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# Unconventional secretion of viral proteins

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## Abstract

Although largely less numerous and characterized than bacterial secreted effectors, several viral virulence factors are secreted by virus infected cells. However, their mode of secretion only starts to be studied at the molecular level. Several of these viral effectors are secreted using an unconventional secretion pathway, *i.e.* despite the lack of signal sequence. We here review recent results illustrating the diversity of these pathways. In the case of HIV-1 proteins Tat and matrix (p17) proteins, secretion directly takes place at the plasma membrane level following binding to PI(4,5)P<sub>2</sub>. The secretion of HTLV-I Tax was found to partly rely on exocytic pathway intermediates. The secretion pathways of VP22 of Herpes simplex virus type I and VP40 of the Ebola virus are less well characterized but VP40 can be recruited to the plasma membrane by PI(4,5)P<sub>2</sub> that thus appears as a key partner enabling the unconventional secretion of many viral proteins. Several studies indicated that circulating retroviral transactivating proteins Tat and Tax are involved in the development of AIDS and HTLV-I associated myelopathy/tropical spastic paraparesis, respectively.

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## Introduction

Most viruses modify infected cells so that these pathogens can easily and safely multiply intracellularly. Accordingly, several viral effectors have an intracellular target that they will either activate or neutralize. For instance, HIV-1 Nef protein prevents MHC class-I molecules transport to the cell surface thereby preventing infected cell lysis by cytotoxic CD8 T-cells [1]. However, viral proteins are often multifaceted proteins and the role of some of them is not limited to their intracellular function. Infected cells indeed actively secrete some viral effectors, and some key virulence factors are exported in the absence of signal sequence. They therefore follow an unconventional secretion pathway. This is the case of retrovirus transactivating proteins Tat (HIV-1) [2] and Tax (HTLV-1) [3], of Ebola virus VP40 matrix protein [4] and of herpes virus VP22 [5]. They therefore follow an unconventional protein secretion (UPS) pathway.

UPS pathways of signal peptide lacking proteins fall in three types. Type I UPS refers to cargo translocation across the plasma membrane, either through self translocation, or with the help of other protein complexes and pores. Type II refers to the use of ABC transporter, and Type III corresponds to secretion of cytoplasmic proteins that reach the lumen of a membrane-bound organelle or that are associated with a membrane-bound organelle during their export route. In addition, they can be released via extracellular vesicles, microvesicles shedding from the plasma membrane and/or by exosomes [6].

In this review we will present and discuss the evidences for the unconventional secretion of viral proteins, the role of these secreted proteins in viral multiplication and their possible association with the development of associated disease(s).

### \*HIV-1 Tat

HIV-1 transactivating protein (Tat) is a small (10 kDa, 86-102 residues) and basic protein that is crucial for viral transcription. Although essentially observed at the plasma membrane level in primary CD4<sup>+</sup> T-cells (the main targets of the virus), Tat concentrates into the nucleus of most cell lines [7, 8]. However, although devoid of signal peptide, Tat was detected in the extracellular medium in the absence of cell lysis [2]. Moreover, Tat secretion was not inhibited by brefeldin A [9], a drug that disrupts the Golgi and inhibits the ER to Golgi conventional secretion pathway [10]. Collectively, these observations indicated that Tat is secreted using an unconventional pathway. This secretion process was found to rely on Tat recruitment to the plasma membrane by phosphatidylinositol (4,5) bisphosphate (PI(4,5)P<sub>2</sub>) (Fig.1) [8], as it is the case for FGF2 secretion [11]. Tat has a very strong affinity for PI(4,5)P<sub>2</sub>, much stronger than that of cellular proteins. This is because to bind PI(4,5)P<sub>2</sub> Tat not only uses the 49-51 basic-residue triplet within its basic domain to recognize PI(4,5)P<sub>2</sub> head group, but also inserts the side chain of its single Trp (W11) in the membrane. In fact, Tat requires a lipid membrane to bind PI(4,5)P<sub>2</sub>. Hence, contrarily to FGF2 [11], Tat does not recognize PI(4,5)P<sub>2</sub> when spotted on nitrocellulose [8]. The key role of Tat Trp in PI(4,5)P<sub>2</sub> binding was confirmed using conservative mutations such as Tat-W11Y that strongly affected Tat binding to PI(4,5)P<sub>2</sub>. Trp side chain insertion into the membrane upon PI(4,5)P<sub>2</sub> binding is a first and key step of Tat translocation to the *trans*-side of the membrane. Accordingly, Tat mutants unable to bind PI(4,5)P<sub>2</sub> such as Tat-(49-51)A or Tat-W11Y failed to be secreted. Moreover, PI(4,5)P<sub>2</sub> depletion using 5-phosphatases or PI(4,5)P<sub>2</sub> masking using neomycin (a polycationic antibiotic) inhibited Tat secretion, thereby confirming the crucial role of PI(4,5)P<sub>2</sub> in Tat unconventional secretion [8].

Recently, as reported earlier for FGF2 [12], Tat and Tat-fusion proteins were found to bind to the alpha subunit of the Na<sup>+</sup>/K<sup>+</sup> ATPase. Accordingly, just as FGF2 [13], Tat secretion by HEK cells was inhibited by ouabain, a well-known inhibitor of the Na<sup>+</sup>/K<sup>+</sup> ATPase [14]. Nevertheless, ouabain did

not affect Tat secretion by T-cells [7] and the role of the Tat-Na<sup>+</sup>/K<sup>+</sup> ATPase interaction in Tat secretion is therefore unclear.

Following PI(4,5)P<sub>2</sub> binding, membrane insertion and translocation to the *trans* side of the membrane, Tat interacts with heparin sulfate proteoglycans (HSPG) that likely facilitate the refolding of secreted Tat [9], as it is the case for FGF2. Indeed, HSPGs are well known to bind extracellular Tat [15] and FGF2 [16]. These interactions are most likely electrostatic driven since HSPGs are highly sulfated anionic polymers [17], while Tat and FGF2 are basic proteins with pIs of 10 and 11, respectively. Tat translocation step *per se* is poorly understood (Fig.1) and it is still not clear whether it requires a cytosolic chaperone. It might need unfolding since, contrarily to VP22, Tat is unable to export  $\beta$ -galactosidase (a huge 476 kDa protein) from cells [18]. An *in vitro* study using PI(4,5)P<sub>2</sub> liposomes and purified Tat indicated that Tat can form pores into membranes [19], as does FGF2 [20]. Liposome destabilization was followed by the release of encapsulated calcein and Tat mutants unable to bind PI(4,5)P<sub>2</sub> were only weakly active in this assay [19]. Whether Tat can form pores in the plasma membrane for secretion is not known yet.

Several evidences indicate that extracellular, circulating Tat is involved in HIV-1 pathogenesis. First, Tat strongly increases the expression of HIV-1 co-receptors CXCR4 and CCR5 at the surface of CD4<sup>+</sup> T-cells, thereby facilitating viral infection [8, 21]. Extracellular Tat also triggers the production of immunoregulatory cytokines such as IL-10 by lymphocytes, monocytes and macrophages, thereby contributing to immune suppression [21, 22]. Tat is also present in the central nervous system. It can either cross the blood-brain barrier or be produced *in situ* by infected microglial cells [23]. Tat was found to be toxic both for neurons [24] and astrocytes [25]. Tat also affects endothelial cells, thereby damaging the blood brain barrier [26]. Hence, Tat is suspected to be associated with the development HIV-1 associated neurological disorders which are among the most severe sources of morbidity for AIDS patients, even under highly active antiretroviral therapy [27].

#### **\*HIV-1 matrix protein (p17)**

HIV-1 Gag precursor (55 kDa) contains on its N-terminal side the matrix protein (p17 or MA) that is key viral component. Indeed, both MA myristoylation and interaction with PI(4,5)P<sub>2</sub> are required for HIV-1 assembly at the plasma membrane [28]. Gag is thought to be cleaved by the viral protease to generate the MA and capsid (p24; CA) proteins only after budding and most Gag is unprocessed within infected cells [29]. Nevertheless, MA unconventional secretion was observed using HIV-1 infected cells or Gag-transfected cells. This secretion was found to require PI(4,5)P<sub>2</sub> binding. When using Gag-transfected cells, *i.e.* in the absence of viral protease, cellular aspartyl protease(s) cleaved the Gag precursor between MA and CA thereby allowing MA secretion [30] (Fig.1). MA secretion is only observed upon expression of the complete Gag protein. Indeed, when expressed alone, MA adopts a folded conformation preventing membrane targeting [31] and secretion [30].

Circulating MA was found to affect the function of several cell types, inducing the adhesion and migration of monocytes for instance and might therefore be involved in immune system perturbation by HIV-1 [32].

#### **\*Ebola virus VP40**

The Ebola virus is responsible for the Ebola virus disease. The capsid protein, VP40 is a key component of the virion that is able to bind RNA, multimerize and recruit ESCRT proteins for budding [4]. Although VP40 is devoid of signal peptide, the monomeric form of VP40 was found to be secreted in a brefeldin A-independent manner, indicating that VP40 can be unconventionally secreted. Interestingly, VP40 is recruited to the plasma membrane by PI(4,5)P<sub>2</sub> that also enables the VP40 oligomerization required for the budding of virus-like particles [33]. It is nevertheless not clear

yet whether VP40 unconventional secretion requires PI(4,5)P<sub>2</sub> binding. Regarding the biological significance of VP40 secretion, circulating monomeric VP40 could be responsible for the early appearance of anti-VP40 antibodies in the *sera* of Ebola infected patients [4].

### **\*Herpes virus VP22 protein**

Herpes simplex virus type I is a widespread virus responsible for blisters. VP22 is a 38 kDa protein present in the tegument region, *i.e.* between the envelope and the capsid of the virus. VP22 does not have a signal peptide and it was found to be secreted by infected cells in a brefeldin A-independent manner [5], thereby indicating that VP22 is secreted using an unconventional secretion pathway. Moreover, VP22 was found to be taken up by neighboring cells and concentrate into their nucleus [5], indicating that VP22 might be used to deliver cargos intracellularly as it is the case for HIV-1 Tat [34]. Indeed, VP22 was able to vectorize GFP or thymidine kinase into various cell types [18]. Nevertheless, some groups were unable to reproduce these findings that seemed to be a fixation artefact, at least in some cases [35]. Although the capacity of VP22 to transport cargos into cells is a matter of debate, VP22 secretion was confirmed in a later study showing that a fusion protein between VP22 and  $\beta$ -galactosidase was efficiently secreted [18]. The mechanistic details of VP22 secretion nevertheless remains poorly documented and the possible implication of this process in viral multiplication is elusive.

### **\*HTLV-1 Tax**

Human T-cell leukemia virus type I (HTLV-I) is a retrovirus responsible for adult T-cell leukemia and HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-I transactivating protein Tax (39.5 kDa) contains a leucine-rich nuclear export signal that enables its export from the nucleus to the cytoplasm, but no signal peptide. Tax concentrates into the nucleus but is also observed in the cytosol [36]. Accordingly, a Tax-YFP fusion protein essentially localized to the nucleus but was also present at the level of the ER and Golgi apparatus [3]. Time lapse microscopy confirmed this localization of Tax-YFP in the secretory pathway. Depending on cell types 16-53% of Tax was secreted, showing that a significant fraction of cellular Tax is exported. Brefeldin A inhibited Tax export by ~60%, indicating that Tax secretion is partly but not entirely unconventional [3]. Tax was later found, using antibody arrays, to interact with proteins regulating ER to Golgi transport ( $\beta$ COP and COPII), and movement from the Golgi to the plasma membrane (SNAP23, SCAMP1 and SCAMP2). Accordingly, silencing COPII, SNAP23 or SCAMP1 inhibited Tax secretion by ~60%. Sequence analysis indicated that Tax exhibits four putative secretion signals (312YTNI315, 319LL320, 330DHE332, and Val353). A point mutation approach showed that the YTN motif is critical both for Tax localization to the exocytic pathway and for secretion [37]. A YXX $\emptyset$  motif has indeed been found to enable localization to the *trans*-Golgi network of transmembrane proteins that own this signal on their cytosolic tail [38]. Nevertheless, this motif is also responsible for recognition by the AP-2 adaptor complex for clathrin-mediated receptor endocytosis [39] and it is therefore not clear how it could mediate the specific recruitment to the TGN of a cytosolic protein such as Tax. Tax secretion is original since it takes place despite the lack of signal sequence although largely relying on the ER/Golgi secretion apparatus (Fig.1). Tax identified partners are present on the cytosolic leaflet of the ER, Golgi apparatus, the plasma membrane and even endosomes for  $\beta$ COP [40]. It is not clear yet at which stage(s) of the exocytic pathway Tax undergoes translocation to the *trans* side of the membrane to reach the lumen of the organelle or the outside medium (Fig.1). The brefeldin A independent Tax secretion pathway remains to be characterized but it likely involves direct targeting to the plasma membrane, maybe following interaction with SCAMP1 or SNAP23 that concentrate at this level [41, 42].

Tax secretion was observed using cultures of T-cells from HAM/TSP patients [43], and extracellular Tax was detected in the cerebrospinal fluid of HAM/TSP patients [37]. Circulating Tax indeed triggers hyperstimulation of the immune system and the release of several proinflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$  by microglial cells and TNF- $\alpha$  by neurons. These cytokines might facilitate the maintenance of an inflammatory state and contribute to the demyelination of neurons that is observed during disease progression [37]. Secreted Tax is thus likely involved in the pathogenic processes associated with HAM/TSP.

## Conclusion

Viral proteins often have multifaceted activities [44] and this is the case for viral proteins that are secreted using an unconventional secretion pathway. They either have a key structural role, as in the case of VP22, VP40 and MA (HIV-1) or a crucial transcriptional activity for Tax and Tat. They are additionally secreted to fulfill their extracellular or extra-viral role, which remains to be clearly established for some of them.

The unconventional pathways used by viruses to achieve secretion of these proteins are diverse (Fig.1). They nevertheless all seem to be self-translocating proteins, indicating that they follow a type I pathway of unconventional secretion [6]. This is particularly clear for HIV-Tat which secretion process showed several similarities with that of FGF2, another well studied Type I UPS cargo [45]. There is a striking common membrane targeting molecule, PI(4,5)P2 that is required for the secretion of Tat [7] FGF2 [46], MA [30], and for VP40 membrane association [33]. It is still not known whether Tat, MA and VP40 secretion exhibit other similarities. A common characteristic of all these viral proteins secreted using unconventional pathways is that they are all expressed early upon cell infection [5, 47-49], indicating that secretion can potentially begin as soon as viral protein synthesis starts. Overall these secretion processes are poorly documented. For instance, whether cellular proteins facilitate or regulate these unconventional secretion pathways is still unclear. Further work should enable to understand how these viral proteins can cross membranes to reach the extracellular milieu.

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**Figure 1.** Unconventional secretion pathways of viral proteins. HTVL-I Tax can be secreted both by a brefeldin A dependent route, *i.e.* following the conventional ER/Golgi pathway or by a brefeldin A-insensitive pathway, presumably involving direct transmembrane transport. HIV-1 Tat is exported directly through the plasma membrane following PI(4,5) P2-mediated recruitment at this level (inset). HIV-1 matrix protein (MA; p17) secretion requires first binding of the Gag precursor to PI(4,5)P2, then proteolysis by the viral or a cellular protease to release p17 from p24 (the capsid protein), enabling p17 export. VP40 is also recruited at the plasma membrane by PI(4,5)P2, and was found to be unconventionally secreted. VP22 secretion mechanism is poorly documented. See the text for details. TGN, *trans* Golgi network.



