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Tat peptides inhibit neprilysin

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Dementia associated with human immunodeficiency virus (HIV) infection occurs commonly in the aging population and amyloid depositions are noted in the brains of patients with HIV infection in younger age groups. This suggests a dysregulation of amyloid processing in the setting of HIV infection. The Tat protein of HIV has been implicated in the neuropathogenesis of HIV infection due to its neurotoxic and glial activation properties. However, Tat protein and Tat-derived peptides were recently also shown to inhibit neprilysin, the major amyloid β peptide degrading enzyme in brain, in a cell aggregate system. This effect could contribute to the observed accumulation of amyloid in the brain of HIV-infected patients. The authors report here that peptides derived from the Tat protein, but not Tat protein itself, inhibit homogeneous recombinant neprilysin. This inhibition was found to be competitive and reversible and therefore does not involve covalent bond formation. Tat peptides and Tat protein were slowly hydrolyzed by neprilysin. Thus the accumulation of Tat-derived proteolytic fragments may serve to inhibit neprilysin and increase amyloid β peptide levels. *Journal of NeuroVirology* (2006) **12,** 153–160.

Keywords: amyloid β peptide; catabolism; inhibition; neprilysin; Tat-derived peptides

Introduction

Human immunodeficiency virus (HIV) infection frequently causes a dementing illness, which is more profound and frequent in the aging population. This dementia is associated with neuronal dysfunction, which is pathologically characterized as a loss of synapses, shortening of neurites, dendritic abnormalities, as well as neuronal loss (McArthur *et al*, 2005). A number of viral proteins and host factors have been implicated in this neuronal injury (Mattson *et al*, 2005).

Several studies have noted marked increases in diffuse amyloid plaques in HIV infected brains compared to age-matched controls (Green *et al*, 2005; Rempel and Pulliam, 2005; Esiri *et al*, 1998; Izycka-Swieszewska *et al*, 2000) and in patients who had been exposed to antiretroviral therapy (Green *et al*, 2005). It has been shown that some antiretroviral drugs can block transporters on the brain endothelial cells that are involved in amyloid β peptide (Aβ) transport thus contributing to increases in Aβ within the brain. Neuroasymptomatic HIV patients have increased expression of amyloid precursor protein (APP) in neurons (Mankowski *et al*, 2002). Furthermore, cytokines such as interleukin-1, tumor necrosis factor-α, and interferon-α are elevated in the brain of patients with HIV dementia (Wesselingh *et al*, 1994). These cytokines increase the activity of the γ-secretase, which processes amyloid precursor protein (APP) to the neurotoxic Aβ fragments. Furthermore, similar to patients with Alzheimer's disease (Skoog *et al*, 2003; Vanderstichele *et al*, 2005), the levels of Aβ_{42} are diminished in the cerebrospinal fluid (CSF) of patients with HIV dementia (Brew *et al*, 2005).

Current antiretroviral therapy is targeted against either the viral protease or viral reverse transcriptase. These modes of therapy have no impact on

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Tat production once the cell is infected and proviral DNA has been formed. This may be particularly important in the brain where the virus may be sequestered in glial cells and macrophages and may evolve over a period of time. Hence understanding the interactions of Tat within the aging brain may be critically important. Tat is a nonstructural HIV protein that is essential for viral replication. It is a nonglycosylated protein formed from two exons. The first exon contributes to 72 amino acids and most functional activities have been attributed to this region. The second exon is variable in sequence and size but in most HIV strains is 29 amino acids in size. Tat transactivates the long terminal region of the HIV genome to initiate viral replication (reviewed in Brady and Kashanchi, 2005; Jeang *et al*, 1999). However, Tat is released extracellularly from HIV infected cells via a leaderless secretion pathway (Chang *et al*, 1997). Infected glial cells produce much larger amounts of *tat* transcripts as compared to that of p24 or gp41. (Tornatore *et al*, 1994). Extracellularly, Tat has the opportunity to interact with several membrane receptors including low-density lipoprotein receptor-related protein (LRP) to which it binds at multiple sites. Through interactions with LRP, Tat blocks the uptake of both APP and Aβ, which are the natural ligands for LRP, thus potentially increasing their extracellular concentrations (Liu *et al*, 2000). Further, Tat has been shown to bind to the Aβ degrading enzyme neprilysin (NEP), which is extracellularly localized on the plasma membrane, suggesting yet another mechanism for accumulation of Aβ in the brain of HIV-infected patients (Rempel and Pulliam, 2005).

The mechanism by which Tat inhibits neprilysin is not clear. Because the cysteine rich domain was considered to be the inhibitory region, possible mechanisms that have been considered include tight noncovalent complex formation with the active site of neprilysin with cysteines of Tat, possibly complexing with the active site zinc or formation of a covalent dimer by cystine bond formation (Nath and Hersh, 2005).

In this study we have examined the interaction of Tat protein and Tat derived peptides with purified neprilysin extracellular catalytic domain. We find that Tat-protein itself does not inhibit neprilysin; but that Tat-derived peptides from the cysteine-rich region of Tat are potent inhibitors. The mechanism of this inhibition is explored.

Results

For these studies a recombinant human neprilysin was obtained as its extracellular catalytic domain by secretion of the enzyme from HEK 293 cells as described in Materials and Methods. The enzyme was judged to be greater than 90% pure by Coomassie staining of an sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel (Figure 1).

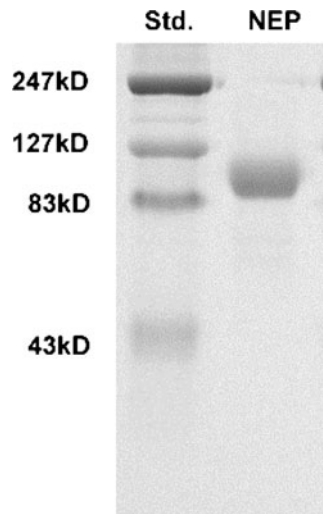

Figure 1 Purified recombinant NEP analyzed by SDS-PAGE. Recombinant extracellular domain of neprilysin was purified as described in Materials and Methods and analyzed on a 10% polyacrylamide gel stained with Coomassie blue.

We made numerous unsuccessful attempts to inhibit neprilysin activity with intact Tat protein 1–72, testing up to a concentration of 333 μg/ml in preincubation experiments and 33 μg/ml added directly to the assay. In these experiments the Tat protein concentration was 300 times higher in preincubation experiments and 30 times higher in the direct assay than used by Rempel and Pulliam (2005) where they noted inhibition of neprilysin activity in their aggregate cell system. Similarly, we attempted to inhibit neprilysin activity with intact Tat peptide 1–101 added to the assay, but again were unsuccessful at concentrations of 1.0 μg/ml and 5.0 μg/ml, which are 10 and 50 times higher concentrations than used by Rempel and Pulliam (2005).

We next tested a series of Tat peptides that spans the entire 102 amino acids of the Tat protein (Table 1). Shown in Figure 2 is the inhibition profile for the full set of Tat peptides. It should be noted that the inhibitory peptides span the same region of Tat found to be inhibitory by Rempel and Pulliam (2005), and include the cysteine-rich region-containing peptides. We measured the concentration dependence for the more inhibitory peptides, which is illustrated in Figure 3. These peptides show a hyperbolic response typical of inhibitors that follow classical Michaelis-Menton kinetics.

Because neprilysin contains cystine residues and the inhibitory peptides contain cysteine residues, we considered the possibility that these peptides inhibit by forming a covalently linked mixed disulfide with neprilysin. If this occurred, inhibition would be irreversible under our assay conditions because the covalently bound peptide would not be able to dissociate

Table 1 Tat peptides used in this study

Peptide amino acid sequence	*NIH AIDS Research and Reference HIV-1 clade B consensus Tat peptide code name*	*Tat peptide sequence*
MEPVDPRLEPWKHPG	5113	1–15
DPRLEPWKHPGSQPK	5114	5–19
EPWKHPGSQPKTACT	5115	9–23
HPGSQPKTACTNCYC	5116	13–27
QPKTACTNCYCKKCC	5117	17–31
ACTNCYCKKCCFHCQ	5118	21–35
CYCKKCCFHCQVCFT	5119	25–39
KCCFHCQVCFTTKGL	5120	29–43
HCQVCFTTKGLGISY	5121	33–47
CFTTKGLGISYGRKK	5122	37–51
KGLGISYGRKKRRQR	5123	41–55
ISYGRKKRRQRRRAP	5124	45–59
RKKRRQRRRAPQDSQ	5125	49–63
RQRRRAPQDSQTHQV	5126	53–67
RAPQDSQTHQVSLSK	5127	57–71
DSQTHQVSLSKQPAS	5128	61–75
HQVSLSKQPASQPRG	5129	65–79
LSKQPASQPRGDPTG	5130	69–83
PASQPRGDPTGPKES	5131	73–87
PRGDPTGPKESKKKV	5132	77–91
PTGPKESKKKVERET	5133	81–95
KESKKKVERETETDP	5134	85–99
KKVERETETDPVDQ	5135	89–102

Note. The peptides shown in bold are inhibitors of neprilysin activity.

from the enzyme. On the other hand, if the inhibition were simply the result of inhibitor binding, it would be reversible. We thus conducted an experiment in which neprilysin was preincubated for up to 30 min with 11.4 μM (20 μg/ml) Tat peptide 21–35 (code number 5118). The enzyme was then diluted 100-fold into an assay mix, and neprilysin activity determined. As shown in Table 2, although the concentration of Tat peptide preincubated with neprilysin inhibits the enzyme by more than 60% when included directly in the assay, the neprilysin activity was essentially the same as if no Tat peptide were present when the peptide was diluted 100-fold into the assay. This experiment shows that the Tat peptide can dissociate from neprilysin by simply lowering its concentration. Thus Tat peptide inhibition of neprilysin is reversible and does not result from disulfide bond formation.

Table 2 Reversible inhibition of neprilysin by Tat peptide 21–35 (ACTNCYCKKCCFHCQ)

Assay condition	*Rate of hydrolysis (μmols/min-mg neprilysin)*
Neprilysin alone	1.24
Neprilysin + 20 μg/ml peptide 21–35 in the assay	0.44
Neprilysin + 0.2 μg/ml peptide 21–35 in the assay	1.13
Neprilysin preincubated with 20 μg/ml peptide 21–35 and then diluted 10 fold into the assay	1.08

Note. Assays mixtures contained 20 mM MES buffer, pH 6.5, 100 μM glutaryl-Ala-Ala-Phe-MNA, ~0.1 μg aminopeptidase, and 80 ng of neprilysin. In the preincubated sample, the Tat peptide 21–35 concentration was originally 20 μg/ml but was diluted to 0.2 μg/ml in the assay. Similarly the neprilysin concentration was originally 8 μg/ml but was diluted to 80 ng/ml in the assay.

Because the Tat peptide acted as a reversible inhibitor, we determined the type of inhibition by measuring the effect of the Tat peptide 21–35 on the K_M and V_{max} of the neprilysin reaction. As shown

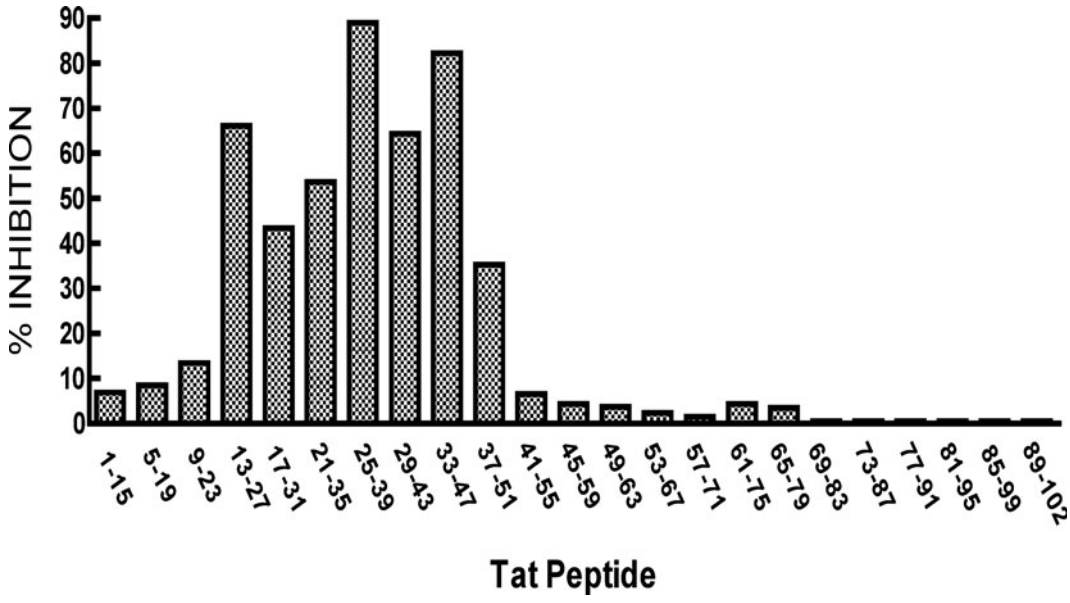

Figure 2 Inhibition profile for Tat peptides. Each peptide was tested at a concentration of 20 μg/ml with 100 μM glutaryl-Ala-AlaPhe-MNA as substrate in 20 mM MES buffer, pH 6.5. The numbers of the peptide corresponds to the Tat peptide sequence (see Table 1).

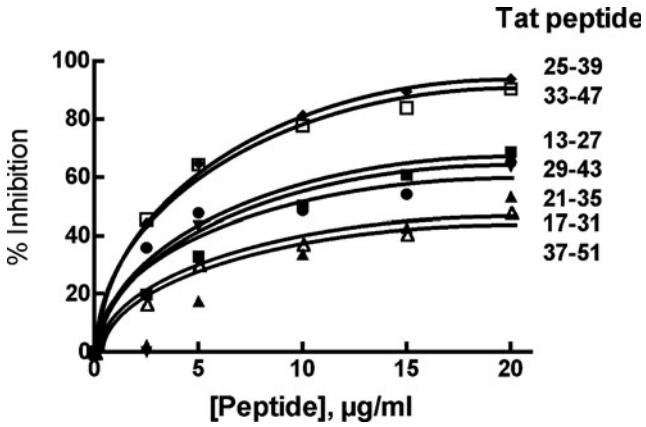

Figure 3 Inhibition of neprilysin (NEP) activity by Tat peptides. Neprilysin activity was measured in the presence of the various Tat peptides. Assay mixtures contained 100 μM glutaryl-Ala-Ala-Phe-MNA in 20 mM MES buffer, pH 6.5, 0.1 μg of neprilysin, and the indicated concentration of Tat peptide. The reaction was measured fluorometrically as described in Materials and Methods.

in Figure 4, inhibition by Tat peptide 21–35 was competitive.

We next determined whether the Tat peptides 21–35 (ACTNCYCKKCCFHCQ) and 33–47 (HCQVCFTTKGLGISY) were substrates for neprilysin by measuring their hydrolysis by high-performance liquid chromatography (HPLC). We compared the hydrolysis of the Tat protein or Tat peptide to Leu-enkephalin, a known endogenous substrate for neprilysin. We found that 25 μM Tat peptide 21–35 and 20 μM Tat peptide 33–47 were indeed hydrolyzed by NEP. This is illustrated in Figure 5. We also determined if intact Tat protein 1–101 could be hydrolyzed by neprilysin. As shown in Figure 5, the intact Tat protein is in fact hydrolyzed by neprilysin, but very slowly relative to Leu-enkephalin. Table 3 summarizes the rates of hydrolysis of the two Tat peptides and intact Tat protein, and as a reference the rate of hydrolysis of Leu-enkephalin is given. It can be seen that Tat protein is hydrolyzed at ~2% the rate of Leu-enkephalin, whereas Tat peptide 21–25 is hydrolyzed at ~4% the rate of Leu-enkephalin. On the other hand, Tat peptide 33–47 is a good substrate, being hydrolyzed at ~43% the rate of Leu-enkephalin.

Table 3 Rates of hydrolysis of Tat peptide 21–35 (peptide 5118), Tat peptide 33–47 (peptide 5121), and Tat protein 1–101 compared to Leu-enkephalin

Peptide	*Rate of hydrolysis in µmols/min-mg neprilysin (Rate relative to Leu-enkephalin)*
Tat protein 1–101	0.003 (0.022)
Peptide 21–35 (ACTNCYCKKCCFHCQ)	0.005 (0.036)
Peptide 33–47 (HCQVCFTTKGLGISY)	0.060 (0.43)
Leu-enkephalin	0.139 (1.00)

Note. Rates were calculated from the data in Figure 5.

Discussion

Rempel and Pulliam (2005) reported that Tat protein and Tat-derived peptides from the cysteine-rich domain of the Tat protein inhibit cellular neprilysin in a human aggregate cell system. This system was composed of all the cells of the central nervous system (CNS) and is reported to contain neurons, astrocytes, microglia, and oligodendrocytes with accompanying myelin within an extracellular matrix. The consequence of this inhibition was an elevated level of amyloid β peptide. In addition, the authors were able to show an increase in Aβ burden in HIV-1-seropositive patients, suggesting a correlation with neprilysin inhibition. In the present study, we have confirmed their findings that cysteine-rich peptides derived from the Tat protein are inhibitors of

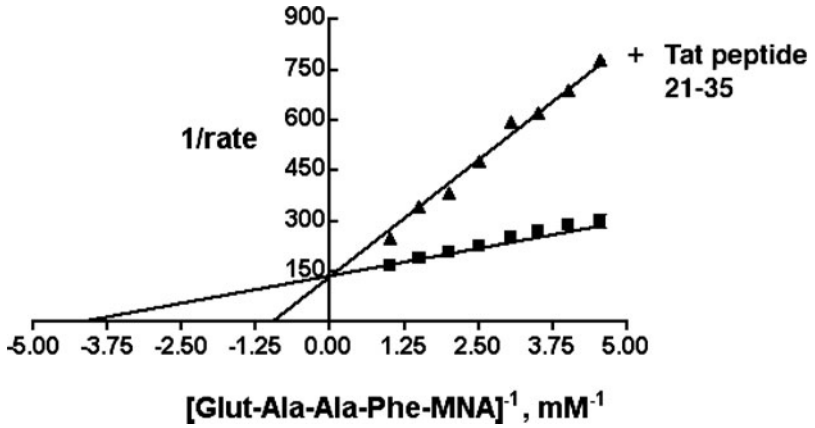

Figure 4 Lineweaver-Burke analysis of the inhibition of neprilysin (NEP) activity by Tat peptide 21–35. Assay mixtures contained variable concentrations of glutaryl-Ala-Ala- Phe-MNA in 20 mM MES buffer, pH 6.5, 0.1 μg of neprilysin, ~0.1 μg of aminopeptidase, and when added 30 μg/mL (10 μM) of Tat peptide 21–35. The reaction was measured fluorometrically as described in Materials and Methods with rates expressed as nmols/min. The data are shown plotted according to the Lineweaver-Burke equation.

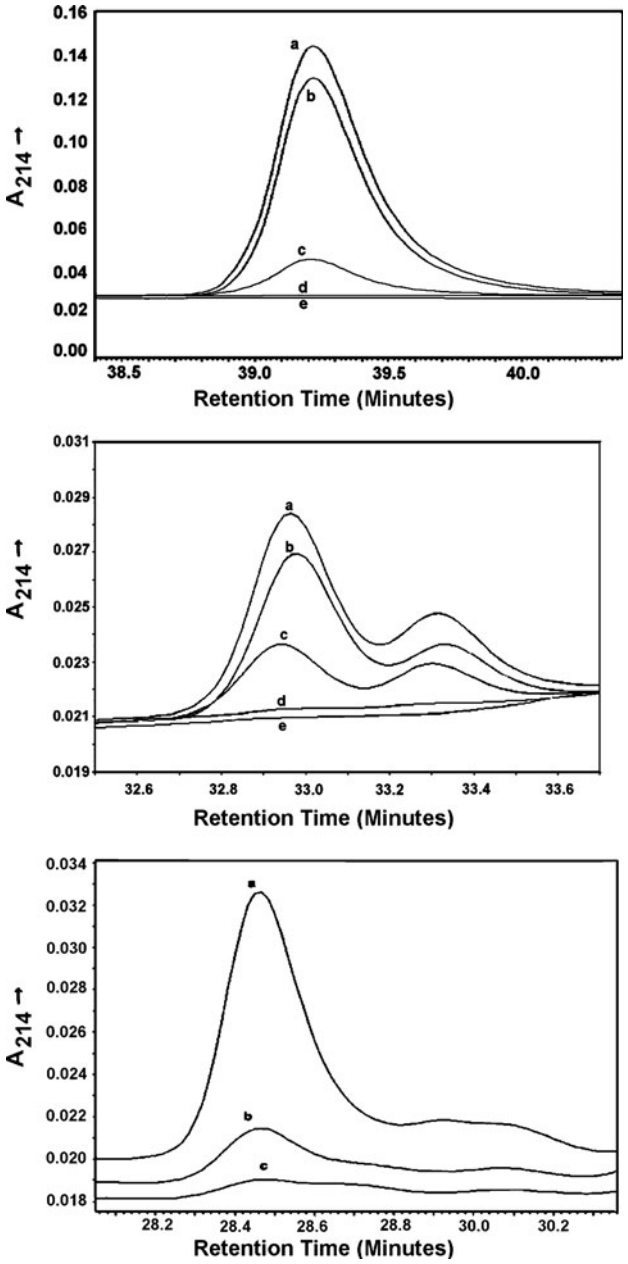

Figure 5 Hydrolysis of Tat-101, Tat peptide 5121, and Leu-enkephalin by neprilysin as analyzed by HPLC. Reaction mixtures containing phosphate-buffered saline, 1 mg/ml BSA, 0.1 mM dithiothreitol, the indicated substrate and neprilysin were incubated for 2 h at 37°C. Samples were analyzed by gradient HPLC as described in Materials and Methods. (Top) Reaction with 25 μM Leu-enkephalin (a) No enzyme, (b) 20 ng of neprilysin, (c) 200 ng of neprilysin, (d) 2000 ng of neprilysin, (e) buffer only. (Middle) Reaction with 12.5 μM Tat protein 1–101. (a) No enzyme, (b) 200 ng of neprilysin, (c) 2000 ng of neprilysin, (d) 20,000 ng of neprilysin, and (e) buffer only. (Bottom) Reaction with 20 μM Tat peptide 33–47. (a) No enzyme, (b) 200 ng of neprilysin, (c) 2000 ng of neprilysin.

neprilysin. On the other hand, we were unable to reproduce their finding that intact Tat protein is a neprilysin inhibitor even though we tried several different types and sources of full length Tat protein. We suggest this apparent discrepancy might arise from the fact that Rempel and Pulliam used a human aggregate cell system containing a number of proteins including neprilysin, whereas we used homogeneous recombinant neprilysin. Thus it is possible that in the human aggregate cell system, there are proteases that degrade intact Tat protein to generate inhibitory Tat peptides.

The mode of inhibition of the Tat peptides is shown to be reversible and competitive. We estimate from the data in Figure 3 that the K_i for all of the Tat peptides is in the range of 10 μM, which indicates a good affinity relative to neprilysin peptide substrates. Although a concentration of 10 μM Tat peptide might at first glance seem high, a known physiological substrate for neprilysin, Leu-enkephalin exhibits a K_M of ~50 μM (Hersh and Morihara, 1986) and it too is not found in high levels. In addition, because a number of Tat-derived peptides are inhibitors, these could produce an additive effect, making their effective concentration higher.

The Tat peptides can bind to the active site of neprilysin and block substrate entry. On the other hand not all of the inhibitory Tat peptides are good substrates for neprilysin. From the data in Figure 3 and Table 3, it can be seen that Tat peptide 33–47 and Tat peptide 21–35 are similar in their inhibition profile (Tat peptide 33–47 produces 1.5-fold more inhibition at 20 μg/ml), yet Tat peptide 33–47 is hydrolyzed by neprilysin more than 10 times faster than Tat peptide 21–35. This apparent discrepancy is related to the complex substrate specificity of NEP (Hersh and Morihara, 1986; Quay *et al*, 1994). The sequence of the peptide determines its ability to bind to neprilysin and to serve as a substrate, but these two parameters don't necessarily correlate. This is illustrated by the finding that the peptide Z-Ala-Leu-Ala is cleaved ~8 times faster than Z-Gly-Leu-Ala, yet the glycine-containing peptide binds with a two-fold higher affinity (Hersh and Morihara, 1986). Both of these peptides are cleaved on the amino side of the leucine residue. The biologically relevant concentration of Tat in the extracellular space remains unknown. However, serum concentrations in the nanomolar range have been reported (Westendorp *et al*, 1995). Thus the concentrations of Tat in the close vicinity of HIV-infected cells would be expected to be several fold higher. Further, Tat is more efficiently released extracellularly in low serum conditions (Chang *et al*, 1997) such as that present in the brain. Further, Tat transcripts are present at much higher levels compared to gag transcripts in glial cells (Tornatore *et al*, 1994).

The ability of the Tat peptides to inhibit neprilysin in the initial rate assays used in this study would not be expected to correlate with their ability to serve as a substrate, because the Tat peptides are not significantly consumed in the brief time interval used to conduct the initial rate assay used in this study. Because Tat peptide 21–35 is rather slowly

hydrolyzed, it would remain available longer to inhibit neprilysin, whereas Tat peptide 33–37 being more rapidly hydrolyzed would act as an alternate substrate inhibitor, with its inhibitory potency decreasing as it is hydrolyzed.

We have confirmed *in vitro* a direct protein–protein interaction between Tat peptides and neprilysin. *In vivo*, other factors could facilitate or diminish this interaction. Peptides derived from the region 17–47 of Tat inhibit neprilysin activity. These 15-mer peptides contained anywhere from 2 to 6 cysteine residues. All the peptides without cysteine residues failed to bind to neprilysin, whereas those with a single residue showed minimal binding. These findings suggest that the cysteine residues are critical for binding to neprilysin and that the site of location of these residues within the Tat protein may not be that important. However, we clearly demonstrated that the cysteine residues of the Tat peptides are not involved in covalent disulfide bond formation. The most likely explanation is that the cysteine residues of the Tat peptides interact with the catalytic zinc and block catalysis. Secondarily, the rest of the sequence of the peptide determines whether it is slowly or rapidly hydrolyzed. The relatively large size of Tat protein likely makes it a poor inhibitor, as neprilysin is known to prefer small substrates and to not work efficiently on proteins.

In this study, we used Tat protein derived from a single strain of HIV clade B virus. Interestingly, each of the cysteine residues are highly conserved in clade B virus, hence our findings are widely applicable to clade B virus. In clade C virus that infects the largest populations in Asia and Africa, the cysteine residue in position 31 is mutated to a serine (Ranga *et al*, 2004). The impact of this mutation on binding to neprilysin needs to be determined.

Processing of cellular proteins to produce bioactive compounds is a well-established phenomenon; examples of which include pro-MMP (matrix metalloproteinase) to active forms, proinsulin to insulin, and the processing of APP. Further, it was shown that under neuroinflammatory conditions, SDF-1 (stromal cell derived factor-1) may be cleaved to produce a neurotoxic molecule (Zhang *et al*, 2003). However, such processing of viral proteins to produce toxic peptides has not been shown before. Tat protein itself has been shown to be neurotoxic and may be cleaved by MMP-1 to form smaller peptides (Rumbaugh *et al*, 2005). The biological properties of these peptides had not been studied before, except that peptides derived from the arginine-rich region have the unique property of being efficiently transported across the cell membrane to the nucleus, a phenomenon also present in the intact Tat protein (Ma and Nath, 1997). This property has been exploited to drag other large proteins into cells, a phenomenon termed protein transduction (Schwarze *et al*, 1999). We now report that peptides derived from a viral protein can acquire new properties that could be detrimental to the host. In particular, we show that peptides from the cysteine-rich region can bind to neprilysin and inhibit its function. These observations may have important implications for viral pathogenesis and also for vaccine strategies, particularly subunit vaccines.

Materials and methods

Tat proteins (1–71, 1–86, and 1–101) were generated from their cDNAs and purified or were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, Maryland). The HIV-1 clade B consensus Tat 15-mer peptides were also obtained from the NIH AIDS Research and Reference Reagent Program. Stock solutions of intact Tat proteins were prepared in phosphate-buffered saline (PBS) with 1 mg/ml bovine serum albumin (BSA) and 0.1 mM dithiothreitol (DTT), whereas Tat peptides were prepared in the same solution containing 10% dimethylformamide (DMF).

Recombinant human neprilysin was obtained as its extracellular catalytic domain through secretion of the enzyme from lentivirus transduced HEK 293 cells (Shinall *et al*, 2005). The medium from these cells was collected after 5 to 7 days of culture in serum-free medium, concentrated ~10-fold, and then dialyzed against 20 mM Tris-HCl, pH 7.5. The dialyzed sample was applied to an anion exchange column (Source 15Q quaternary ammonium resin; Amersham Biosciences) in 20 mM Tris-HCl, pH 7.5. Neprilysin was eluted with a linear salt gradient from 0 to 0.5 M NaCl and the active fractions pooled and further purified on a Superdex 200 16/60 molecular sieve column (Pharmacia) in 20 mM Tris-HCl pH 7.5. Peak fractions containing neprilysin activity were pooled and concentrated. The purity of the enzyme was determined by SDS-PAGE on a 10% gel.

The inhibition of neprilysin by Tat protein or Tat peptide was tested by two methods. In the first method Tat protein or Tat peptide was added directly to the neprilysin assay mixture and activity determined as described below. In the second method Tat protein or Tat peptide was preincubated with neprilysin and then an aliquot was removed and assayed for enzyme activity. In this latter protocol the original concentration of the Tat protein or Tat peptide was diluted 100-fold in the assay reaction.

Neprilysin activity was determined with the fluorogenic substrate glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide (Li and Hersh, 1995) in a continuous assay. In this assay the glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide is cleaved by neprilysin to release Phe-4-methoxy-2-naphthylamide, which in the presence of an aminopeptidase is converted to the fluorescent 4-methoxy-2-naphthylamine. Reaction mixtures (200 μl) contained 100 μM glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide in 20 mM MES buffer, pH 6.5, and included ~1 μg of purified recombinant human puromycin-sensitive aminopeptidase

(Thompson *et al*, 2003). The formation of 4-methoxy-2-naphthylamine was measured continuously in a SpectraMax Gemini XS plate reader (Molecular Devices) at an excitation wavelength of 340 nm and an emission wavelength of 425 nm. Measuring the reaction in a continuous manner permits linear initial rates to be determined as compared to the two-step assay used by others in which the reaction is run for a fixed time period, stopped, and then an aminopeptidase is added. In this study the rates we determined were proportional to the amount of neprilysin in the assay, the reactions were inhibited by phosphoramidon, a specific neprilysin inhibitor, and the addition of more aminopeptidase to the assay had no effect on the rate.

In order to determine the type of inhibition produced by the Tat peptides, we performed a kinetic analysis in which the substrate glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide was varied in the presence or absence of Tat peptide 21–35. The data were plotted as 1/rate versus 1/[substrate] according to the Linewaever-Burke equation $\{1/v = 1/V_{max}(K_M/[S] + 1)\}$ where v is the observed rate, V_{max} is the maximal rate, [S] is the concentrated of substrate added, and K_M is the substrate K_M. In the presence of a competitive inhibitor the slope of the line will be increased but the intercept on the y axis will be unchanged.

The rate of cleavage of Tat protein, Tat peptides, and Leu-enkephalin was determined in reaction mixtures containing phosphate-buffered saline, 1 mg/ml BSA, and 0.1 mM dithiothreitol. Following incubation for 2 h at 37°C, the reaction was analyzed by HPLC using a C4 column and a linear gradient from 0.1% trifluoroacetic acid in 95% water/5% acetonitrile to 0.1% trifluoroacetic acid in 50% water/50% acetonitrile. Peptides and Tat protein were followed by absorbance at 214 nm. The rate of hydrolysis was quantified by measuring the decrease in the peak area of the substrate as a function of time and neprilysin concentration, and was inhibited by the neprilysin inhibitor phosphoramidon.

References

Brady J, Kashanchi F (2005). Tat gets the "green" light on transcription initiation. *Retrovirology* **2:** 69.

Brew BJ, Pemberton L, Blennow K, Wallin A, and Hagberg L (2005). CSF amyloid beta42 and tau levels correlate with AIDS dementia complex. *Neurology* **65:** 1490–1492.

Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B (1997). HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparin sulfate proteoglycan through its basic region. *AIDS* **11:** 1421–1431.

Esiri MM, Biddolph SC, Morris CS (1998). Prevalence of Alzheimer plaques in AIDS. *J Neurol Neurosurg Psychiatry* **65:** 29–33.

Green DA, Masliah E, Vinters HV, Beizai P, Moore DJ, Achim CL (2005). Brain deposition of beta-amyloid is a common pathologic feature in HIV positive patients. *AIDS* **19:** 407–411.

Hersh LB, Morihara K (1986) Comparison of the subsite specificity of the mammalian neutral endopeptidase 24.11 (enkephalinase) to the bacterial neutral endopeptidase thermolysin. *J Biol Chem* **261:** 6433–6437.

Izycka-Swieszewska E, Zoltowska A, Rzepko R, Gross M, Borowska-Lehman J (2000). Vasculopathy and amyloid beta reactivity in brains of patients with acquired immune deficiency (AIDS). *Folia Neuropathol* **38:** 175–182.

Jeang KT, Xiao H, Rich EA (1999). Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J Biol Chem* **274:** 28837–28840.

Li C, Hersh, LB (1995). Neprilysin: assay methods, purification, and characterization, *Methods Enzymol* **248:** 253–263.

Liu Y, Jones M, Hingtgen CM, Bu G, Laribee N, Tanzi RE, Moir RD, Nath A, He JJ (2000). Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. *Nat Med* **6:** 1380–1387.

Ma M, Nath A (1997). Molecular determinants for cellular uptake of Tat protein of human immunodeficiency virus type 1 in brain cells. *J Virol* **71:** 2495–2499.

Mankowski JL, Queen SE, Tarwater PM, Fox KJ, Perry VH (2002). Accumulation of beta-amyloid precursor protein in axons correlates with CNS expression of SIV gp41. *J Neuropathol Exp Neurol* **61:** 85–90.

Mattson MP, Haughey NJ, Nath A (2005). Cell death in HIV dementia. *Cell Death Differ* **12**(Suppl 1): 893–904.

McArthur JC, Brew BJ, Nath A (2005). Neurological complications of HIV infection. *Lancet Neurol* **4:** 543–555.

Nath A, Hersh LB (2005). Tat and amyloid: multiple interactions. *AIDS* **19:** 203–204.

Quay T, Slaughter C, Davis TP, Merrill BJ, Hersh LB (1994). Positional effects in the neprilysin (neutral endopeptidase) reaction. *Arch Biochem Biophys* **308:** 133–136.

Ranga U, Shankarappa R, Siddappa NB, Ramakrishna L, Nagendran R, Mahalingam M, Mahadevan A, Jayasuryan N, Satishchandra P, Shankar SK, Prasad VR (2004). Tat protein of human immunodeficiency virus type 1 subtype C strains is a defective chemokine. *J Virol* **78:** 2586–2590.

Rempel HC, Pulliam L (2005). HIV-1 Tat inhibits neprilysin and elevates amyloid beta. *AIDS* **19:** 127–135.

Rumbaugh J, Galey D, Turchan-Cholewo J, Conant K, St. Hillaire C, Anderson C, Slevin R, Nath A (2005). Interaction of HIV proteins and matrix metalloproteinases in HIV neuropathogenesis: new host defense mechanism. *Ann Neurol* **56:** S70–71.

Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF (1999). In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **285:** 1569–1572.

Shinall H, Song E-S, Hersh LB (2005). Susceptibility of amyloid beta peptide degrading enzymes to oxidative damage: a potential Alzheimer's disease spiral. *Biochemistry* **44:** 15345–15350.

Skoog I, Davidsson P, Aevarsson O, Vanderstichele H, Vanmechelen E, Blennow K (2003). Cerebrospinal fluid beta-amyloid 42 is reduced before the onset of sporadic dementia: a population-based study in 85-year-olds. *Dement Geriatr Cogn Disord* **15:** 169–176.

Tornatore C, Meyers K, Atwood W, Conant K, Major EO (1994). Temporal patterns of human immunodeficiency virus type 1 transcripts in human fetal astrocytes. *J Virol* **68:** 93–102.

Thompson MW, Govindaswami M, Hersh LB (2003). Mutation of active site residues of the puromycin-sensitive aminopeptidase: conversion of the enzyme into a catalytically inactive binding protein,*Arch Biochem Biophys* **413:** 236–242.

Vanderstichele H, De Meyer G, Andreasen N, Kostanjevecki V, Wallin A, Olsson A, Blennow K, Vanmechelen E (2005). Amino-truncated beta-amyloid42 peptides in cerebrospinal fluid and prediction of progression of mild cognitive impairment. *Clin Chem* **51:** 1650–1660.

Wesselingh SL, Glass J, McArthur JC, Griffin JW, Griffin DE (1994). Cytokine dysregulation in HIV-associated neurological disease. *Adv Neuroimmunol* **4:** 199–206.

Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH (1995). Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* **375:** 497–500.

Zhang K, McQuibban GA, Silva C, Butler GS, Johnston JB, Holden J, Clark-Lewis I, Overall CM, Power C (2003). HIV-induced metalloproteinase processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. *Nat Neurosci* **6:** 1064–1071.