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Internalization of Jembrana disease virus Tat: Possible pathway and implication

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Abstract

Jembrana disease virus (JDV) is a lentivirus highly related to the bovine immunodeficiency virus (BIV). It causes an acute disease with high mortality rate within 1–2 weeks. JDV encodes the most potent Tat (JTat) of any of the lentiviruses. JTat can transactivate all LTRs and functionally substitute for HIV Tat in the viral genome and may function as a pivotal regulator in the acute pathogenesis of JDV. The goal of this paper is to study JTat internalization by cells, the mechanisms involved in internalization, and the effect of JTat on neighbouring cells. By quantification and fluorescence microscopy, we found that the internalization of extracellular EGFP-JTat fusion protein was both time and dose-dependent, but endocytosis and energy independent. We identified that arginines which were responsible for the internalization. Internalized JTat was distributed in both the nucleus and the cytoplasm, could transactivate JDV LTR and modulate cellular gene expression. Based on our findings, we propose that secretion and internalization of JTat may be a way for JDV to influence neighbouring cells and make the cellular environment more amenable to viral infection.

Keywords: JDV Tat; EGFP; Internalization; Arginine-rich domain

1. Introduction

Jembrana disease was first recognized in 1964 as an acute and infectious disease affecting cattle in the Jembrana district of Bali island in Indonesia (Budiarso and Hardjosworo, 1976; Wilcox et al., 1997). Although the sequence analysis of the etiological agent suggested that Jembrana disease virus (JDV) is a lentivirus highly related to the bovine immunodeficiency virus (BIV) (Chadwick et al., 1995a, b), it causes an acute disease in infected animals after a short incubation period, and its characteristics are quite different from those of most lentivirus infections (Budiarso and Hardjosworo, 1976; Wilcox et al., 1995). For example, while human immunodeficiency virus (HIV) causes immunodeficiency and ultimately leads to the death of infected patients several years after infection (Coffin et al., 1997), JDV rapidly replicates on extremely high titers in blood of infected animals and can lead to death of the animal within only 1–2 weeks (Wilcox et al., 1995, 1997). The mortality rate is about 17% (Chen et al., 1999).

Lentiviruses are complex retroviruses, which encode a number of accessory proteins that play important regulatory roles in the viral life cycle (Dignam et al., 1983). One of these accessory proteins, Tat, can strongly activate viral long terminal repeat (LTR) and is essential for efficient transcription of viral genes and for viral replication. Tat is encoded by two exons, exon1 and 2. The protein encoded by exon 1 is a strong transactivator, whereas the role of exon 2 encoded Tat is still unknown (Chen et al., 1999). Tat binds to the transactivation response (TAR) element and recruits the positive transcription elongation factor b (P-TEFb), which relieves a transcriptional block (Mancebo et al., 1997; Marshall et al., 1996; Zhu et al., 1997). JDV encodes a Tat closely related to BIV Tat, which can strongly activate not only the JDV LTR but also the LTRs of other lentiviruses, including HIV and BIV (Chen et al., 2000; Cheng-Mayer et al., 1991). JDV Tat can specifically recognize the heterologous TAR RNAs using very different mechanisms (Smith et al., 2000). Moreover, JDV Tat can functionally substituted for the HIV Tat when the JDV tat gene is introduced into the HIV genome (Chen et al., 2000), suggesting similar mechanisms may be shared between JDV and HIV Tat (HTat) proteins.

Viruses can influence neighbouring uninfected cells by secreting certain proteins, modulating a number of cellular genes, and making the cellular environment amenable for viral

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replication (Brigati et al., 2003). HIV Tat has been shown to act as a pleiotropic factor for a number of functions both inside and outside of the cell (Brigati et al., 2003). Extracellular Tat also possesses the unusual property of being able to enter the cells and translocate to the nucleus (Green and Loewenstein, 1988; Mann and Frankel, 1991). This unusual characteristic requires a nine-amino acid arginine-rich sequence (RKKRRQRRR). This basic region of the protein is also contains the nuclear localization signal and the transactivation-responsive region binding domain of the protein (Marcello et al., 2001; Jeang et al., 1999). Several other proteins, such as Drosophila Antennapedia homeoprotein, and HSV-1 VP22 have subsequently been shown to traverse the cell membrane. Many types of molecules can be internalized by cells after they are covalent coupled to short cell permeable peptides. These structures include peptides (Bonny et al., 2001; Dostmann et al., 2000), proteins (Jo et al., 2001; Peitz et al., 2002), oligonucleotides (Astriab-Fisher et al., 2000), and high macromolecular weight liposomes (Torchilin et al., 2001), polymeric particles (Lewin et al., 2000), phages (Eguchi et al., 2001) and adenoviruses (Gratton et al., 2003). These short cell permeable peptides (CPPs) share little sequence or structural homology except for high arginine content, and yet there is competition for cellular uptake among them (Suzuki et al., 2002) indicating the possible existence of common internalization mechanisms of CPPs (Suzuki et al., 2002). It is not clear whether tat peptides interact with cell surface sulfated polysaccharides (Hallbrink et al., 2001; Tyagi et al., 2001; Violini et al., 2002), or are internalized by endocytosis (Suzuki et al., 2002; Silhol et al., 2002; Richard et al., 2003).

It is thought that Tat released by infected cells exerts autocrine and paracrine activities that are beneficial to HIV survival and spread (Noonan and Albini, 2000). Tat can be taken up by bystander cells, reach the nucleus, and transactivate cellular genes such as interleukin 6 (IL-6), IL-2 and TNFα (Goldstein, 1996), and some endothelial adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 (Dhawan et al., 1997). Thus, some of the reported effects of Tat may be mediated by Tat-induced cytokines or, possibly, chemokines.

We were interested in determining the possible role of exogenous JDV Tat in the pathogenesis of JDV infections. We have examined the internalization ability of JDV Tat fusion proteins expressed in vitro and their effects on neighbouring uninfected cells. Analogous to HIV Tat, JDV Tat fusion proteins expressed in prokaryotic cells can translocate through the plasma membrane in an endocytosis-independent pathway and accumulate primarily in the cytoplasm. We found the responsible JDV Tat sequence to be **R**HDG**RR**KK**R**GT**R**GKG**R**. Extracellular JDV Tat cannot only translocate through the plasma membrane and transactivate JDV LTR, but also can directly activate cellular promoters such as the NFκB response element. This is of interest because HIV Tat transactivation involves the direct, TAR-independent activation of NFκB (Demarchi et al., 1996). Our results provide evidence that the JDV Tat is a pleiotropic factor in both infected and uninfected cells. Further characterization of the mechanism involved in pathogenesis by JDV Tat may benefit the research of JDV as a special acute lentivirus.

2. Materials and methods

2.1. Plasmids

The cDNA transcript of JDV *tat* was amplified via PCR from a eukaryotic expression plasmid pJtat (Liu et al., 2003). The resultant PCR product was digested and subcloned into pEGFP-C3 (Clontech) in frame with a EGFP open-reading frame to generate a plasmid named pEGFP-Jtat, the eukaryotic expression plasmid of EGFP-JTat fusion protein.

pETH (Yuan et al., 2004) was the parental plasmid for construction of various prokaryotic expression vectors. Using plasmids pJtat, pHtat, pEGFP-C3 and pEGFP-Jtat bearing the JDV *tat*, HIV *tat*, EGFP and EGFP-Jtat fusion protein genes as templates, the cDNA transcripts were PCR amplified and subcloned into pETH to create prokaryotic expression plasmids named pETH-Jtat, pETH-Htat, pETH-EGFP and pETH-EGFP-Jtat. pETH-EGFP-Jtat was *Bgl*II-*Xho*I-digested, eliminated the JDV *tat* coding region, the remaining plasmid was named ETH-EGFP. HIV *tat* was subcloned, *Bgl*II-*Xho*I-digested and ligated with ETH-EGFP to create pETH-EGPF-Htat. All Tat proteins used in this article were encoded by exon 1. pETH-EGFP-JR and pETH-EGFP-JRm were constructed to express the Arg-rich domain (amino acids 62–77 including 6 arginines) of JTat or 2 arginines mutated to lysines within this domain (4 arginines left) and fused to EGFP as follows. First, two oligonucleotides were synthesized, annealed and incubated with *Ex* DNA polymerase (Takara) and dNTP at 72 °C for 15 s to generate a doublestranded oligonucleotide encoding the 16 amino acids of JTat Arg-rich domain or mutant. The sequences for JTat Arg-rich domain were (top strand) 5′-GGGAGATCTCAGACATGATGGAAGAAGGAAGAAA-AGAGGAACCAG-3′ and (bottom strand) 5′-GGGCTCGA-GGATCCTCCCCTTTCCTCTGGTTCCTCTTTTCTTCC-3′. The sequences for JTat Arg-rich domain mutant were (top strand) 5′-GGGAGATCTCAAGCATGATGGAAGAAGGAA-GAAAAGAGG_AACCAAG-3′ and (bottom strand) 5′-GGGC-TCGAGGATCCTCCCCTTTCCCTTGGTTCCTCTTTTCTT-CC-3′. pETH-EGFP-HTatR was constructed to express the Arg-rich domain (amino acids 49–57 including 6 arginines) of HTat and fused to EGFP in a similar way. The double-stranded oligonucleotides were *Bgl*II-*Xho*I-digested and ligated with ETH-EGFP to generate pETH-EGFP-JR, pETH-EGFP-JRm and pETH-EGFP-HTatR. The sequencing of these plasmids was carried out by Shanghai Bioaisa Bio-technology LTD.

The JDV promoter constructs, pJDVLTR-*luc* was constructed by insertion of wide type JDV LTR in front of a luciferase report gene. pJDVLTR-RRE-*luc* was constructed by substitution of the HIV Rev response element (RRE) with JDV LTR TAR(nucleotide [nt] 0 to +59, encompassing the intact JDV TAR sequence) in pJDVLTR-*luc*. Additional NFκB and AP1 promoter constructs named pNFκB*3-*luc* and pAP1*3-*luc* were constructed by insertion of three tandem NFκB or AP1 response elements in front of luciferase report gene, these were used previously to test their responsiveness to BICP0 or ICP0 transactivation. pJtat-Rev was eukaryotic expression plasmid for JTat-Rev fusion protein (data not shown).