**1F1γcWT cells were extracted in buffer containing 0.5% Nonidet P-40, phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μg/ml), aprotonin (10 μg/ml), iodoacetamide (10 mM), sodium orthovanadate (1 mM), and sodium fluoride (1 mM) as described previously (15) and precipitated with anti-STAT5 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) associated to protein G-Sepharose.
The precipitated material was resolved by 10% SDS-polyacrylamide electrophoresis under reducing conditions, transferred to nitrocellulose, and blocked by incubation with 5% nonfat dried milk in PBS, pH 7.2, (5% milk) for 1 h. The nitrocellulose was subsequently incubated with either anti-phospho-STAT5 (Upstate Biotechnology, Lake Placid, NY) or anti-STAT5 antisera in 5% milk for 90 min, washed three times with PBS containing 0.1% Nonidet P-40, and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit Ig for 60 min followed by washing twice with PBS containing 0.1% Nonidet P-40 and once with PBS alone. Bands were visualized by chemiluminescence using ECL Western blotting detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RESULTS

Effect of Proteasome Inhibitors on the Internalization and Degradation of IL-2—After IL-2 binds to the high affinity IL-2R, ~50% is detected intracellularly (I/S = 1) within 10–15 min (3, 4), and a similar rate of internalization has been noted for IL-2R-bearing EL4, including 1F1γcWT (Fig. 1 and Refs. 6 and 13). By 45 min, ~70% of the 125I-labeled IL-2 was detected intracellularly without appreciable degradation (Fig. 1, B and C; Refs. 6 and 13). To test whether proteasome function was required for receptor-mediated endocytosis of IL-2, 1F1γcWT cells were treated with 125I-labeled IL-2 in the cold, and internalization of the surface-associated IL-2 was initiated by culturing the cells at 37 °C in the absence or presence of the proteasome inhibitor MG132 (Fig. 1). During the first 10–15 min, the MG132 minimally affected the initial internalization of IL-2 as measured by the initial loss of cell surface IL-2 (Fig. 1A) or the accumulation of intracellular IL-2 (Fig. 1B). However, an altered I/S ratio was evident for 1F1γcWT 45 min after initiation of endocytosis when the cells were cultured with either MG132 or the highly proteasome specific inhibitor lactacystin (Fig. 2A). This effect was because of an increased level of surface IL-2 that was evident for the MG132-treated cell by 30 min (Fig. 1A). The altered endocytosis by MG132 and lactacystin was specific as the I/S ratio was not affected when 1F1γcWT were treated with the calpain inhibitor antipain or the cysteine protease inhibitor E-64 (Fig. 2A).

After 60 min in culture, an obvious fraction of the IL-2 that was initially associated with the 1F1γcWT cells was detected in the culture supernatant, and at this and all subsequent time points, ~90% of this released IL-2 was soluble in trichloroacetic acid, consistent with its degradation to small peptides. The small amount of IL-2 released during the first 30 min in culture was precipitated by trichloroacetic acid, which most likely reflected the release of IL-2 from the IL-2R prior to its endocytosis. Surprisingly, MG132 substantially inhibited the degradation of IL-2 (Fig. 1C). Like MG132, the related peptide aldehyde proteasome inhibitor P51, but not antipain or E-64, prevented the degradation of IL-2 (Fig. 2B). This finding suggests that the inhibition by these two peptide aldehydes is caused by effects on the proteasome rather than effects on lysosomal cysteine proteases or calpains. Again, the highly proteasome-specific inhibitors lactacystin and clasto-lactacystin β-lactone (not shown) also inhibited the degradation of IL-2 (Fig. 2B). Dose-response studies indicated that 2–5 μM MG132, P51, or lactacystin were required for inhibition of 50% degradation of IL-2 (Fig. 2C). This concentration of inhibitors is consistent with those required to inhibit the proteasome (16–18). Collectively, these data indicate that proteasome function is necessary for normal receptor-mediated endocytosis and subsequent degradation of IL-2.

Relationship Between the Proteasome and Lysosome for Internalization and Degradation of IL-2—Previous studies have demonstrated that upon internalization of the IL-2-IL-2R complex, IL-2 traffics to the lysosome and is degraded (3, 4, 7). We confirmed that degradation of IL-2 by 1F1γcWT occurred in the lysosome as its degradation was inhibited by 50 μM chloroquine (Fig. 3A). The peptide aldehyde proteasome inhibitor MG132 was even a somewhat more potent inhibitor of degradation of IL-2. During the first 15 min of culture, time-course studies revealed that MG132 only minimally blocked internalization of IL-2 (Figs. 1A and 3B). However, after a 20–30 min culture in the presence of MG132, internalization of IL-2 reached a maximal level, and after that time the fraction of surface and intracellular IL-2 remained constant. By contrast, chloroquine, which directly inhibits lysosomal function, showed much lower capacity to affect the internalization of IL-2 with consistently a higher I/S ratio than MG132-treated cells (Fig. 3B). This finding in conjunction with the complete inhibition of degradation of IL-2 by proteasome and lysosome inhibitors suggests that impairment of IL-2 internalization by the proteasome inhibitors may be secondary because of the blockade in trafficking of IL-2 to the lysosome, preventing the degradation of IL-2.

Proteasome Function Is Not Required for Initial Internalization of IL-2—From the preceding data, only the initial endocytosis of IL-2 was not influenced by MG132. However, this apparently normal internalization might simply be related to a short delay for MG132 to effectively block the proteasome. To directly test whether the entry of IL-2 into the cells was unaffected by proteasome inhibitors, 1F1γcWT cells were pretreated with proteasome inhibitors prior to initiation of receptor-mediated endocytosis (Table I). When analyzing the continuous internalization of IL-2 for 45 min (Table I, Experiment 1), the fraction of surface and intracellular IL-2 was comparable for cells pretreated with either the Me₂SO vehicle or proteasome inhibitors. In the latter two experiments (Table 1, Experiments 2 and 3), the cellular distribution of IL-2 was assessed 3 h after initiation of endocytosis of IL-2-pulsed cells. In this scenario, most of the cell-associated IL-2 was found in
as described in the legend to Fig. 2 and assayed after 2–3 h (Experiments and indicated time (B).

The internalization and degradation of IL-2. Cells were then pulsed with 125I-labeled IL-2 at 4 °C for 30 min. After lactacystin for 120 min at 37 °C and then cooled on ice for 15 min. The maintained at 4 °C (Experiment 1) or by assay before shifting to 37 °C associated prior to initiation of endocytosis as determined by cells washing, the cells were cultured at 37 °C for 180 min with further addition of PSI or lactacystin. 70–85% of the cpm were cell surface-associated prior to initiation of endocytosis as determined by cells maintained at 4 °C (Experiment 1) or by assay before shifting to 37 °C (Experiments 2 and 3).

Table I

| Experiment | Inhibitor | Time assayed (min) | Surface | Intracellular | Degraded |
|------------|-----------|--------------------|--------|--------------|----------|
|            |           |                    |        |              | % 125I/IL-2 |
| 1          | None      | 45                 | 31     | 68           | NT       |
|            | MG-132    | 36                 | 64     | NT           |          |
| 2          | None      | 180                | 8      | 10           | 82       |
|            | PSI       | 20                 | 73     | 7            |          |
| 3          | None      | 180                | 13     | 35           | 52       |
|            | Lactacystin | 180            | 25     | 62           | 11       |

* NT, not tested.

Fig. 2. Comparison of proteasome and protease inhibitors on the internalization and degradation of IL-2. Effect on internalization (A) or degradation (B) of IL-2. C, dose-response of proteasome inhibitors. 1F1cWT cells were pulsed with 125I-labeled IL-2, treated with 50 μM indicated inhibitors as described under "Experimental Procedures," and cultured for 45 min (A) or 180 min (B and C) at 37 °C. In A, the fraction of cell-associated IL-2 that was intracellular (I) versus cell surface (S) was determined and plotted as the IS ratio where 1 represents 50% of the cell-associated IL-2 within an intracellular compartment. Data in A are the and range of two independent determinations, except for lactacystin. In B, degraded IL-2 was assessed by the fraction of trichloroacetic acid-soluble cpm in the culture medium and is the mean ± S.D. of 3–6 determinations, except for antipain.

Fig. 3. Comparison of proteasome and lysosome inhibitors on the internalization and degradation of IL-2. Effect on degradation (A) and internalization (B) of IL-2. These experiments were performed as described in the legend to Fig. 2 and assayed after 2–3 h (A) or the indicated time (B). Data in A represent the mean ± S.D. of three experiments and B is representative of three experiments.

In Experiment 1, 1F1cWT cells were pretreated with MG132 (50 μM) for 60 min at 37 °C prior to adding 125I-labeled IL-2 without washing. The cells were further cultured at 37 °C for 45 min, and cell surface- and intracellular-associated cpm were determined. In experiments 2 and 3, 1F1cWT cells were pretreated with PSI (50 μM) or lactacystin for 120 min at 37 °C and then cooled on ice for 15 min. The cells were then pulsed with 125I-labeled IL-2 at 4 °C for 30 min. After washing, the cells were cultured at 37 °C for 180 min with further addition of PSI or lactacystin. 70–85% of the cpm were cell surface-associated prior to initiation of endocytosis as determined by cells maintained at 4 °C (Experiment 1) or by assay before shifting to 37 °C (Experiments 2 and 3).

TABLE I

Pretreatment of 1F1cWT with proteasome inhibitors does not impair initial internalization of IL-2

In the presence of this agent (Table I, Experiment 3) strongly supports the notion that initial endocytosis of IL-2 was unaffected by blockade of the proteasome.

After a 30-min culture of IL-2-treated 1F1cWT, usually 65–75% of the cell-associated IL-2 was detected as an intracellular fraction of ligand (see Figs. 1B and 2R and Ref. 6). The addition of MG132 at this time resulted in inhibition of degradation of IL-2 to a level that was comparable when MG132 was added at the initiation of the culture (Fig. 4A). Substantially less inhibition was noted when MG132 was added 60 min after initiation of endocytosis of IL-2, a time when degradation of IL-2 is beginning to be detected in the control cells. Furthermore, when compared with MgSO4 vehicle-treated cells, an accumulation of intracellular IL-2 occurred when MG132 was added at culture initiation (t0) or 30 min later. The increased fraction of intracellular IL-2 was most prominent when the proteasome inhibitor was delayed for 30 min, as normal levels of IL-2 were allowed to first be internalized before blocking the proteasome. Delayed addition of MG132 similarly affected the degradation of 125I-labeled IL-2 by C57BL/6 T blasts (Fig. 4B), demonstrating that proteasome function for IL-2 degradation was not unique to only IL-2R-bearing EL4 cells, but was also required for a normal population of T cells. Collectively, these data suggest that blockade of the proteasome inhibits a relatively early step in the intracellular traffic of the IL-2/IL-2R complex, but a step later than required for the initial endocytosis of the receptor-ligand complex.

IL-2-induced Signaling. Degradation of IL-2, and Proteasome Function—Normal internalization of IL-2 is dependent upon the cytoplasmic tail of the γc-subunit of the IL-2R (5, 6). For the 1F1c284-transfected variant of EL4, which expresses wild-type IL-2R α- and β-chains but the cytoplasmic tailless γ-chain, the rate of internalization of IL-2 is ~2–3-fold slower than 1F1cWT, with a corresponding lower fraction of degraded IL-2 after 2–3 h in culture (Table II and Ref. 6). By contrast, the CXβt-transfected variant of EL4, which expresses wild-type IL-2Rα and -γc, but a cytoplasmic tailless β-chain, demonstrated essentially normal rates of internalization and degradation of IL-2 (Ref. 13 and Table II). Importantly, MG132 very efficiently inhibited the degradation of the fraction of IL-2 that was internalized by the IL-2R on 1F1cWT or CG/β (Table II). These findings indicate that the proteasome-dependent pathway for degradation of IL-2 is independent of the cytoplasmic tail of IL-2Rβ or -γc, including the respective IL-2R-associated signaling molecules.
Several studies have implicated the proteasome in the regulation of Jak-STAT signal transduction, including IL-2-induced activation of STAT5 (19, 20). In 1F1cyWT, IL-2 induced tyrosine phosphorylation of STAT5 (Fig. 5a, lane 1 versus 3), and upon extended culture without IL-2, the level of phosphorylated STAT5 was considerably reduced (Fig. 5a, lane 3 versus 5 and 7). Furthermore, as expected, MG132 stabilized IL-2-induced activation of phosphorylated STAT5, which was especially evident after 2 h in culture (Fig. 5a, lane 7 versus 8). To investigate whether this enhanced signaling might be related to the proteasome-dependent blockade of the IL-2 degradation pathway, we examined the ability of delayed addition of MG132 to potentiate STAT5 phosphorylation in 1F1cyWT (Fig. 5b).

Because the IL-2 degradation pathway was nearly completely independent determinations.

**FIG. 4. Effect of delayed addition of MG132 on the degradation and internalization of IL-2.** 1F1cyWT (A) or C57BL/6 T blasts (B) were pulsed with 125I-labeled IL-2 and cultured at 37 °C for 3 h. MG132 was added at various times after the initiation of internalization by culture at 37 °C. The cpm associated with the cell surface, an intracellular pool, or released as IL-2 trichloroacetic acid-soluble cpm in the supernatant were determined. Data are the mean and range of two independent determinations.

**TABLE II**

| Cells          | Experiment | Degradation of 125I-IL-2 | % controlb | % inhibited by MG132b |
|----------------|------------|--------------------------|------------|-----------------------|
| 1F1cy284       | 1          | 41.1                      | 96.1       |
|                | 2          | 42.8                      | 94.3       |
| CXjkt     | 3          | 82.5                      | 75.8       |
|                | 4          | ND                       | 96.3       |

a Comparison to 1F1cyWT cells.

b Comparison to MeSO-treated 1F1cy284 (Experiments 1 & 2) or CXjkt (Experiments 3 and 4).

c ND, not determined.

**FIG. 5. Effect of the proteasome inhibitor MG132 on IL-2-induced STAT5 activation.** 1F1cyWT cells were treated with IL-2 (400 units/ml) at 4 °C for 30 min in complete medium, washed three times, and cultured at 37 °C for the time indicated (time assayed). MG132 (50 μM) or MeSO (0.5%) was added at the initiation (A) (t0) of this 37 °C culture or delayed (B), as indicated. Nonidet P-40 extracts were prepared from these cells, precipitated with anti-STAT5 antiserum, and subjected to Western blot analysis using antiserum specific for phosphorylated STAT5 (upper panel). The blot was stripped and reprobed with antiserum to STAT5 protein (lower panel) to verify the levels of STAT5 in each lane. Data are representative of three experiments.
These results strongly suggest that the cytoplasmic portion of the IL-2R or their associated signaling components are unlikely to be targets for the proteasome. Consistent with this notion, we showed distinct kinetic requirements for proteasome function for IL-2-mediated endocytosis and IL-2-induced STAT5 tyrosine phosphorylation.

A number of cell surface receptors are ubiquitinated upon ligand activation. In mammalian cells, the platelet-derived growth factor receptor (22), growth hormone receptor (23), TCR, (24), and c-kit (25, 26) were among the first receptors shown to undergo ligand-induced ubiquitination of their cytoplasmic tails. In these cases, the lysosome appears to be the primary target for degradation of these receptors. In Saccharomyces cerevisiae the G-protein coupled α-factor receptor undergoes enhanced mono- or short chain ubiquitination upon binding α-factor which facilitates endocytosis and subsequent degradation by the lysosome (27, 28). The use of yeast defective in ubiquitin-conjugating enzymes established a role for this protein modification in endocytosis (27, 28). So far, there has been little direct evidence that proteasome function is involved in this process, and it has been suggested that ubiquitination may serve as a targeting sequence for entry into the endocytic pathway leading to down-regulation of the cell surface receptor (10). One exception appears to be endocytosis of the growth hormone receptor. Similar to our results on the IL-2R, proteasome activity appears to be required for ligand-induced endocytosis and lysosomal degradation of growth hormone receptor that is independent of ubiquitination of the cytoplasmic tail of growth hormone receptor (29). The fate of the receptor-associated ligand was not determined in that study. Proteasome inhibitors have also been reported to affect the levels of platelet-derived growth factor receptor (22) and the Met kinase receptor (30), but these effects have not been ascribed to regulation of the endocytic pathway. If our findings on the IL-2R are generalized, perhaps the role for proteasome function in regulating these cell surface receptors is to gain entry into a cellular compartment for lysosomal/vacuolar degradation that is independent of ubiquitination of the cytoplasmic tail of the receptor.

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