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Human immunodeficiency virus-1 Tat protein interacts with distinct proteasomal α and β subunits

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Abstract The human immunodeficiency virus-1 (HIV-1) Tat protein was previously reported to compete the association of PA28 regulator with the α rings of the 20S proteasome and to inhibit its peptidase activity. However, the distinct interaction sites within the proteasome complex remained to be determined. Here we show that HIV-1 Tat binds to α4 and α7, six β subunits of the constitutive 20S proteasome and the interferon-γ-inducible subunits β2i and β5i. A Tat–proteasome interaction can also be demonstrated in vivo and leads to inhibition of proteasomal activity. This indicates that Tat can modulate or interfere with cellular proteasome function by specific interaction with distinct proteasomal subunits.

Key words: Proteasome; Proteasome inhibition; Antigen presentation; Major histocompatibility complex class I; Human immunodeficiency virus

1. Introduction

Human immunodeficiency virus-1 (HIV-1), a retrovirus of the lentivirus subgroup, causes the debilitating and generally fatal disease AIDS (acquired immune deficiency syndrome). The HIV-1 Tat protein, an 86 amino acid protein, is essential for virus replication. It is an RNA binding protein and an activator of transcription. Tat has been shown to be involved in the progression of HIV infection by immunosuppression, induction of apoptosis of T cells, inhibition of phagocytosis of apoptotic tumour cells by accessory cells, and by suppression of antigen-driven T-cell proliferation [1]. Possibly, HIV proteins including Tat may prevent the correct processing and presentation of tumour-associated and viral antigens by the major histocompatibility (MHC) class I pathway.

An important component of the MHC class I immune response is the ubiquitin–proteasome system, the central proteolytic system of eukaryotic cells [2]. Besides generating antigenic peptides, the major functions of the proteasome are the selective ATP-dependent degradation of cytosolic, nuclear and membrane-bound proteins [3], as well as the regulation of important cellular processes such as cell cycle progression, transcription, differentiation, apoptosis and the cell stress response [4–6].

The proteasome is a multicatalytic protease complex consisting of a 20S catalytic core bound to regulatory complexes. The 20S core particle, exhibiting the proteolytic activity, consists of seven different α and seven different β subunits arranged in four heptameric rings giving an α7β7β7α7 order. Three of the seven β subunits (β1, β2, β5) in the two inner rings are catalytically active and can be replaced by their cytokine-inducible counterparts β1i (LMP2), β2i (Mecl1) and β5i (LMP7) [7]. Incorporation of immunosubunits into the complex can modulate the proteolytic characteristics of the 20S proteasome representing an important mechanism for the efficient generation of antigenic peptides [4].

The larger 26S proteasome consists of the central 20S core particle capped on both ends by a 19S regulatory complex that is essential for recognition and degradation of ubiquitin–protein conjugates in an ATP-dependent manner [8]. Eukaryotic cells also contain another particle called the 19S-20S-PA28 hybrid proteasome. This complex consists of the central 20S core particle capped on one end by the 19S regulator and on the other by a complex known as PA28 activator. PA28, whose synthesis is induced by interferon-γ (IFNγ), was previously shown to play an important role in antigen processing by the MHC class I pathway [4].

In previous work it was shown that the Tat protein inhibits the catalytic activity of the 20S proteasome in vitro [9], interferes with proteasomal antigen processing by competing the PA28 binding to the 20S proteasome and that it shares a common binding site with the two subunits of PA28 on the 20S proteasome [10].

However, so far the subunits of the 20S proteasome that interact with Tat still remain to be defined. Here we present the first experimental evidence that HIV-1 Tat interacts specifically with two of the seven α subunits (α4 and α7), several β subunits of the constitutive proteasome and two β subunits of the immunoproteasome. Furthermore, our in vivo experimental data show that Tat affects the catalytic activity of the proteasome complex through direct binding.

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Abbreviations: HIV-1, human immunodeficiency virus-1; MHC, major histocompatibility complex; IFNγ, interferon-γ; GST, glutathione *S*-transferase; GSH, glutathione; GFP, green fluorescent protein

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2. Materials and methods

2.1. Preparation of expression vectors

The seven human α subunit cDNAs were amplified by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into the pCite vector (Novagen) in the correct reading frame resulting in constructs pCite-α_{1-7}. The cDNAs of the 10 different human β subunits were obtained by RT-PCR and ligated in frame into the *Not*I and *Bam*HI sites of plasmid pIVEX2.3_MCS (Roche Molecular Biochemicals) for expression of untagged proteins. The HIV-Tat cDNA was cloned into *Bam*HI sites of vectors pET41 (Novagen) and pEGFP-C1 (Clontech), ensuring selection of the correct reading frame. All resulting constructs were sequenced to confirm identity and PCR fidelity.

2.2. Expression, purification and binding analysis of recombinant proteins with proteasomes

Transformed *Escherichia coli* strain BL21 (Stratagene) was grown at 37°C in kanamycin-containing LB medium until the logarithmic phase (OD_{600} at 0.6) and induced with 1 mM IPTG for 1 h. Cells were harvested by centrifugation, resuspended in phosphate-buffered saline containing 1% (v/v) Tween and were disrupted by a French press. Glutathione *S*-transferase (GST)- or GST-Tat-containing protein extracts were incubated with glutathione (GSH)-Sepharose beads (Amersham Pharmacia Biotech) and purified according to the manufacturer's protocols.

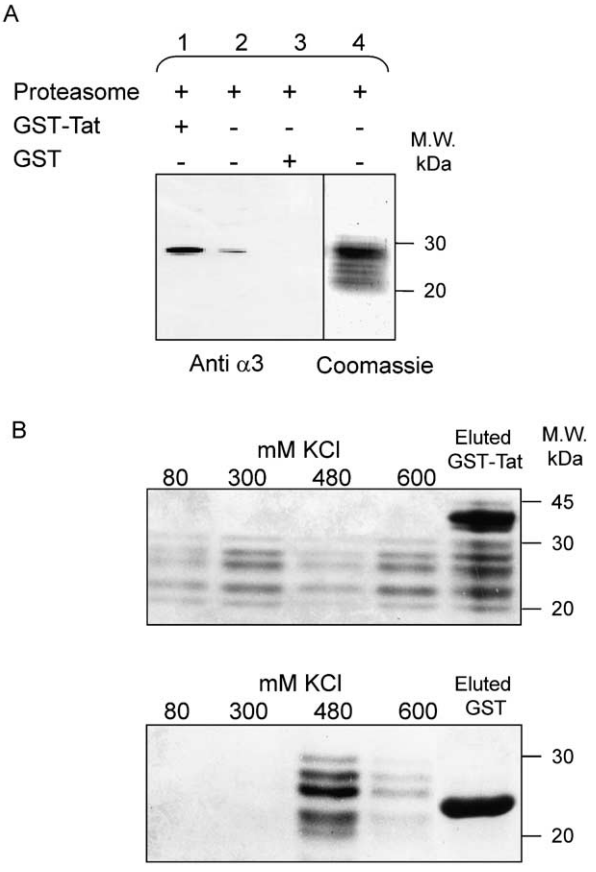

Fig. 1. Interaction of HIV-1 Tat protein with 20S proteasome in vitro. A: Immobilised GST-Tat (lane 1) or GST (lane 3) was incubated with 20S proteasomes from T2 cells (proteasome loading control; lane 2) and eluted proteins were detected by Western blot with an anti-α3 antibody (lanes 1–3). Coomassie staining control of 20S proteasomes (lane 4). B: Immobilised GST-Tat (upper panel, lanes 1–5) or GST (lower panel, lanes 1–5) was incubated with purified 20S proteasomes from calf liver and washed consecutively with 80, 300, 480, or 600 mM KCl, and finally eluted with GSH. Proteins were separated by SDS–PAGE and detected by Coomassie staining with respective molecular weight markers on the right.

For in vitro transcription/translation the single tube protein system 3 (STP3) kit (Novagen) was used according to the manufacturer's instructions.

For binding studies with radiolabelled subunits, equal amounts (9 μg) of GST or GST-Tat proteins were immobilised onto GSH-Sepharose beads and processed as described [11]. For interaction studies of the 20S proteasome and Tat, immobilised GST or GST-Tat proteins were incubated 2 h at 4°C with 50 μg of 20S proteasome, washed with different concentrations of salt as indicated and finally eluted from the beads using GSH.

The synthesised or eluted proteins were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [12] followed by Western blot, Coomassie staining or phospho-imager analyses (Fujifilm FLA-2000R).

2.3. Cell culture and transfection

Human HeLa and T2 cells were cultivated under standard conditions in RPMI medium containing 10% foetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. HeLa cells were transfected with the green fluorescent protein (GFP)-Tat expression plasmid or the GFP control plasmid using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals).

2.4. Sucrose gradient fractionation, immunoprecipitation, and Western blot analyses

Cell extracts were fractionated by velocity gradient ultracentrifugation as described [13]. Equal amounts of protein were subjected to immunoprecipitation and processed according to a previously described protocol [14]. The precipitates were separated by SDS–PAGE, transferred onto polyvinylidene difluoride membranes by electroblotting and immunodetected as described [13].

Antibodies against proteasomal subunits were kindly provided by K. Hendil (Copenhagen). The anti-proteasome antiserum MP3 is laboratory stock and has been described previously [13]. The monoclonal anti-GFP is available from BabCo. The polyclonal anti-Tat antibody was raised against a peptide corresponding to the core region of the Tat protein ($_{48}$GRKKRRQRRRPPQGSQ$_{63}$).

2.5. 20S proteasome preparation and measurement of proteolytic activity

20S proteasomes were purified from calf liver and human T2 cells by standard procedures [15]. Chymotryptic activity of the proteasome was assessed using the synthetic peptide substrate Suc-Leu-Leu-Val-Tyr-aminomethylcoumarin as described [10].

3. Results

3.1. Interaction of the GST-Tat protein with the 20S proteasome

To demonstrate an interaction between the HIV-1 Tat protein and the 20S proteasome, we expressed a recombinant GST-Tat protein in bacteria. The GST fusion protein and GST alone were immobilised on GSH beads and incubated with purified 20S proteasomes. The beads were then washed and the eluted proteins were analysed by Western blotting (Fig. 1A). As evidenced by Coomassie staining and immunoblotting with an antibody directed against the anti-α3 subunit, GST-Tat is able to trap 20S proteasomes while GST alone shows no binding (Fig. 1A).

To dissect the specificity and stringency of the Tat–proteasome interaction the beads loaded with GST-Tat and bound proteasome were washed with increasing concentrations of KCl (80 mM, 300 mM, 480 mM, and 600 mM). Proteins that remained bound were eluted from the beads by GSH and analysed by SDS–PAGE. Even at a concentration of 600 mM KCl the proteasomes still remained bound to the HIV-1 Tat protein supporting the specificity of the interaction (Fig. 1B). The results confirm the previous observations that Tat protein directly interacts with the 20S protea-

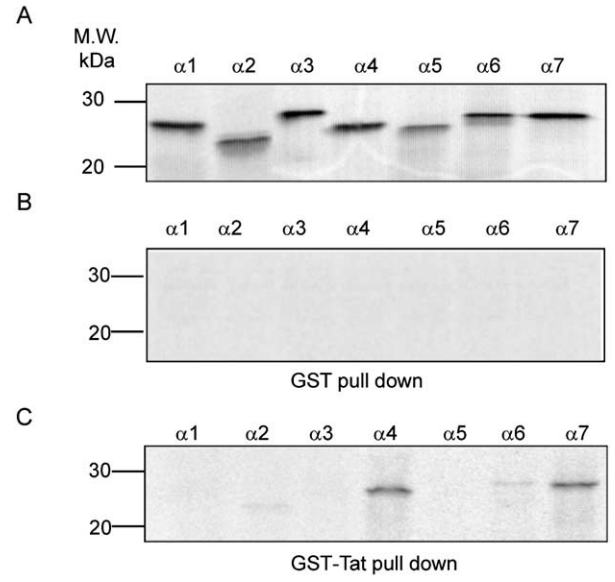

Fig. 2. Interactions of GST-Tat protein and proteasomal α subunits. A: In vitro translated ^{35}S-radiolabelled α subunits. B,C: The binding of in vitro translated ^{35}S-radiolabelled α subunits to immobilised GST (B) or GST-Tat (C). The positions of relevant molecular weight markers are shown on the left.

some [9] and that our recombinant GST fusion protein is functional.

3.2. Interactions of immobilised GST-Tat with distinct proteasomal α and β subunits in vitro

Based on the observation that Tat competes PA28 binding it had been proposed that the interaction of HIV-Tat with 20S core particle occurs via the α ring structure. To dissect which of the 20S proteasome subunits binds to Tat each individual α and β subunit was tested for interaction with the Tat protein. With this aim all proteasomal α and β subunits were cloned into expression vectors and translated in vitro in the presence of [^{35}S]methionine using a transcription/translation system (Figs. 2A and 3A). To identify the subunits that are able to interact with Tat, in vitro translated ^{35}S-radiolabelled α subunits were incubated with immobilised GST-Tat (Fig. 2C) or with GST as control (Fig. 2B). Immobilised Tat protein bound specifically to the α4 and α7 subunits while a weak interaction was also found with the α2 and α6 subunits (Fig. 2B). No interaction was detected with the other α subunits or GST alone (Fig. 2B,C).

Surprisingly, immobilised Tat bound strongly to six constitutive β subunits (β1, β2, β3, β5, β6 and β7) and two immuno-β subunits (LMP7/β5i and Mecl1/β2i) (Fig. 3C). No interactions were found with β4 and LMP2/β1i or GST alone (Fig. 3B). This result was completely unexpected, since so far Tat was thought to bind only to the α ring subunits [10].

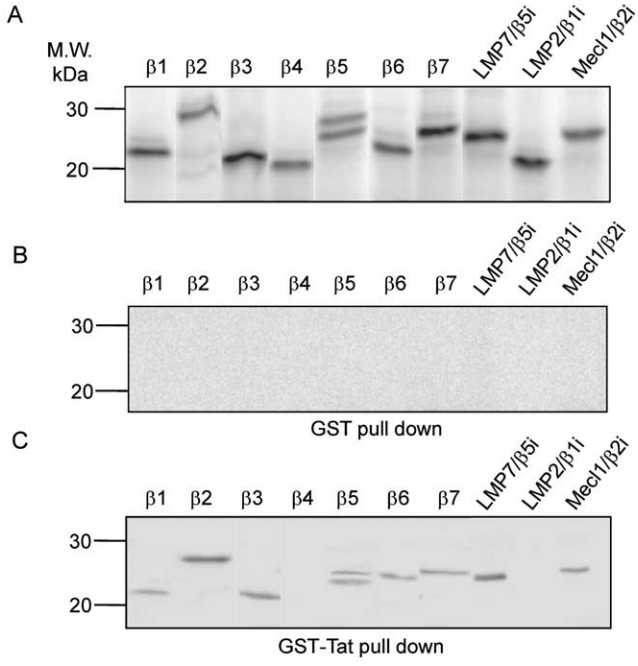

Fig. 3. Interactions of GST-Tat protein and proteasomal β subunits. A: In vitro translated ^{35}S-radiolabelled β subunits. B,C: The binding of in vitro translated ^{35}S-radiolabelled β subunits to immobilised GST (B) or GST-Tat (C) was evaluated. The positions of relevant molecular weight markers are shown at the left.

3.3. HIV-Tat interacts with 20S proteasomes and inhibits peptidase activity in vivo

To confirm the observed Tat–subunit interactions and the possible consequences for proteasome function in vivo human HeLa cells were transiently transfected with a GFP-tagged version of HIV-Tat or with GFP as a control. Cellular lysates

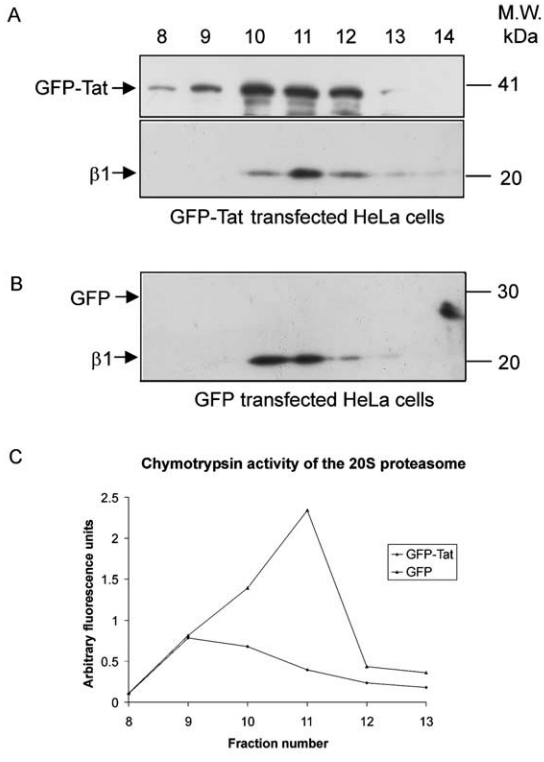

Fig. 4. Co-migration of GFP-Tat with 20S proteasomes and inhibition of proteolytic activity. Sucrose gradient fractionation of cellular extracts from GFP-Tat (A) and GFP-transfected cells (B). Fraction 1 corresponds to the top (10% sucrose) and fraction 20 to the bottom (40% sucrose) of the gradient. Gradient fractions were analysed by Western blotting for β1 and GFP. C: Chymotrypsin-like activity from 10 μl of each gradient fraction containing 20S proteasomes from GFP-Tat-transfected (rhombi) and GFP-transfected cells (triangles).

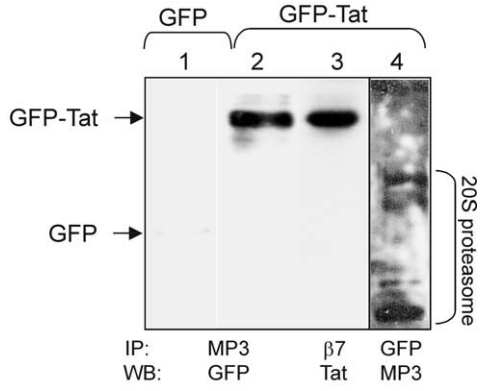

Fig. 5. Co-immunoprecipitation of GFP-Tat and 20S proteasomes in vivo. Proteasomes were immunoprecipitated (IP) from proteasome-containing fractions 9–11 from GFP- or GFP-Tat-transfected HeLa cells (see Fig. 4) with anti-20S (MP3) (lanes 1 and 2), anti-β7 (lane 3) or anti-GFP antibodies (lane 4). Bound proteins were separated by SDS–PAGE and analysed by Western blotting (WB) with anti-GFP (lanes 1 and 2), anti-Tat (lane 3) or anti-20S antibodies (lane 4).

of these transfected cells were fractionated by sucrose gradient ultracentrifugation and fractions were analysed by immunoblotting for GFP-Tat and 20S proteasomes. GFP-Tat indeed co-migrated with 20S proteasomes in fractions 10–12 (Fig. 4A) whereas GFP alone did not migrate in these 20S fractions (Fig. 4B) and was only found on the top of the gradient (data not shown). Similar results were obtained with IFNγ-stimulated cells where GFP-Tat sedimented in the same fractions with LMP7 (data not shown). Assaying the proteolytic activity in the gradient fractions reveals that cellular interaction of GFP-Tat with proteasomes results in maximum 80% inhibition for the chymotryptic activity of 20S proteasomes (Fig. 4C).

For direct proof of an in vivo interaction pull-down experiments were carried out using a polyclonal anti-proteasome antibody (MP3) and a monoclonal anti-β7 antibody with pooled gradient fractions containing 20S proteasomes and Tat. The proteins co-immunoprecipitated from the fractions with MP3 were further analysed by immunoblotting using a monoclonal anti-GFP antibody. Immunoblot analysis of MP3-precipitated proteins from gradient fractions 9–11 revealed a band of approximately 41 kDa corresponding to the GFP-Tat fusion protein (Fig. 5, lanes 2 and 3). No co-immunoprecipitated proteins were found in HeLa cells transfected with GFP alone (Fig. 5, lane 1). Identical results were obtained after immunoprecipitation using the anti-β7 antibody and immunoblot analysis with a polyclonal anti-Tat antibody (Fig. 5). The anti-GFP antibody vice versa immunoprecipitated proteasomal subunits detected in immunoblots by the MP3 antibody (Fig. 5, lane 4). Together these data show that HIV-Tat when expressed in HeLa cells associates with 20S proteasomes and impairs proteasomal activity.

4. Discussion

The HIV-1 Tat protein has pleiotropic roles in infected cells. The manipulation of the 26S proteasome by Tat is one of the intracellular activities of Tat [9,10]. A recent study has reported that Tat and the subunits of the PA28 regulator share a similar site for binding the α subunits of the 20S proteasome [10]. However, the distinct subunits of the 20S proteasome that bind to Tat had not been identified. We have now demonstrated a direct interaction of two α subunits, six constitutive β subunits and two immuno-β subunits with Tat in vitro (Figs. 2 and 3).

The binding of the Tat complex to the α subunits blocks binding of the PA28 regulator thereby interfering with antigen presentation [10]. Our results extend these findings by identifying the α subunits that bind directly to Tat (Fig. 2). Interestingly, the crystal structure of a PA28–20S complex shows that seven tail-like projections of the 11S regulator bind equally to pockets located between proteasomal α subunits forcing conformational changes in the proteasomal α subunits incompatible with the closed gate conformation [16]. These interactions might be prevented by Tat binding to the α4 and α7 subunits of the proteasome. Interestingly, the hepatitis B virus HbX protein also binds to the α4 subunit and interferes with PA28 regulator binding [17,18] indicating an important role for α4 in proteasome function.

Moreover, our experiments show that Tat binds to six β subunits of the constitutive 20S proteasome and two β subunits of the immunoproteasome (Fig. 3). The binding of Tat to β subunits may influence proteasomal catalytic activity for instance as an allosteric effector or by affecting its cellular distribution. However, mapping of the interaction site within the β subunits or the complex will be a future challenge.

Several other viral proteins bind to proteasomal β subunits. HIV Nef interacts with β7 and down-regulates intracellular β7 levels [19]. Furthermore, NF-κB/p105 processing by the proteasome is affected by the human T cell leukaemia virus Tax protein which binds to β7 as well as to α3 [20]. The combined observations suggest that viral proliferation and morphogenesis may capitalise on the manipulation of proteasomal catalytic activity as we observed a dramatic decrease of the chymotrypsin activity of the 20S proteasome in GFP-Tat-transfected cells in vivo (Fig. 4C). Proteasome inhibition by GFP-Tat is also mirrored by the stabilisation of proteasomal substrates such as IκBα (our unpublished results).

In summary, HIV-Tat influences proteasome function in two ways: (1) by interfering with binding of the PA28 regulator to the α ring by interaction with α4 and α7, and (2) by blocking the proteolytic activity of the proteasome in vivo by direct interaction with the β subunits thereby contributing directly to the immune escape of the virus.

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