Furin cleavage of the HIV-1 Tat protein

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Abstract Extracellular human immunodeficiency virus-1 (HIV-1) Tat protein and Tat-derived peptides are biologically active but mechanisms of Tat processing are not known. Within the highly conserved basic region of HIV-1 Tat protein (amino acids, a.a. 48–56), we identified two putative furin cleavage sites and showed that Tat protein was cleaved in vitro at the second site, RQRR↓ (a.a. 53–56↓). This in vitro cleavage was blocked by a monoclonal antibody that binds near the cleavage site or by the furin inhibitor α-1 PDX. Monocytoid cells rich in furin also degraded Tat and this process was slowed by the furin inhibitor or the specific monoclonal antibody. Furin processing did not affect the rates for Tat uptake and nuclear accumulation in HeLa or Jurkat cells, but the transactivation activity was greatly reduced. Furin processing is a likely mechanism for inactivating extracellular HIV-1 Tat protein.

Keywords: Tat; HIV; Furin; Protease; Processing

1. Introduction

The Tat protein of human immunodeficiency virus-1 (HIV-1) is a transcriptional activator for the provirus. Sequence-specific interactions of Tat protein with host factors and binding of these complexes to the transactivation-responsive sequence element in human immunodeficiency virus RNA relieve a block to transcript elongation and increase viral gene expression [1]. Tat is present in sera from infected individuals [2] and in Kaposi's sarcoma lesions where it was associated with endothelial spindle cells. Cells acutely infected with HIV supply Tat to cocultured spindle cells and increase their growth rate [3]. Extracellular Tat can rescue virus expression in some latently infected cells [4] and increase the permissivity of monocytes and lymphocytes for HIV-1 by increasing surface expression of the viral coreceptors, CXCR4 [5] and CCR5 [6].

Peptides corresponding to the cysteine-rich domain of Tat will activate monocytes [7] and induce tumor necrosis factor-related apoptosis-induced ligand (TRAIL) – mediated CD4+ T-cell killing [8]. Peptides containing the basic domain stimulated cytokine production in T-cells [9] and mediated protein transduction in many cell types [10]. Clearly, fragments or peptides from Tat retain a variety of activities, but whether these peptides are released in vivo, and the mechanisms for processing the intact protein, have not been described.

The Lys/Arg-rich sequence near the middle of Tat is required for nuclear entry and transcription. We noted the presence of recognition sites for furin-like endoproteases within this part of the protein. Furin is expressed in a broad range of tissue and cells, including T-cells and macrophages [11], and endoproteolytic cleavage occurs at the carboxyl terminus of the consensus sequence Arg-X-Lys/Arg-Arg↓. Furin cycles between the trans-Golgi network and the cell surface [12], and is also active in conditioned medium [13]. Here, we studied furin recognition and cleavage of HIV-1 Tat. We show that Tat cleavage by furin occurs in the most conserved region of the protein and furin-like proteases may be involved in processing of Tat in vivo.

2. Materials and methods

2.1. Monoclonal antibodies, Tat protein, and cell lines

Monoclonal antibodies that prevent nuclear accumulation and neutralize extracellular Tat activity in T-cells were reported previously [14]. The TR1 antibody binds to the amino terminal peptide (a.a. 1–15) and the 9A11 antibody recognizes the epitope RPPQ at a.a. 57–60. Tat protein (86 a.a.) from HIV BH10 was from Advanced Biosciences Laboratory, Inc., Kensington, MD or Aventis Pasteur, Paris, France. A full-length 101 a.a. Tat protein was from Xeptagen, S.p.A., Naples, Italy. The monocytoid cell line J774A.1 was obtained from the ATCC collection.

2.2. Incubation of extracellular Tat with J774A.1 cells

Examination of the HIV-1 Tat amino acid sequence revealed two putative furin cleavage sites (a.a. 49–50↓ and a.a. 53–56↓) within the basic region. The myeloid J774A.1 cells are known to express furin on the cell surface [11]. We incubated 10^6 J774A.1 cells in 50 μl of 0.15 M NaCl with 20 mM HEPES, pH 7.4, and 3 mM Ca^{2+} with 15 ng of recombinant Tat for different periods of time at 37 °C, in the presence or absence of 8 μg/ml of furin-like protease inhibitor, α-1 antitrypsin Portland (α-1 PDX). In some experiments, Tat was incubated with J774A.1 cells in the presence of 2 μg of 9A11 antibody or IgG1 control antibody. Cell free fluids were collected, separated by gel electrophoresis, and detected by Western blotting with the TR1 monoclonal antibody.

2.3. In vitro cleavage of Tat protein

In most experiments, we used the recombinant 86 a.a. form of HIV IIIB Tat. In some experiments, the 101 a.a. form of Tat was used. Tat was incubated with furin (Affinity Bioreagents, Golden, CO) at 1 μg of Tat to 1 U of furin in 20 μl of 200 mM HEPES, pH 7.2, containing 3 mM Ca^{2+}, for 2 h at 37 °C. Tat cleavage was prevented by a specific inhibitor of furin, α-1 PDX [15] (Fig. 1B). Products of the Tat cleavage were detected by either Coomassie staining of the polyacrylamide gel or Western blotting with the monoclonal antibody TR1, that recognizes the amino terminus.

2.4. NH_2-terminal sequencing

The 56 a.a. amino terminal Tat fragment was isolated by reverse phase HPLC on the Xterra MS C18 reverse phase column (Waters,

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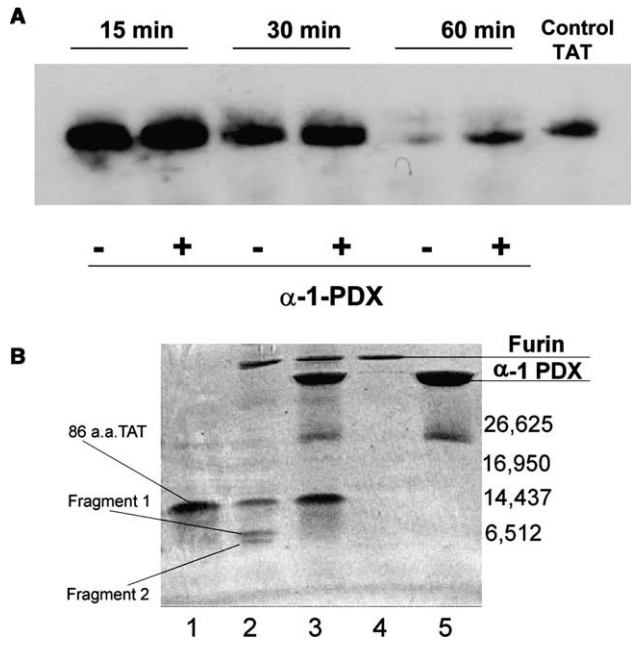

Fig. 1. (A) The furin inhibitor α-1 PDX slows Tat degradation in cell culture. Tat degradation was reduced significantly in the presence of α-1 PDX (+) compared to controls without inhibitor (−). The results are representative of three independent experiments. (B) Cleavage of Tat (86 a.a., HIV-1 III B) by furin in vitro. Coomassie staining of the polyacrylamide gel is shown. Furin treatment of Tat reduced the level of full-length cleaved Tat (14 kDa) and generated two cleaved products. The reaction is inhibited by adding the specific furin inhibitor α-1 PDX. Lane 1: non-treated Tat, lane 2: furin-treated Tat, lane 3: inhibition of Tat cleavage by α-1 PDX, lane 4: furin alone, lane 5: α-1 PDX alone.

Milford, MA). The proteins were eluted with a linear 5–65% gradient of acetonitrile in 0.1% TFA. The truncated 56 a.a. Tat was present in fractions containing 41–45% acetonitrile. Cleavage products were separated in 16.5% Tris–Tricine polyacrylamide gels to isolate the C-terminal Tat fragment. This fragment was submitted for sequencing by Edman's degradation (N-terminal sequencing) to confirm the cleavage site.

2.5. Tat internalization and Tat transactivation assays

Jurkat cells at 5×10^6 per ml were incubated with recombinant Tat (86 a.a. ABL, Kensington, MD) at 1 μg/ml, in RPMI supplemented with 0.1% Ultrapure BSA (Panvera, Madison, WI) for 1 h at 37 °C. In some cases, Tat was preincubated with monoclonal antibodies 9A11, TR1 or IgG controls at the ratio of 1 μg Tat to 25 μg IgG. After incubation, the cells were washed three times, then nuclear extracts were prepared using NE-PER kit (Pierce, Rockford, IL). The transactivation assay used a HeLa T4 cell line, containing a Tat-deficient integrated provirus (HeLa T4 HIV Tat-minus cells) [16] and was performed as described [14]. Briefly, HeLa T4 HIV Tat-minus cells were seeded into 96-well plates, at 20 000 cells per well, and incubated overnight. The wells were then incubated for 90 min with different Tat concentrations in RPMI, containing 0.1% Ultrapure BSA (Panvera, Madison, WI). The Tat solution was replaced with DMEM containing 10% FBS and 96 h later supernatants were tested for p24 content using a commercial ELISA (R&D Systems). In some experiments, after incubation with intact or furin-treated Tat HeLa T4 HIV Tat-minus cells were lyzed and nuclear extracts were analyzed in Western blot with monoclonal antibody TR1 to measure nuclear accumulation of Tat.

2.6. Western blotting

Western blotting was performed as described previously [14]. Briefly, 12.5 μg of each nuclear extract was separated by polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Protein transfer efficiency was routinely controlled by membrane staining with Ponceau Red (Sigma, St. Louis, MO). TR1 monoclonal antibody (0.5 μg/ml) and subsequent goat anti-mouse alkaline phosphatase conjugate (Sigma) were used to detect Tat on the blots.

3. Results

3.1. Tat degradation in cell culture is slowed by a specific inhibitor of furin-like proteases or a monoclonal antibody to the basic domain of Tat

Tat protein is not internalized by monocytoid cells and is degraded rapidly in culture. To test whether Tat cleavage may be mediated by furin-like proteases, we incubated Tat with the myeloid cell line J774A.1, that is known to express furin on its surface [11]. Tat was incubated with J774A.1 for different periods of time in the presence or absence of α-1 PDX, anti-Tat antibody 9A11 or IgG1 control antibody.

Degradation of Tat was significantly reduced by α-1 PDX, (Fig. 1A) or Tat-specific 9A11 antibody (Fig. 2B). We could not detect Tat accumulation in the cytoplasm or nuclei of J774A.1 cells in agreement with our previous finding that uptake of extracellular Tat is not detected in myeloid cells [8]. The experiment was repeated three times with similar results.

3.2. Furin cleavage sites in HIV-1 Tat

Entropy plot analysis of more than 1300 Tat sequences identified a highly conserved region between a.a. 44 and 56 [14]. This region includes a lysine/arginine rich sequence designated the basic domain [17]. Within the basic domain we identified two putative furin cleavage sites. The predicted

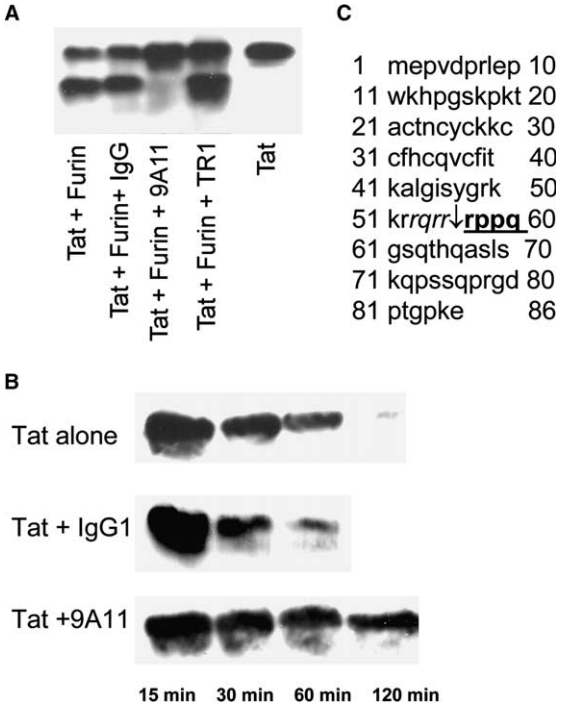

Fig. 2. (A) Monoclonal antibody 9A11 blocks furin cleavage of Tat in vitro. The 9A11 antibody recognizes the 57RPPQ60 sequence immediately downstream from the second putative furin recognition site (a.a. 53–56). (B) Tat degradation by J774A.1 cells was slowed in the presence of antibody 9A11. (C) Within the Tat sequence a furin cleavage site is indicated by arrow and the 9A11 epitope is highlighted in bold type.

recognition and scissile bond sites are: a.a. 49–52/53 and a.a. 53–56/57. Importantly, these sites are highly conserved in all HIV-1 sequences including multiple clades, and the second site (53–56/57) abuts the epitope for our 9A11 monoclonal antibody at a.a. 57–60 [14].

3.3. Furin cleaves Tat in vitro

Commercial 86 a.a. Tat was incubated with a purified furin. The cleavage produced two fragments of Tat (Fig. 1B) corresponding approximately to 7 and 5 kDa portions. Cleavage efficiency was routinely around 70%, although a minor fraction of non-cleaved material always remained. This may be due to the interaction between Tat molecules [18] or conformational changes that inhibit enzyme access to the cleavage site.

Cleaved Tat was purified by reverse phase HPLC and then subjected to electrospray mass spectrometry. The mass spectrometry identified a larger fragment of 6.6 kDa, that corresponds to cleavage at the second putative site, 53RQRR/R57. To confirm the cleavage site assignment, we separated fragments with a 16.5% Tris–Tricine polyacrylamide gel, then eluted the small fragment (carboxy terminal fragment) for Edman's degradation sequencing. The sequencing confirmed that cleavage occurred at the second putative furin site, placing the scissile bond between a.a. 56 and 57. We have also confirmed that furin cleaves full-length (101 a.a.) Tat protein between a.a. 56 and 57; irrespective of sequence differences at a.a. 57.

3.4. Monoclonal antibody 9A11 inhibits Tat cleavage by furin

The furin cleavage site in Tat abuts the minimal recognition site for our neutralizing monoclonal antibody 9A11 (a.a. 57–60). We next tested whether this monoclonal antibody would specifically block furin-mediated cleavage.

Tat protein was incubated with monoclonal antibody 9A11 or the control monoclonal antibody TR1 that recognizes the amino terminus. These complexes were exposed to furin and cleavage was monitored by polyacrylamide gel separation of products followed by Western blotting with the amino-terminal specific detection antibody TR1. Furin cleavage was blocked by 9A11 but not by the amino terminus-specific TR1 monoclonal antibody (Fig. 2A).

3.5. Uptake of the Tat protein by Jurkat cells and inhibition by monoclonal antibodies

We showed previously that exogenous Tat added to Jurkat T-cells readily enters and translocates to the nucleus [14]. This process was blocked by preincubating Tat with monoclonal antibody TR1 (reacting with an epitope within a.a. 1–15) or 9A11 (recognizing amino acids sequence 57–60). Here, we showed that the amino terminal Tat fragment generated by furin cleavage readily enters cells and penetrates the nucleus, similarly to 86 a.a. Tat (Fig. 3). The 9A11 antibody does not inhibit uptake of the furin-truncated form of Tat, because the antibody epitope is destroyed by the cleavage reaction (Fig. 3).

3.6. Furin cleavage reduces Tat activity in a cell-based transactivation assay

We asked whether furin cleavage altered the biological activity of Tat protein. Tat was treated with furin for 2 h at 37 °C and tested in the transactivation assay. Sequential 1:1 dilutions of cleaved Tat were tested for their ability to induce p24 production in HeLa T4 HIV Tat-minus cells. As a control, Tat was incubated in buffer without furin for 2 h at 37 °C and then overlayed on the indicator cells. Furin-treated Tat had a markedly reduced transactivation activity that was decreased approximately eight times compared to mock-treated controls (Fig. 4A). Furin cleavage reduced the transactivation activity of Tat without preventing Tat uptake and entry into the nucleus (Fig. 4B).

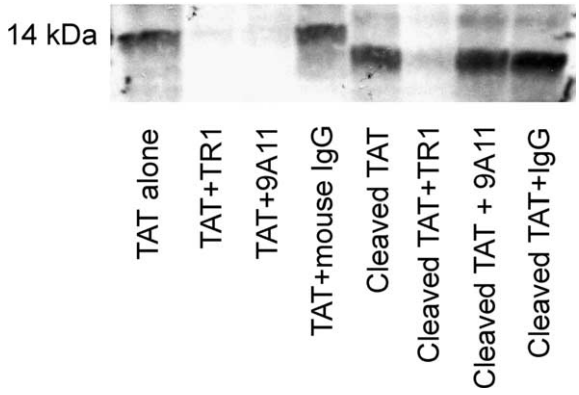

Fig. 3. Uptake of non-cleaved and furin-cleaved Tat by Jurkat T-cells. Both non-cleaved and cleaved Tat accumulated in the nuclei of Jurkat cells as shown by Western blotting of nuclear extracts with the monoclonal antibody TR1. TR1 recognizes an N-terminal part of Tat and only binds the larger cleavage product. Both monoclonal antibodies, TR1 (recognizing the N-terminal epitope) and 9A11 (recognizing the 57RPPQ60 sequence), inhibit uptake of intact Tat. Antibody TR1 but not the antibody 9A11 inhibited the uptake of cleaved Tat, since the epitope for 9A11 is separated by the furin reaction from the basic region sequences that control cellular penetration and nuclear localization.

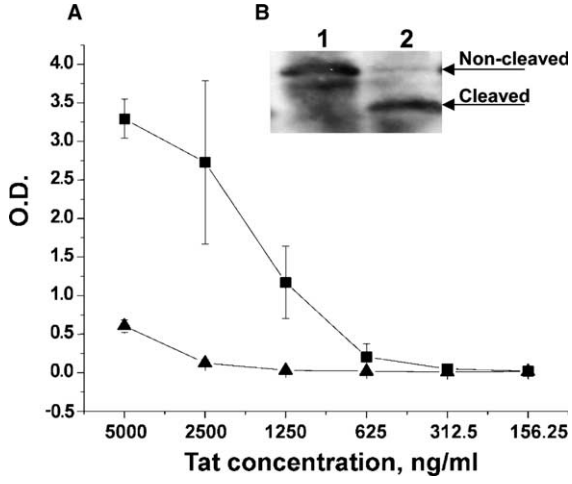

Fig. 4. (A) Inhibition of Tat transactivation activity by furin cleavage. The furin-treated Tat (triangles) had a reduced capacity to activate the Tat deficient integrated HIV provirus compared to mock-treated Tat (squares). Data are expressed as means ± S.D. of the optical density readings from quadruplicate capture ELISA assays. (B) Both intact and furin cleaved. Tat accumulate in the nuclei of HeLa T4 HIV Tat-minus cells in similar manner, as determined by Western blotting of nuclear lysates.

4. Discussion

The primary function of Tat is to regulate transcription from the HIV long terminal repeat. However, other evidence suggests that extracellular Tat contributes to HIV disease through its effects on bystander cells but mechanisms for eliminating extracellular Tat are not known. We showed that furin present on cell surfaces or in conditioned medium participates in the proteolytic processing of HIV-1 Tat. In vitro assays mapped the cleavage site to the second of two possible positions and showed that furin cleavage was not affected by Tat sequence variation at the carboxyl-terminus proximal position in the recognition site. Furin cleavage significantly reduced Tat protein potency for transactivating virus expression.

There is little information in the literature about post-translational modifications or processing of Tat. It is known that acetylation of lysines 50 and 51 stimulates Tat transcriptional activity [19]. Recently, the first report on Tat proteolytic processing showed that the HIV-1 protease was capable of degrading this protein in vitro [20]. We showed that Tat is degraded by contact with monocytoid cells and the process involves furin proteases in addition to other cellular proteases.

In vitro studies confirmed that Tat protein can be cleaved by furin, a subtilisin-like, Ca^{2+}-dependent endoprotease expressed in many eukaryotic cell types. Furin recognition at a.a. 52–56 matched the consensus recognition sequence (Arg-X-Lys/Arg-Arg↓) that is also recognized by a family of kexin/subtilisin-like serine proteinases (so-called convertases) found in many cells and tissues. The family of proteases includes furin, PC1/3, PC2, PACE4, PC4, PC5/6, PC7/LPC, PACE4, PC5/6 and PC7/LPC that are widely distributed among tissue and cell types [11]. So far, we have only tested furin in the Tat cleavage system but other family members may also be active.

Analysis of Tat sequences obtained from the NCBI database showed that a.a. 43–56, corresponding to the basic domain of Tat, are highly conserved among different HIV isolates [14]. This sequence is known to be important for Tat biological activity and represents an important target for the development of therapeutic agents. However, we found recently that this amino acid sequence is poorly immunogenic compared with non-conserved downstream sequences or the amino terminus (Tikhonov et al., unpublished). Specific recognition of furin cleavage sequences in the basic domain of Tat may be useful for developing molecular tools that inactivate Tat by targeting the most conserved part of this viral regulatory molecule.

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