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Exosome-associated release, uptake, and neurotoxicity of HIV-1 Tat protein

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Abstract

HIV-1 Tat is an indispensable transactivator for HIV gene transcription and replication. It has been shown to exit cells as a free protein and enter neighboring cells or interact with surface receptors of neighboring cells to regulate gene expression and cell function. In this study, we report, for the first time, exosome-associated Tat release and uptake. Using a HIV-1 LTR-driven luciferase reporter-based cell assay and Western blotting or in combination with exosome inhibitor, OptiPrep gradient fractionation, and exosome depletion, we demonstrated significant presence of HIV-1 Tat in exosomes derived from Tat-expressing primary astrocytes, Tat-transfected U373.MG and 293T, and HIV-infected MT4. We further showed that exosome-associated Tat from Tat-expressing astrocytes was capable of causing neurite shortening and neuron death, further supporting that this new form of extracellular Tat is biologically active. Lastly, we constructed a Tat mutant deleted of its basic domain and determined the role of the basic domain in Tat trafficking into exosomes. Basic domain-deleted Tat exhibited no apparent effects on Tat trafficking into exosomes, while maintained its dominant-negative function in Tat-mediated LTR transactivation. Taken together, these results show a significant fraction of Tat is secreted and present in the form of exosomes and may contribute to the stability of extracellular Tat and broaden the spectrum of its target cells.

Keywords

HIV-1 Tat; Exosomes; Tat release; Tat uptake; Biological activity

Introduction

HIV-1 Tat is one of the three early encoded HIV-1 proteins and translated from the multiply spliced viral RNA transcripts. The *tat* gene is extremely conserved at the amino acid level. Tat protein is encoded by two exons and contains 86–101 amino acids (aa), depending on HIV-1 isolates (Kulberg 1990). Examination of Tat primary amino acid sequence reveals four distinct domains (Bayer et al. 1995; Rice and Carlotti 1990; Sodroski et al. 1985): N-terminal activation domain (aa 1–20); central cysteine-rich domain (aa 21–37); core domain

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

for Tat binding to TAR RNA (aa 38–48); basic domain essential for nuclear localization, TAR binding, and transactivation (aa 49–57); and the second C-terminal exon of variable lengths. The C terminus of Tat also contains a highly conserved tripeptide sequence Arg-Gly-Asp, known as an RDG sequence, which has been found important for Tat binding to cell surface via integrin-mediated cell adhesion (Brake et al. 1990). In HIV transcription, Tat first binds to cyclin T1 and recruits cyclin T1/CDK9 complex termed positive transcription elongation factor b (P-TEFb) to the HIV-1 LTR promoter (Wei et al. 1998; Wei and Fultz 1998), a process that requires binding of Tat to the TARRNA bulge and of cyclin T1 to the TAR loop. The cyclin T1-associated CDK9 kinase then phosphorylates the C-terminal domain of RNA polymerase II (Pol II), leading to a critical transition of Pol II from a non-processive state to a processive state and, hence, transcription of HIV genome and synthesis of full-length HIV RNA transcripts. A number of cellular factors have been identified to interact with Tat and regulate HIV-1 replication (Hottiger and Nabel 1998; Kamine et al. 1996; Veschambre et al. 1995; Weeks et al. 1993; Xiao et al. 1998).

In addition to being a transcriptional regulator of HIV-1 replication, intact and biologically active HIV-1 Tat secretes out of HIV-infected cells (Westendorp et al. 1995; Xiao et al. 2000) and Tat-expressing cells (Albini et al. 1998b; Chang et al. 1997; Ensoli et al. 1990; Milani et al. 1993; Morgavi et al. 1997; Zauli et al. 1993, 1995). In the central nervous system (CNS), Tat is detected in the brain of patients with HIV-associated dementia (Hudson et al. 2000); it can derive from HIV-infected microglia/macrophages and astrocytes in the CNS or HIV-infected monocytes/macrophages and infiltrated CD4⁺ T lymphocytes (Banks et al. 2005; Chang et al. 1997; Chen et al. 1995; Ensoli et al. 1993). HIV-1 Tat secretion occurs through unconventional secretory pathways since the gene encoding Tat does not contain a signal sequence (Chang et al. 1997; Morgavi et al. 1997). Such alternative secretory pathways have been documented for other growth factors, such as interleukin-1 β and basic epidermal growth factor (Morgavi et al. 1997). Several studies including ours have demonstrated that HIV Tat or Tat-derived peptides are capable of entering cells cultured and transactivating the HIV LTR promoter (Bonifaci et al. 1995; Frankel and Pabo 1988; Green et al. 1989; Liu et al. 2000; Mann and Frankel 1991; Viscidi et al. 1989). Tat peptides have also been utilized to successfully deliver protein cargos into cell targets (Fawell et al. 1994; Schwarze et al. 1999; Vives et al. 1997). Basic peptides have been found to enhance Tat protein internalization (Green et al. 1989; Schwarze et al. 1999; Vives et al. 1997); the fact that Tat is a basic protein and contains strongly basic domains could possibly explain non-discriminative Tat uptake into cells. We have demonstrated that Tat specifically interacts with LRP on neurons, which leads to Tat neuronal uptake in its biologically active form (Liu et al. 2000), suggesting that Tat may affect neuronal functions through altering neuronal gene expression.

A number of functions have been proposed for extracellular Tat protein, resulting from transactivation of either LTR or cellular genes following direct Tat uptake, or as a consequence of intracellular signaling following the interaction of Tat with cell surface receptors. These include activated production and expression of growth factors (Gibellini et al. 1994) and cytokines (Cupp et al. 1993; Nabell et al. 1994; Sastry et al. 1990) and of cytokine receptors (Ganju et al. 1998; Pocsik et al. 1992; Puri and Aggarwal 1992), stimulation of cell proliferation and migration (Albini et al. 1996b; Singhal et al. 1995) and

protease production, chemoattractant activity of monocytes (Albini et al. 1998c; Huang et al. 1998), and angiogenic activity (Albini et al. 1994, 1995, 1996a; Barbanti-Brodano et al. 1994; Corallini et al. 1996). Extracellular Tat also transforms and immortalizes keratinocytes in cultures (Schuurman et al. 1993). In the CNS, Tat negatively impacts neurons in both direct and in indirect fashions. Direct Tat exposure alters integrity, homeostasis, neuroexcitatory property, endoplasmic reticulum calcium load, and oxidative state in neurons (Aprea et al. 2006; Brailoiu et al. 2006; Caporello et al. 2006; Kruman et al. 1998; Norman et al. 2007; Orsini et al. 1996). Tat could affect neuron survival indirectly by recruiting monocytes/ macrophages and lymphocytes into the CNS (Albini et al. 1998a; Benelli et al. 2000; de Paulis et al. 2000; Jones et al. 1998; Lafrenie et al. 1996; Park et al. 2001) or by altering neuronal gene expression profiles and intracellular signaling cascades (Eugenin et al. 2007; Liu et al. 2000; Peruzzi 2006). Tat can affect neuronal integrity and survival through its interaction with astrocytes (Fan et al. 2011, 2015; Kim et al. 2003; Liu et al. 2000; Zhou et al. 2004; Zou et al. 2010). Extracellular Tat induces apoptosis in peripheral blood mononuclear cells (Li et al. 1995), indicating its involvement in the depletion of CD4⁺ T cells and progression to AIDS. Extracellular Tat has also been implicated in the initial outburst of HIV-1 replication (Dunne et al. 1994) and for increased HIV-1 gene expression in glial cells through induction of cell arrest at G1 phase (Kundu et al. 1998). Finally, extracellular Tat represses the transcription of the MHC class I gene and negatively affects on the immune system (Barton et al. 1996; Carroll et al. 1992), human mannose receptor (Caldwell et al. 2000), and interleukin-12 (Ito et al. 1998). Tat has been shown to activate expression of the murine cytomegalovirus promoter (Kim and Risser 1993) and the JC virus (Chowdhury et al. 1990, 1992; Tada et al. 1990), suggesting that Tat can alter the replication of other viruses, probably through interaction with upstream enhancer elements. Thus, these findings support the important role of extracellular Tat in the progression of HIV/AIDS and HIV-associated pathologies including HIV-associated neurocognitive diseases.

In this study, we investigated the possibility of exosome-associated release and uptake of HIV-1 Tat protein. Using a HIV-1 LTR-driven luciferase reporter-based cell system and an OptiPrep-based exosome purification protocol, we demonstrated, for the first time, Tat association with exosomes and the biological activity of exosome-associated Tat. In addition, we attempted to determine the roles of Tat basic domain in Tat association with exosomes and Tat release from exosomes following cellular uptake.

Materials and methods

Cells and transfection

Human astrocytoma cell line U373.MG, human neuroblastoma cell line SHSY-5Y, and human kidney epithelial cells 293T were purchased from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD) supplemented with 10 % FBS (Hyclone, Logan, UT) and 1 % penicillin-streptomycin-glutamine (Lonza). Human CD4⁺ T cell line MT4 were obtained from NIH AIDS Reagent Program (kindly donated by Dr. Douglas Richman of the University of California, San Diego) (Larder et al. 1989) and maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640) (Lonza). TZM-b1 cells were obtained from NIH AIDS Reagent

Program (contributed by Dr. John C. Kappes and Dr. Xiaoyun Wu of Tranzyme Inc.) (Wei et al. 2002) and maintained in DMEM. Primary astrocytes were isolated from 18.5-day-old embryonic brain tissue of the doxycycline-inducible astrocyte-specific Tat transgenic mice (iTat) or C57B/L6 wild-type (WT) mice as described previously (Kim et al. 2003). Exosome-free media were obtained by ultracentrifugation of the complete media (serum with antibiotic) at 100,000 *g* for 16 h and removal of the exosomes and used in all studies involving exosomes. All transfections were performed using the standard calcium phosphate precipitation method.

Plasmids

cDNA3 and EGFP-N3 plasmids were purchased from Clontech (Mountain View, CA). Tat.Myc, Tat.His, and Tat.GFP plasmids were previously described (Liu et al. 2000). BD Tat mutant was constructed using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) with Tat. His as the template and primers 5'-GGC ATC TCC TAT GGC CCT CAA GGA TCC-3' and 5'-GGA TCC TTG AGG GCC ATA GGA GAT GCC-3'. The mutant deleted of the basic domain region (aa 49–57) was verified by sequencing.

Cell culture supernatant and exosome preparation

Cell culture supernatant and exosome preparation were performed as we previously described (Luo et al. 2015). Briefly, cells were cultured in exosome-free culture medium for 72 h. The culture media (36 ml) were collected, cleared of cell debris by centrifugation at 3000*g* for 10 min and filtration through a 0.22- μ m filter, and saved as supernatants. The cells were harvested and lysed in RTPA; whole cell lysates were prepared for Western blotting. The supernatants were further centrifuged at 100,000*g* for 70 min, the exosome pellets were washed and were either lysed in RIPA buffer [10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 % SDS, 140 mM NaCl, 2 mM PMSF, and 1 $\times$ protease inhibitor cocktail (Roche, Indianapolis, IN)] for Western blotting or suspended in 500 μ l phosphate-buffered saline PBS (exosomes). OptiPrep gradient fractionation was performed to fractionate exosomes as previously described (Li and Donowitz 2008; Tauro et al. 2012). Briefly, a 5 ml 6–18 % OptiPrep iodixanol (Sigma, St. Louis, MO) was prepared using a Hoefer SG15 gradient maker (Hoefer Inc., Holliston, MA). Exosomes (500 μ l) were loaded on top of the gradient, followed by centrifugation at 250,000*g* for 90 min. At the end of this centrifugation, a total of 11 fractions (500 μ l each) were collected from top to bottom and further analyzed for Tat transactivation activity and acetylcholinesterase (AChE) activity or subjected to trichloroacetic acid precipitation to recover the proteins from the fractions for Western blotting. In case of GW4869 treatment, cells were cultured in the presence of 10 μ M GW4869 (SantaCruz Biotechnology, Santa Cruz, CA) for 24 h prior to collection of culture media for supernatant and exosome preparation.

Western blotting

Protein concentration of whole cell lysates, exosomes, and TCA precipitates of exosome fractions was determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Whole cell lysates, exosomes, and TCA precipitates were electrophoretically separated by a

10 % SDS-PAGE gel, blotted, and detected using a home-made enhanced chemiluminescence kit. Antibodies for β -actin and TSG101 were from Sigma, CD63 from System Biosciences (Mountain View, CA), HIV-1 p24 monoclonal antibody (183-H12-5C) from NIH AIDS Reagents Program (donated by Dr. Bruce Chesebro of NIAID) (Chesebro et al. 1992), antibody for GFP from Clontech (Mountain View, CA), and sheep anti-mouse IgG-horseradish peroxidase (HRP) and donkey anti-rabbit IgG-HRP from GE Healthcare (Marlborough, MA).

Slot blotting for ganglioside GM1 detection

Exosome fractions (150 μ l each) from OptiPrep gradient fractionation were directly blotted onto nitrocellulose membrane using a slot blotting manifold (Schliecher and Schuell, Keene, NH). Ganglioside GM1 in each fraction was probed with HRP-conjugated Cholera toxin subunit B (Sigma) (Spiegel 1990). The GM1 level in each fraction was determined using the ImageJ (NIH) software and calculated as a percentage of the total GM1 in all fractions.

TZM-b1 assay

TZM-b1 assay was performed as previously described (Takeuchi et al. 2008). Briefly, TZM-b1 cells were plated in a 24-well plate at a density of 50,000 cells/well and cultured for 24 h. Supernatants, exosomes, and exosome fractions (200 μ l each unless otherwise stated) were added to each well and continued to culture at 37 °C for 24 h. The cells were washed with PBS and lysed in 1 $\times$ firefly luciferase lysis buffer (Promega, Madison, WI). Lysates were briefly centrifuged and mixed with firefly luciferase substrate (Promega), and the luciferase (Luc) activity was measured by an Opticomp Luminometer (MGM Instruments, Hamden, CT).

AchE activity assay

AchE activity assay was performed as we previously described (Luo et al. 2015). Briefly, exosome fractions (15 μ l each) obtained from OptiPrep gradient fractionation were mixed with 85 μ l 1.25 mM acetylcholine (Sigma) and 100 μ l 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma) in a 96-well plate. The mixtures were incubated at room temperature until yellow color was developed. The optical density (OD) at the wavelength of 450 nm was recorded using a Bio-Rad 96-well plate reader (Bio-Rad).

Neurotoxicity assay

SH-SY5Y was differentiated as previously described (Encinas et al. 2000). Differentiated cells were plated in a 24-well plate in a density of 75,000 cells/well and cultured for 24 h. Supernatants or exosomes (200 μ l each) were added into the cell culture and incubated for 48 h. Then, bright field images were taken for evaluation of morphology and neurite formation and length, followed by the (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. Briefly, MTT (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The medium was carefully removed, and the formazan crystals were solubilized in acidified isopropanol. The mixtures (150 μ l each) were transferred to a clean 96-well plate, and the plate was read at a test wavelength of 570 nm and a reference wavelength of 630 nm using a microplate reader.

HIV infection and RT assay

HIV infection and RT assay were performed as we previously described (Luo et al. 2015). Briefly, MT4 (1×10^6) were incubated with NL4-3 equivalent to 30,000 counts per minute (cpm) RT virus in 1 ml complete RPMI at 37 °C for 2 h. The cells were then pelleted down, washed with PBS to remove the input virus, added 2×10^6 fresh MT4, and continued to culture for 6 days. The culture media were collected to prepare supernatants and exosomes or used for RT assay as previously described (Luo et al. 2015). Briefly, supernatants (1 ml) were centrifuged at 14,000g for 1 h, and the pellets were suspended in 10 μ l dissociation buffer (50 mM Tris-HCl, pH 7.5, 0.25 % Triton X-100, 20 % glycerol, 1 mM DTT, and 0.25 MKC1), followed by three rounds of freezing and thawing. Then, 35 μ l RT assay buffer (50 mM Tris-HCl pH 7.5, 1 mM DTT, 10 mM MgCl₂, and 0.25 % Triton X-100), 5 μ l of 1 mg/ml poly(A) $\times$ (dT) 15 (Roche, Indianapolis, IN), and 1 μ l [³H]-thymidine 5'-triphosphate tetrasodium salt (ICN, Irvine, CA) were added to the virus lysate and incubated at 37 °C for 1 h. The mixtures were then spotted onto DE81 ion-exchange chromatographic disks (Whatman, Clifton, NJ). The disks were washed with 2 $\times$ SSC (0.3 M NaCl, 30 mM Na citrate pH 7.0) three times, 5 min each. The disks were then dehydrated with 100% ethanol, air-dried, and analyzed for ³H incorporation using a scintillation counter (Perkin Elmer, Waltham, MA). The RT activity was recorded as cpm per milliliter.

Exosome immunodepletion

Protein-A agarose beads (Millipore, Billerica, MA) were used to capture AchE+ exosomes as described previously (Subra 2013). Briefly, protein-A agarose beads (25 μ l) were coated with 1.6 μ g anti-AchE antibody (Sigma, St. Louis, MO) or isotype control IgG by shaking 1 h at room temperature. After washing with 1 $\times$ PBS, the antibody-coated beads were incubated at room temperature for 1 h with 200 μ l exosomes produced from culture supernatants of iTat and WT primary mouse astrocytes with or without doxycycline treatment and U373.MG transfected with pcDNA3 or pTat.MYC and mock- or NL43-infected MT4. Following this step, the bead-exosome complexes were pelleted down by centrifugation and the exosome-depleted supernatants were tested for Luc activity using the TZM-b1 assay as described above.

Results

Detection of HIV-1 Tat in exosomes derived from Tat-expressing cells

To determine whether HIV-1 Tat is secreted in the exosomes, we first took advantage of the doxycycline-inducible astrocyte-specific Tat transgenic mice (iTat), which was created in our laboratory (Kim et al. 2003; Zhou et al. 2004). Primary mouse astrocytes were isolated from those mice and treated with doxycycline to induce Tat expression. Tat presence in the supernatants of Tat-expressing astrocytes was confirmed using the HIV-1 LTR promoter-driven luciferase (Luc) reporter gene-based TZM-b1 assay (Fig. 1a). Exosomes derived from the very same Tat-expressing primary astrocytes showed transactivation of the Luc reporter gene expression in the same assay (Fig. 1b). To ascertain Tat presence in the exosomes, exosomes were further fractionated through a 6–18 % OptiPrep density gradient, and all fractions were analyzed using the TZM-b1 assay. Only a background level of Luc activity was detected in all fractions of exosomes from Dox-treated WT primary astrocytes (Fig. 2a).

Exosome marker analysis confirmed that OptiPrep gradient successfully separated the exosomes into AchE+CD63+ and TSG101 + subpopulations (Fig. 2b). In comparison, the highest Luc activity was detected in the first fraction of exosomes from Dox-treated iTat astrocytes, and all other fractions also showed a higher Luc activity (Fig. 2d). There were also AchE+CD63+ and GST101+ subpopulations of exosomes detected in those fractions (Fig. 2e). These results together suggest that HIV-1 Tat is associated with exosomes, particularly enriched in the AchE+ CD63+ exosomes.

AchE and CD63 are often associated with lipid microdomains (Van Deun et al. 2014). Thus, biogenesis of AchE+ CD63+ exosomes is likely ceramide-dependent. To further investigate Tat association with AchE+CD63 exosomes, Dox-treated iTat astrocytes were treated with GW4869, an inhibitor of neutral sphingomyelinase, which converts sphingomyelin to ceramide; exosomes were then prepared and fractionated through the same OptiPrep gradient. GW4869 treatment led to significantly lower Luc activity in the first fraction but higher Luc activity in all other fractions (Fig. 2g). In parallel, there were decreases in AchE +CD63+ exosomes (Fig. 2h). Decreases of ganglioside GM1, a glycosphingolipid abundant in lipid microdomains in AchE+ CD63+ exosome fractions, supported the notion that those exosomes were rich in lipid microdomains (Fig. 2c, f, i). These results not only further confirm Tat presence in the exosomes with more in AchE+CD63+ exosomes, but also suggest that AchE+CD63+ exosomes are generated in a ceramide-dependent manner.

Similarly, Tat was also detected in the exosomes from Tat-transfected U373.MG (Figs. 3 and 4) and in the exosomes from Tat-transfected 293T (Figs. 5 and 6). In addition to the Luc activity assay, Tat was detected in the exosomes from both Tat-transfected U373.MG and 293T by Western blotting, in which the exosome purity was verified by lack of cytochrome c in the exosomes (Figs. 3a and 5a). Moreover, one other important difference was noted. Compared to primary astrocytes (Fig. 2d), higher Luc activity was detected in the first three fractions in U373.MG (Fig. 4d) and little Luc activity above the background was detected in the first three fractions in 293T (Fig. 6d). This difference is likely due to the different levels of AchE and CD63 expression among those cells.

Tat is detected in exosomes from HIV-1-infected CD4+ T cells

Next, we determined whether Tat is present in the exosomes in the context of HIV infection. MT4 were infected with NL4-3, culture media were collected, and supernatants and exosomes were prepared. RT activity assay of the supernatants confirmed successful HIV infection in those cells (Fig. 7a). A significant Luc activity was detected in exosomes from NL4-3-infected cells (Fig. 7b). HIV-1 and exosomes are similar in size and density, and exosome preparations have been shown to contain HIV (Cantin et al. 2008). Thus, the significant Luc activity of exosomes derived from NL4-3-infected cells could likely be due to both transactivation of Tat in the exosomes on the HIV LTR promoter and HIV infection of TZM-bl. To distinguish these two contributing factors, exosomes from mock- and NL4-3-infected cells were further fractionated through the same OptiPrep gradient. Compared to the mock infection (Fig. 8a), significant Luc activity was detected in all fractions of exosomes from NL4-3-infected MT4 (Fig. 8d). As reported previously (Luo et al. 2015), there was no HIV, measured by Western blotting for p24 in the fractions of exosomes from mock

infection (Fig. 8b), while there was HIV detected around single fraction no. 8 (Fig. 8e). Exosome markers AchE, CD63, and TSG101 showed similar fractionation patterns as before, regardless of HIV infection (Fig. 8c, f). A clear separation of HIV from exosomes by the OptiPrep gradient (Luo et al. 2015) and a significant Luc activity detected in fractions without HIV indicate that Tat was also present in exosomes derived from HIV-1-infected CD4+T cells.

Significant levels of Tat in AchE+CD63+ exosomes

AchE is a GPI-anchored protein located at the surface of exosomes (Hicks et al. 2011), and anti-AchE antibodies have successfully been used to immunodeplete AchE+ exosomes (Subra 2013). Thus, to determine the relative contribution of AchE+CD63+ exosomes to Tat association with exosomes, we immunodepleted AchE+CD63+ exosomes using anti-AchE-coated agarose beads and performed the Luc activity assay. Exosomes from Tat-expressing primary astrocytes, Tat-transfected U73.MG, and HIV-infected MT4 were used. Compared to IgG control, anti-AchE antibody depletion decreased the Luc activity of exosomes from Tat-expressing astrocytes by 43 % (Fig. 9a), exosomes from Tat-transfected U373.MG by 40 % (Fig. 9b), and exosomes from HIV-infected MT4 by 70 % (Fig. 9c). These results provide additional evidence to support Tat presence in exosomes, particularly in AchE +CD63+ exosomes from astrocytes and CD4+ T cells.

Shortened neurites and decreased neuron survival by Tat-containing exosomes from Tat-expressing astrocytes

HIV-1 Tat expression in astrocytes causes neurite shortening and neuron death (Chauhan et al. 2003; Kim et al. 2003; Liu et al. 2000; Singh et al. 2005; Zhou and He 2004; Zou et al. 2007). To further characterize the biological activity of exosome-associated Tat, we cultured differentiated SHSY-5Y in the presence of exosomes from Tat-expressing primary astrocytes and determined effects of the exosomes on neurites and neuron survival. Exosomes were first tested for their transactivation activity on HIV-1 LTR promoter as before. Compared to mock and exosomes from primary astrocytes without Tat expression, more exosomes from Tat-expressing astrocytes gave rise to a higher Luc activity (Fig. 10a), meanwhile more neurons with shorter or no visible neurites (Fig. 10b) and more neuron death (Fig. 10c). Next, we used anti-AchE antibody-coated agarose beads to immunodeplete AchE+ exosomes from supernatants and determined effects of AchE+ exosome depletion on neurites and neuron survival. As shown before (Fig. 10b, c), only supernatants and exosomes from Tat-expressing primary astrocytes showed shortened neurites (Fig. 11a) and neuron death (Fig. 11b). Depletion of AchE+ exosomes from supernatants from Tat-expressing primary astrocytes significantly improved neurite morphology (Fig. 11a) and neuron survival (Fig. 11b) and, as expected, resulted in lower Luc activity (Fig. 11c). These results together provide further evidence to support that exosome-associated Tat is biologically active.

Role of Tat basic domain on Tat trafficking into exosomes

Tat basic domain has been shown to be responsible for binding to the endolysosomal membranes (Hui et al. 2012), which likely provides Tat access to the multivesicular bodies in exosome biogenesis. To address this possibility and to understand the molecular mechanisms of Tat association with exosomes, we constructed a Tat mutant BD deleted of

the basic domain (aa 48–57). U373.MG were transfected with Tat or BD. Supernatants and exosomes from Tat- and BD-transfected cells were prepared and tested for the LTR transactivation activity. As expected (Roy et al. 1990), both supernatants and exosomes from BD-transfected cells showed little Luc activity (Fig. 12a). However, BD and Tat were detected in the exosomes at a comparable level (Fig. 12b). BD functions as a dominant negative for Tat in the LTR transactivation (Fraisier et al. 1998; Meredith et al. 2009; Orsini and Debouck 1996). Consistent with these findings, co-transfection of Tat and BD into TZM-b1 showed significantly lower Luc activity than Tat transfection alone, by 70 % (Fig. 12c). Interestingly, exosomes from Tat- and BD-co-transfected cells showed a lower Luc activity than those of Tat transfection alone but, to a much lesser extent, only by 16 % (Fig. 12d). These results do not support any significant roles of the basic domain in Tat trafficking to exosomes and cannot completely rule out roles of the basic domain in regulation of Tat release from exosomes.

Discussion

In this study, we first demonstrated Tat association with exosomes derived from Tat-expressing primary cells (Fig. 1), Tat-transfected U373.MG (Fig. 3), Tat-transfected 293T (Fig. 5), and HIV-infected MT4 (Fig. 7). Tat association with exosomes, particularly AchE +CD63+ exosomes, was further confirmed by OptiPrep gradient fractionation (Figs. 2, 4, 6, and 8) and AchE antibody-based immunodepletion of exosomes (Fig. 9). In addition to the HIV-1 LTR promoter-based cell assay, Tat in the form of exosomes was shown to be biologically active, demonstrated by exosome-associated Tat-induced neurotoxicity such as shortened neurites and decreased neuron survival (Figs. 10 and 11). Lastly, we showed that deletion of the basic domain from Tat exhibited no apparent effects on Tat trafficking into exosomes, despite its dominant-negative function remained (Fig. 12). Taken together, these results provide strong evidence to support exosome-associated Tat release and uptake, a previously unrecognized pathway for Tat.

Exosomes are bi-layered extracellular vesicles with a size range of 50–150 nm and constitutively secreted by many cells and carry significant RNA and protein cargos and serve as intercellular messengers to regulate physiological and pathophysiological processes in target cells (Bellingham et al. 2012; Corrado et al. 2013; Simons and Raposo 2009). They are generated by inward budding of the multivesicular body (MVB) membrane, formation of intraluminal vesicles, fusion of the MVB membrane with the plasma membrane, and release of the intraluminal vesicles from MVB into the extracellular environment (Raposo and Stoorvogel 2013). The intraluminal vesicles in the MVB could be formed by endosomal sorting complex required for transport (ESCRT)-dependent pathway and ceramide-dependent pathway (Colombo et al. 2014). The ESCRT-dependent pathway involves the sorting of monoubiquitinated cargos on the MVB membrane and the packaging of cargos into the vesicles (Tanaka et al. 2008). The ceramide-dependent pathway mostly relies on lipids such as ceramide to induce inward vesicle budding of the MVB membrane and lipid raft formation (Megha 2004; Trajkovic et al. 2008). Thus, exosomes derived from those two pathways can be identified by specific markers: TSG101 for exosomes from ESCRT pathway (Tanaka et al. 2008; Théry et al. 2002) and GM1, CD63, and AchE for lipid raft-rich exosomes from ceramide pathway (Hicks et al. 2011; Johnstone 1992; Kerem et al.

1993; Megha 2004; Nichols et al. 2001; Pols and Klumperman 2009; Record et al. 2014; Schweitzer 1993). Our results demonstrated the presence of Tat in exosomes derived from both ESCRT and ceramide pathways (Figs. 2d–f, 4d–f, 6d–f, and 8d–f). More Tat was detected in exosomes from ceramide pathway than those from ESCRT pathway in astrocytes and HIV-infected CD4 T cells (Figs. 2d–f, 4d–f, and 8d–f), while Tat was exclusively detected in ESCRT exosomes in 293T (Fig. 6d – f). These results are in agreement with the significant demand of astrocytes for major lipid raft components such as ceramide in their metabolism (Blázquez et al. 2001) and production of ceramide-rich exosomes in astrocytes (Wang et al. 2012). Subsequent studies showed that disruption of ceramide synthesis by GW4869 abrogated Tat activity in ceramide-derived exosomes in astrocytes (Figs. 2g – i and 4g–i) but did not have any effects on ESCRT-derived exosomes in 293T (Fig. 6g – i) and that anti-AchE antibody depletion of AchE+/ CD63+ exosomes significantly decreased Tat activity (Fig. 9) and astrocyte-mediated Tat neurotoxicity (Figs. 10 and 11). These results further support Tat presence in both ceramide-and ESCRT-derived exosomes.

Unconventional secretion of Tat protein outside the cell has been attributed to Tat interaction with phosphatidylinositol 4, 5-bisphosphate head groups in the phospholipid membranes and with negatively charged lipid groups in the membranes such as dioleoylphosphocholine head groups and lipid raft domains through its positively charged basic domain (Akabori et al. 2014; Futaki et al. 2001; Rayne et al. 2010a; Tyagi et al. 2001; Xie et al. 2014). Tat has recently been shown to bind endolysosomal membranes resulting in enlarged and dysfunctional lysosomes (Hui et al. 2012). Thus, it is conceivable that following exit from the nucleus or being taken up into the cell, Tat binds lipid raft domains on endocytic membranes that mature into late endosomes and MVB, leading to sorting of Tat into intraluminal vesicles and finally its release through exosomes. To directly determine if the basic domain of Tat is necessary for binding to endocytic membranes and gaining access to the exosome biogenesis machinery, we created a basic domain-deleted Tat mutant and studied its exosomal secretion. Although the basic domain deletion of Tat maintained dominant-negative effects over Tat in Tat-mediated LTR transactivation, this mutant was detected in the exosomes (Fig. 12a – c). However, compared to direct inhibition of Tat-mediated LTR transactivation by the mutant (Fig. 12c), exosomes from cells expressing both Tat and the mutant showed much less inhibitory effects on Tat-mediated LTR transactivation (Fig. 12d). These results indicate that Tat interaction with biological membranes may not be involved in Tat trafficking to the endocytic system and exosome biogenesis apparatus. On the other hand, studies have shown that neutralizing the positive charges of Tat basic domain does not abolish the interaction of Tat with membranes (Rusnati et al. 1998) and that hydrophobic domains of Tat (aa 36–47) are capable of forming bonds with lipid bilayers (Li et al. 2012a). It is clear that additional studies are needed to determine the role of other Tat domains such as the hydrophobic domain in Tat traffic to the MVB.

It is interesting to note that two recent proteomic studies on exosomes from HIV-1-infected T cells have failed to detect Tat protein but have detected either known Tat-interacting cellular proteins such as CD38 and ANAXA5 and other viral components such as Gag, Env, and TAR miRNA in exosomes using the highly sensitive liquid chromatography-linked tandem mass spectrometry (LC-MS/MS) (Li et al. 2012b; Narayanan et al. 2013). However, lack of detection of Tat in exosomes from the infected cells in these two studies cannot

simply be interpreted as absence of the protein. LC-MS/MS has not been detected Tat protein in HIV-producing CD4 T cells (Li 2015; Santos et al. 2012; Saphire et al. 2006) and in sera of HIV-infected patients (Donnelly and Ciborowski 2016). Only one study has detected Tat protein in purified HIV using LC-MS/MS in combination with micro-capillary reversed-phase liquid chromatography (μ RPLC) (Chertova et al. 2006), which has been shown to improve detection of low-abundant proteins such as Tat (Tomlinson and Chiciz 2003). These findings suggest that LC-MS/MS alone is not sufficiently sensitive to detect trace amount of highly basic proteins such as Tat (Ott 2008). In addition, since Tat is not packaged into HIV, the Tat protein detected in the purified virus preparation above is likely derived from exosomes that were co-fractionated with HIV.

Viral proteins and nucleic acids and intact viruses have been shown to be present in exosomes from infected cells. In case of HIV, intact HIV, Gag protein, unspliced HIV-1 RNA, and HIV-derived microRNA have been detected in exosomes from HIV-infected cells (Columba Cabezas and Federico 2013; Fang et al. 2007; Narayanan et al. 2013; Wiley and Gummuluru 2006). Thus, exosomes from HIV-infected cells could contribute to transmission of the virus or work as a mechanism of defense against the virus. In this study, we showed that a fraction of HIV-1 Tat protein is released in the form of exosomes and is biologically active. Incorporation of Tat into exosomes can be considered advantageous for HIV-1 pathogenicity. The majority of Tat protein has been shown to secrete outside the infected cell while a trace amount of the protein remains in the nucleus to ensure efficient transcription of the viral genome (Rayne et al. 2010b). Therefore, exosomal secretion of Tat will not negatively affect the life cycle of HIV-1. On the other hand, the non-discriminative nature of exosome uptake could afford access of exosome-associated Tat to diverse cell types and therefore enhance the adverse effects of HIV-1 infection. In the CNS, exosomes produced from HIV-infected microglia/macrophages and astrocytes can potentially improve the stability of Tat by protecting the protein from degradation and expand the efficient delivery of Tat to other cells in the CNS including neurons. A recent study has shown that Tat is persistently expressed in the CNS in the era of combination antiretroviral therapy (Johnson et al. 2013). Thus, additional studies on therapeutic interventions targeted at exosome-associated Tat protein are warranted and may help mitigate the symptoms of HIV/neuro AIDS.

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Fig. 1.

Transactivation of HIV-1 LTR promoter by exosomes derived from Tat-expressing primary mouse astrocytes. Primary astrocytes were prepared from wild-type (WT) or Tat transgenic mice (iTat) and cultured in the absence of doxycycline (– Dox) or presence of Dox (+ Dox) for 48 h. The culture media were collected, cleared of cell debris, and saved as supernatants or further processed to isolate exosomes. TMZ-b1 were plated in a 24-well plate at a density of 50,000 cells/well, cultured for 24 h, added 200 μ l supernatants (**a**) or exosomes (**b**) prepared from primary astrocytes above, cultured for additional 24 h, and harvested for the luciferase reporter gene assay. The data were mean $\pm$ SD of triplicates and representative of three independent experiments

Fig. 2.

Transactivation of HIV-1 LTR promoter by exosomes derived Tat-expressing primary mouse astrocytes followed by OptiPrep fractionation. Exosomes derived from WT + Dox primary astrocytes (**a–c**) and iTat + Dox astrocytes (**d–f**) in Fig. 1 were further fractionated in 6–18 % OptiPrep. **g–i** Exosomes were derived from iTat primary astrocytes that were treated with 10 μ M GW4869 for 24 h between Dox treatment and collection of culture medium. All fractions were analyzed for their transactivation activity on the LTR promoter activity as in Fig. 1 (**a, d, g**); for exosome markers AchE activity (*top panels* in **b, e, h**), CD63 expression (*middle panels* in **b, e, h**), and TSG101 expression (*bottom panels* in **b, e, h**) by Western blotting; or for GM1 expression by slot blotting, followed by densitometry (**c, f, i**). GM1 expression in each fraction was expressed as a percentage of the total GM1 in all fractions. The data were mean $\pm$ SD of triplicates and/or representative of three independent experiments

Fig. 3.

Detection of Tat in exosomes derived from Tat-transfected U373.MG. U373.MG were transfected with Tat.Myc expression plasmid and cultured for 48 h. cDNA3 plasmid, the cloning backbone of Tat.Myc plasmid, was included as a control. Supernatants (*Supn*) and exosomes (*Exsm*) were prepared as above. Cells were harvested for whole cell lysates (WCL). Tat expression in WCL and Exsm was detected by Western blotting against an anti-Myc antibody (**a**). Cytochrome c (Cyto c) was included in Western blotting to ensure the exosome purity. The transactivation activity of Supn and Exsm was determined as above (**b**).

The data were mean $\pm$ SD of triplicates and/or representative of three independent experiments

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Fig. 4.

Transactivation of HIV-1 LTR promoter by exosomes derived from Tat-transfected U373.MG followed by OptiPrep fractionation. Exosomes derived from cDNA3-transfected U373.MG (**a–c**) or Tat.Myc-transfected U373.MG (**d–f**) in Fig. 3 were further fractionated in 6–18 % OptiPrep. **g–i** Exosomes were derived from Tat.Myc-transfected U373.MG that were treated with 10 μ M GW4869 for 24 h between Dox treatment and collection of culture medium. All fractions were analyzed for their transactivation activity on the LTR promoter activity (**a, d, g**); for exosome markers AchE activity (*top panels* in **b, e, h**), CD63 expression (*middle panels* in **b, e, h**), and TSG101 expression (*bottom panels* in **b, e, h**) by Western blotting; or for GM1 expression by slot blotting, followed by densitometry (**c, f, i**). GM1 expression in each fraction was expressed as a percentage of the total GM1 in all fractions. The data were mean $\pm$ SD of triplicates and/or representative of three independent experiments

Fig. 5.

Detection of Tat in exosomes derived from Tat-transfected 293 T. 293 T were transfected with Tat. GFP expression plasmid and cultured for 48 h. GFP plasmid, the cloning backbone of Tat.GFP plasmid, was included as a control. Supernatants (Supn) and exosomes (Exsm) were prepared as above. Cells were harvested for whole cell lysates (WCL). Tat expression in WCL and Exsm was detected by Western blotting against an anti-Myc antibody (**a**). Cytochrome c (Cyto c) was included in Western blotting to ensure the exosome purity. The

transactivation activity of Supn and Exsm was determined as above **(b)**. The data were mean $\pm$ SD of triplicates and/or representative of three independent experiments

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Fig. 6.

Transactivation of HIV-1 LTR promoter by exosomes derived from Tat-transfected 293T followed by OptiPrep fractionation. Exosomes derived from GFP-transfected 293T (**a–c**) or Tat.GFP-transfected 293T (**d–f**) in Fig. 5 were further fractionated in 6–18 % OptiPrep. **g–i** Exosomes were derived from Tat.GFP-transfected 293T that were treated with 10 μ M GW4869 for 24 h between Dox treatment and collection of culture medium. All fractions were analyzed for their transactivation activity on the LTR promoter activity (**a, d, g**); for exosome markers AchE activity (*top panels* in **b, e, h**), CD63 expression (*middle panels* in **b, e, h**), and TSG101 expression (*bottom panels* in **b, e, h**) by Western blotting; or for GM1 expression by slot blotting, followed by densitometry (**c, f, i**). GM1 expression in each fraction was expressed as a percentage of the total GM1 in all fractions. The data were mean $\pm$ SD of triplicates and/or representative of three independent experiments

Fig. 7.

Transactivation of HIV-1 LTR promoter by exosomes derived from HIV-infected MT4. MT4 were inoculated with NL4-3 at 37 °C for 2 h. A mock infection (mock) was included as a control. After removing the input virus, the cells were cultured for 6 days. The culture medium was collected and used to prepare supernatants and exosomes as above. The supernatants were determined for virus replication using the RT activity assay (**a**); the exosomes were assayed for the transactivation activity of HIV-1 LTR promoter as above (**b**). The data were mean $\pm$ SD of triplicates and representative of three independent experiments

Fig. 8.

Transactivation of HIV-1 LTR promoter by exosomes derived from HIV-infected MT4 followed by OptiPrep fractionation. Exosomes derived from mock-infected MT4 (**a–c**) or NL4-3-infected MT4 (**d–f**) in Fig. 7 were further fractionated in 6–18 % OptiPrep. All fractions were analyzed for their transactivation activity on the LTR promoter activity (**a, d**); for p24 level by Western blotting (**b, e**); or for exosome markers AchE activity (*top panels* in **c, e**), CD63 expression (*middle panels* in **c, e**), and TSG101 expression (*bottom panels* in **c, e**).

e) by Western blotting. The data were mean $\pm$ SD of triplicates and/or representative of three independent experiments

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Fig. 9.

Tat level in the AchE+ exosomes. Exosomes derived from iTat-Dox and iTat+Dox primary mouse astrocytes (**a**), cDNA3- and Tat.Myc-transfected U373.MG (**b**), or mock- and NL4-3-infected MT4 (**c**) were incubated with anti-AchE antibody-coated protein A agarose beads (AchE Ab) or a matched IgG-coated protein A agarose beads. After removing the beads, the remaining exosomes were determined for the transactivation activity of HIV-1 LTR promoter as above. The data were mean $\pm$ SD of triplicates and representative of three independent experiments

Fig. 10.

Effects of Tat-containing exosomes on neurite growth and neuron survival. Exosomes (1×: 10 μ l; 20×: 200 μ l) from iTat-Dox and iTat + Dox primary mouse astrocytes in Fig. 1 were determined for the transactivation activity of HIV-1 LTR promoter as above (**a**); or added into differentiated SHSY-5Y, cultured for 24 h, and imaged for neurite growth (**b**); or cultured for 48 h and determined for neuron survival using MTT assay (**c**). A regular medium was included as a mock control (mock). Neuron survival was calculated using mock as a reference, which was set at 100 %. The data were mean $\pm$ SD of triplicates and representative of three independent experiments

Fig. 11.

Effects of AchE+ exosomes on neurite growth and neuron survival. Supernatants (Supn) and exosomes (Exsm) from iTat-Dox and iTat+Dox primary mouse astrocytes in Fig. 1 and AchE+Ab-depleted exosomes (Supn*) were added into differentiated SHSY-5Y, cultured for 24 h, and imaged for neurite growth (**a**); or cultured for 48 h and determined for neuron survival using MTT assay (**b**); or determined for the transactivation activity of HIV-1 LTR promoter as above (**c**). A regular medium was included as a mock control (mock). Neuron survival was calculated using mock as a reference, which was set at 100 %. The data were mean $\pm$ SD of triplicates and representative of three independent experiments

Fig. 12.

Role of Tat basic domain on Tat sorting into exosomes. **a, b** U737.MG were transfected with Tat.His (Tat) or its basic domain deletion (ΔBD). Supernatants (Supn), exosomes (Exsm), and whole cell lysates (WCL) were prepared as above and determined for transactivation activity of HIV-1 LTR promoter (**a**) and Tat expression by Western blotting against anti-His antibody (**b**). **c** TZM-b1 were transfected with Tat, ΔBD, or Tat/ΔBD and assayed for the Luc activity. **d** 293T were transfected with Tat, ΔBD, or Tat/ΔBD. Exosomes were assayed

for transactivation activity of HIV-1 LTR promoter. The data were mean $\pm$ SD of triplicates and representative of three independent experiments

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