HIV-1 Tat Inhibits the 20 S Proteasome and Its 11 S Regulator-mediated Activation*

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The proteasomal system consists of a proteolytic core, the 20 S proteasome, which associates in an ATP-dependent reaction with the 19 S regulatory complex to form the functional 26 S proteasome. In the absence of ATP, the 20 S proteasome forms a complex with the γ-interferon-inducible 11 S regulator. Both the 20 S proteasome and the 11 S regulator have been implied in the generation of antigenic peptides. The human immunodeficiency virus (HIV)-1 Tat protein causes a number of different effects during acquired immunodeficiency syndrome (AIDS). Here we show that HIV-1 Tat protein strongly inhibits the peptidase activity of the 20 S proteasome and that it interferes with formation of the 20 S proteasome-11 S regulator complex. In addition, it slightly increases the activity of purified 26 S proteasome. These results may explain the mechanism by which HIV-infected cells escape cytotoxic T lymphocyte response and at least in part immunodeficiency in AIDS patients.

The 26 S proteasome (or 26 S proteasome) is a component of the ubiquitin (Ub) pathway involved in cell cycle and in transcriptional regulation (1, 2). The 11 S regulator (3) or PA28 (4) stimulates 20 S proteasomal peptidase activities, and because it is inducible by γ-interferon, we suggested that it would be involved in antigen processing (3). Recently it has been demonstrated that the 20 S proteasome generates antigens presented by MHC class I molecules (5) and is regulated by the 11 S complex in vivo (6).

Cellular data on the effects of HIV-1 Tat are accumulating rapidly. In addition to its transcriptional functions (7), genetic evidence has been provided that Tat might have independent effects in determining infectivity and cytopathicity in a developing HIV-1 infection (8). Tat is produced in large quantities in HIV-1-infected cells. It is secreted and can be taken up rapidly by other cells (9). Exogenous Tat accelerates CD95-mediated, activation-induced T cell apoptosis (10). This mechanism may lead to a depletion of noninfected CD4+ T cells. It has been reported that HIV-1 Tat potentiates TNF-induced NF-κB activation, which stimulates the replication of HIV-1 (11). Moreover, HIV-1 Tat inhibits antigen-induced lymphocyte proliferation (12) possibly by interfering with either antigen processing or presentation. Both NF-κB activation and antigen processing require the proteasomal system (5, 13).

Our interest in Tat was stimulated by the fact that two ATPase subunits of the 19 S regulatory complex, MSS1 and TBP1 (14), directly or indirectly influence Tat action. MSS1 (mammalian suppressor of sgs1) is a modulator of Tat-mediated transactivation (15), and TBP1 (Tat-binding protein 1) was identified by direct protein-protein interaction with Tat (16) and hence could be a potential Tat-binding subunit of the 26 S proteasome. We reasoned that HIV-1 Tat could have an effect on the proteasomal system and performed experiments to test this hypothesis.

EXPERIMENTAL PROCEDURES

The components of the proteasomal system, the 20 S and 26 S proteasomes and the 11 S regulator, were purified from human erythrocytes as described (17, 18).

SucLLVY-AMC cleavage assays were conducted with the electropheretically homogenous protein complexes in a final volume of 100 μl. Final concentrations of the substrate, HIV-1 Tat, and the proteasomal complexes are indicated in the figures. Fluorescence was measured at 37 °C with a microtiter plate reader (Fluoroscan II, Labsystems) at 355 nm excitation and 460 nm emission after a 5-min lag time over a 60-min period in 5-min intervals. During this time period the reactions were linear.

The degradation of Ub-[125I]lysozyme conjugates was measured in the absence and in the presence of ATP. After 30 min of incubation in the presence of 50 μl of Ub-[125I]lysozyme conjugates (~4 × 107 cpm), the reaction was stopped, and the percentage of degradation was determined as described (19).

For Western blotting purified 26 S proteasome (2 μg) was separated by nondenaturing electrophoresis on a 4–15% Phast gel (Pharmacia Biotech Inc.) at 300 volt hours. Under these conditions some of the complex disassembles into the 20 S proteasome and the 19 S regulatory complex. 20 S and 26 S proteasome bands were visualized by substrate complexes are indicated in the figures. Fluorescence was measured at 37 °C with a microtiter plate reader (Fluoroscan II, Labsystems) at 355 nm excitation and 460 nm emission after a 5-min lag time over a 60-min period in 5-min intervals. During this time period the reactions were linear.

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To show Tat binding to the protein complexes, blots were incubated with a Tat solution (0.1 μg/ml) for 60 min. Subsequently the blots were washed with PBS and developed with a polyclonal anti-Tat antibody. The antibody against Tat was made with synthesized protein in rabbits using standard techniques.

The two-exon Tat used in most of the experiments was synthesized as described previously (21). Recombinant Tat was purchased from AGMED.

RESULTS AND DISCUSSION

As an initial experiment we investigated whether HIV-1 Tat could affect peptide hydrolysis by the 20 S proteasome. To our surprise the degradation of a fluorogenic peptide, sucLLVY-AMC, by the 20 S proteasome was inhibited by Tat with an average 50% inhibition value of $K_{i50} = 5 \times 10^{-8}$ M (Fig. 1A). The inhibition is almost complete with a remaining 5–10% activity that differs slightly between preparations. Because the $K_{i50}$ values were independent of substrate concentrations (see Fig. 1A), Tat does not compete with the fluorogenic peptide for binding to the active centers in the lumen of the 20 S proteasome. This is in accordance with earlier results showing that a
FIG. 1. **Kinetics of Tat interactions with the proteasomal system.** A, effect of Tat concentration on the 20 S proteasome suclLVY-AMC cleavage activity. Increasing amounts of Tat were added to 100 ng of isolated 20 S proteasome in the presence of 0 (○), 100 (■), and 200 μM (▲) peptide substrate in a final volume of 100 μl. An average 50% inhibition value ($K_{i}$) of 5 $\times$ 10$^{-7}$ M, which was independent of the substrate concentration, can be estimated. The data are representative for three (50 and 100 μM of substrate) and twelve (200 μM of substrate) independent experiments.

B, dependence of 20 S proteasome inhibition by Tat on 11 S regulator amount. Increasing amounts of Tat were added to 30 ng of isolated 20 S proteasome preincubated for 10 min with 0 (○), 0.8 (▲), and 1.6 μl (△) of isolated 11 S regulator (200 μg/ml). Increasing amounts of 11 S regulator caused a linear increase of $K_{i}$ values (inset) indicating competition. The final concentration of suclLVY-AMC was 200 μM. The data are representative for four independent experiments.

C, effect of Tat on the suclLVY-AMC cleavage activity of the 26 S proteasome in the presence of ATP. Increasing amounts of Tat were added to 100 ng of isolated 26 S proteasome in the presence of 200 μM substrate, 2 mM ATP, and 5 mM MgCl$_2$. The samples were preincubated for 30 min at 37 °C before the fluorescence was measured. An average 50% activation value ($K_a$) of 5 $\times$ 10$^{-7}$ M was estimated. The data are representative for four independent experiments.

FIG. 2. **Tat stimulates the Ub-lysozyme conjugate degradation by the 26 S proteasome.** Samples containing 2.5 μg 26 S proteasome were preincubated in the absence (Control) and presence of 50 μg/ml Tat protein (Tat) for 30 min at 37 °C in 50 μl of buffer with (white columns) and without (black columns) 2 mM ATP and 5 mM MgCl$_2$. Tat increased the ATP-dependent cleavage of Ub-lysozyme conjugates approximately 3-fold. Data are means ± S.E. (n = 4).

FIG. 3. **Interaction of Tat with components of the proteasomal system.** A, Tat binding to immobilized 20 S proteasome, 19 S regulatory complex, and 26 S proteasome. Purified 26 S proteasome (2 μg) was separated by nondenaturing electrophoresis. Under these conditions the complex partially disassembles into the 20 S proteasome and the 19 S regulatory complex. Immobilized complexes were stained with Ponceau (Ponceau). 20 S (20S) and 26 S (26S) proteasome bands were visualized by substrate overlay (Overlay) or detected by immunoblotting with a polyclonal antibody directed against the 20 S proteasome (Anti 20S). The antibody against subunit 4 of the 19 S regulatory complex (Anti S4) was used to stain the 26 S proteasome as well as the 19 S regulatory complex (19S RC). To show Tat binding to the protein complexes, blots were developed with an anti-Tat antibody (Tat). B, displacement of Tat from 20 S proteasome by 11 S regulator. Purified 20 S proteasome (2 μg) was transferred to nitrocellulose from a nondenaturing gel. Immobilized enzyme was incubated with Tat (0.1 μg/ml) in PBS. Nitrocellulose strips were washed in PBS containing 0.1% Tween 20 and further incubated with PBS (Tat) or with 2 mg/ml 11 S regulator (Tat + 11S Reg). The blots were then stained with an anti-Tat antibody using the ECL system (Amersham Corp.). The Control lane is immobilized 20 S proteasome without Tat or 11 S regulator treatment, showing that the anti-Tat antibody does not cross-react with the 20 S proteasome.
folded protein cannot penetrate into the inner compartment of the 20 S proteasome (22). To prove whether the effect was restricted to synthesized Tat, we tested a Tat protein expressed in and isolated from *Escherichia coli* that also caused 20 S proteasome inhibition (data not shown).

Tat decreases antigen-induced lymphocyte proliferation (12), possibly because antigens are not processed and thus not presented. Therefore, it was intriguing to test its effect on the 20 S proteasome-11 S regulator complex, which is involved in the processing of antigens presented by MHC class I (6). The kinetic data shown in Fig. 1B demonstrate that Tat competes with the 11 S regulator for binding sites on the 20 S proteasome. There is a linear increase of the $K_i$ values at increasing 11 S regulator amounts (see Fig. 1B, inset). Sigmoidal kinetics indicate that Tat must displace the 11 S regulator from both of its binding sites, the two $\alpha$-rings of the 20 S proteasome (23), to exert inhibition. The same kinetic profile, although as an activation, was obtained when the 20 S proteasome was incubated with Tat protein prior to the addition of different 11 S regulator amounts (data not shown). Moreover, Western blot analysis demonstrates that the 11 S regulator displaces Tat from its proteasomal binding sites (Fig. 3B).

Next we examined the effect of Tat on the 26 S proteasome. In the presence of ATP, the cleavage of suclLVV-AMC is activated by Tat about 3–5-fold with an average 50% activation value ($K_{50}$) of approximately $5 \times 10^{-7}$ M (Fig. 1C). In experiments with disassembled 26 S proteasome, Tat activation was observed only after 30 min in the presence of ATP (data not shown). This time period corresponds with the ATP-dependent assembly process of the 26 S proteasome from the 19 S regulatory complex and the 20 S proteasome. It is likely that the 19 S regulatory complex displaces Tat from the 20 S proteasome, because it likewise displaces the 11 S regulator (17). Presumably Tat binds to TBP1 and perhaps other ATPases of the 19 S regulatory complex, which leads to this moderate activation of the 26 S proteasome.

We tested the effect of Tat on a more physiologically relevant function of the 26 S proteasome, the degradation of Ub conjugates (Fig. 2). Tat activated ATP-dependent conjugate degradation to the same extent as it stimulated peptide cleavage activity of the 26 S proteasome. In samples without exogenous ATP (Fig. 2, –MgATP) we observed a slight stimulation of proteolysis in the presence of Tat, probably due to residual ATP in the preparation (18).

Although the kinetic data argue strongly that Tat binds to components of the proteasomal system, it is demonstrated directly in Fig. 3. The 26 S proteasome was separated by nondenaturing electrophoresis and localized by substrate overlay and specific antibodies, which also identified the 19 S and the 20 S complexes (Fig. 3A). The occurrence of all three complexes reflects partial dissociation of the 26 S proteasome during electrophoresis. As expected on the basis of our kinetic studies, the lane labeled Tat in Fig. 3A shows binding of Tat to the 26 S proteasome and the 19 S regulatory complex as well as to the 20 S proteasome. Binding to the 19 S complex supports our conclusion of Tat binding site(s) on the 26 S proteasome, independent of that on the 20 S proteasome. Preliminary experiments using immunological probing of streptavidin precipitated biotin-Tat peptide indicate that Tat does not bind the 11 S regulator (data not shown).

To illustrate the competition between Tat and the 11 S regulator for the proteasomal binding sites, isolated 11 S regulator was used to displace Tat bound to immobilized 20 S proteasome (Fig. 3B).

The specific Tat-binding subunits of the 20 S proteasome are currently unknown. However, because the 20 S proteasome subunit C2 is involved in 11 S regulator binding (24), it could also participate in the interaction with Tat.

It is not unique that a protein such as Tat interacts with the proteasomal system. Two other viral proteins, Hbx (25) and Tax (26), also bind to the 20 S proteasome. The consequences of these interactions for the 20 S and 26 S enzymes activities, however, remain speculations.

While inhibiting the 20 S proteasome, Tat stimulates the 26 S proteasome needed for cell cycle progression and transcriptional regulation (1, 2). In this context it is interesting to note

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**FIG. 4. Summary of the interconversion of proteasomal complexes, their reported in vivo functions, and the effects of Tat shown in this paper.** The 11 S regulator (11S Reg) is presented as a heptameric ring. Its exact symmetry, however, is unknown. The presentation of the 19 S regulatory complex (19S RC) is based on earlier structural data (27).
that the 26 S enzyme activates NF-κB (13). It has been shown with different cell lines that Tat enhances tumor necrosis factor-induced activation of NF-κB, which leads to a stimulation of HIV-1 replication (11).

The cartoon in Fig. 4 summarizes Tat effects on the proteasomal system based on the data shown in this paper. The in vivo consequence of 11 S regulator replacement and 20 S proteasome inhibition by Tat should be a decrease in antigen processing and subsequently, a reduced MHC class I presentation by infected and perhaps noninfected cells. The role of the 26 S proteasome in antigen processing is unclear. It has been shown that ubiquitination supports the presentation of antigens derived from ovalbumin (5), and the initial degradation of antigens by the 26 S proteasome can be assumed. Whether the enzyme itself produces peptides that can be presented by MHC class I molecules or whether it makes intermediates that are further processed by the free 20 S proteasome and/or the 20 S proteasome-11 S regulator complex is not yet known. The latter pathway could be inhibited by Tat.

Although the highly basic Tat protein has been reported to bind to a number of different proteins, the cellular data demonstrating the effects of exogenous Tat are consistent with our in vitro effects upon the proteasomal system. The immunosuppressive activity of Tat has been demonstrated with lymphocytes. Tetanus toxoid-induced lymphocyte proliferation was inhibited by exogenous Tat with the same $K_{i50}$ value (12) of 50 nM as obtained in our studies for 20 S proteasome inhibition. In concert with its effects on CD4$^+$ T cells (10), Tat inhibition of antigen processing may at least partially contribute to the profound immunodeficiency in AIDS patients.

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