MECHANISM OF CELLULAR INTERNALIZATION OF THE HIV-1 TAT PROTEIN

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Chapter 1

Introduction

INTRODUCTION

The replication cycle of HIV: an overview

The human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2) are retroviruses belonging to the lentivirus group and are the etiological agents of the acquired immune deficiency syndrome (AIDS). HIV establishes a persistent infection based on its capacity to integrate the proviral genome into chronically infected cells, and through the fast evolution of viral gene products due to a high mutation rate during the viral replication. In addition, the ability to set up a latent infection during early stages of disease facilitates the persistence of HIV even after intensive drug therapy and strong immune responses against the virus.

HIV requires transcription of its proviral genome for completion of the viral replication cycle and the production of viral progeny. HIV transcription depends on different interactions of transcription factors and co-activators with the viral long terminal repeat (LTR) promoter. The regulatory elements within the promoter bind constitutive and inducible transcription factors leading to the assembly of a stable transcription initiation complex that stimulates multiple rounds of transcription by RNA polymerase II.

The genome of HIV-1 is complex, encoding a number of regulatory and accessory proteins not found in other retroviruses (Fig. 1). This complexity is reflected in the HIV-1 replication cycle, which shows mazy regulatory pathways and remarkable mechanisms for viral persistence.

Similar to all retroviruses, the HIV-1 genome carries the gag, pol and env genes that encode the structural proteins necessary for virion assembly as well as the enzymes needed for genome replication, provirus integration and polyprotein processing. The gag, pol and env gene products are synthesized as single polyproteins; subsequently, Gag and Pol are processed by the viral protease whereas Env by a cellular enzyme. The gag gene encodes the structural core proteins (capsid, nucleocapsid and p6) and the matrix protein of the viral particle, the env gene encodes the glycoproteins gp120 and gp41, and the pol

gene encodes the enzymes crucial for viral replication: reverse transcriptase, integrase and protease.

In addition to gag, pol and env gene products, HIV expresses other additional auxiliary proteins: Vif, Vpr, Vpu (only HIV-1), Vpx (only HIV-2), Tat, Rev and Nef.

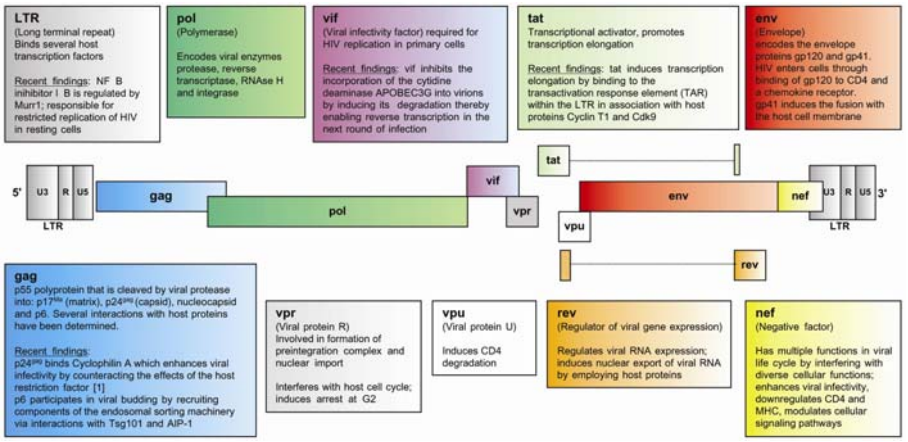


Fig. 1. The image shows the HIV-1 genome structure and the function of its encoded proteins (Trkola, 2004).

HIV-1 has been reported to infect a broad range of cell types *in vitro* , whereas the only cell types that are infected *in vivo* are $CD4^+$ T-lymphocytes and cells of the monocyte-macrophage lineage.

Productive infection starts when HIV-1 fuses its envelope with the cell plasma membrane (Fig. 2). The trimeric surface gp120 protein on the virion binds the CD4 receptor on the host cell, inducing conformational changes and promoting the binding to chemokine receptors (receptors of small proteins which serve as chemoattractants in inflammation) that function as coreceptors for HIV-1 infection (Kwong et al., 1998). On one hand, these coreceptors provide a critical function for virus entry, while on the other they are the main determinants of tropism among $CD4^+$ cell types. Only two of the chemokine receptors are used by HIV-1 for infection: CCR5, which binds macrophage-tropic viruses (R5

viruses), and CXCR4, which binds T cell tropic viruses (X4 viruses). R5 viruses are responsible of both mucosal and intravenous transmission of HIV-1, whereas X4 isolates appear in late stages of infection when the immunodeficiency emerges (Scarlatti et al., 1997).

The surface gp120 is bound to gp41, a transmembrane viral protein that is assembled as a trimer on the virion membrane. The interactions between gp120, CD4 and chemokine receptors induce a conformational change in the trimeric gp41 that expose three peptide fusion domains. Subsequently these domains spear the plasma membrane and promote the viral envelope fusion that leads to the release of the HIV-1 core into the cell cytoplasm (Chan and Kim, 1998).

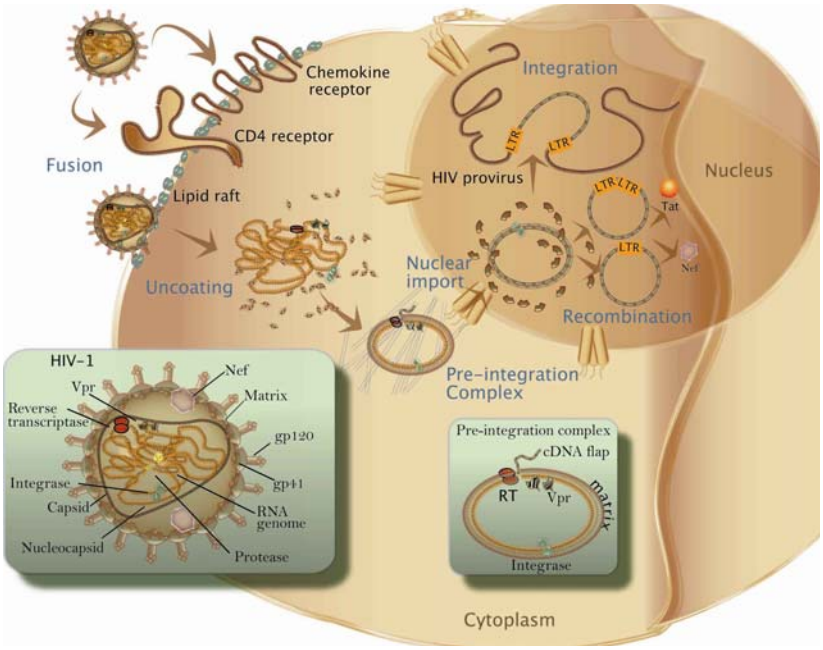


Fig. 2. Schematic description of early events occurring after HIV-1 infection.

Once inside the cell, the following step is the uncoating of the virus that generates the viral reverse transcription complex, comprising the viral genome, reverse transcriptase, integtrase, matrix, nucleocapsid, Vpr and other host

proteins (Karageorgos et al., 1993). Subsequently, phosphorylated matrix binds to actin microfilaments (Bukrinskaya et al., 1998) and Vif stabilizes the reverse transcription complex (Ohagen and Gabuzda, 2000).

Finishing of reverse transcription originates the HIV preintegration complex (PIC), which is composed of viral cDNA, integrase, matrix, Vpr and reverse transcriptase (Miller et al., 1997). In contrast to most retroviruses, HIV is able to infect nondividing cells such as differentiated macrophages, thus the PIC needs to translocate into the nucleus. Vpr, integrase and matrix have a nuclear localization signal and each one has been implicated in mediating nuclear import of the PIC (Bukrinsky et al., 1993; Gallay et al., 1997; Heinzinger et al., 1994). However, it is still unclear whether these proteins function in a cooperative manner or have individual roles in different target cells.

Once inside the nucleus, integrase mediates integration of the viral DNA into the cellular genome. Integrase and other proteins that participate in the formation of the PIC bind specific sequences located at the ends of the viral cDNA (att sites) and integrase removes two nucleotides left by the terminal transferase activity of the reverse transcriptase. In contrast, no strong primary sequence in the cellular genome has been shown as a preferential target site and integration seems to occur at random on DNA molecules.

Alternatively, the viral DNA may follow three different fates, all of which do not lead to the formation of a functional provirus. The ends of viral DNA may join to form a 2-LTR ring or the viral genome may undergo homologous recombination producing a single LTR ring. Finally, the viral DNA may integrate into itself leading to the formation of a rearranged circular structure. These three different circular structures are non infectious even though some of them are transcriptionally active and produce the Tat and Nef proteins (Wu and Marsh, 2001).

Since the integration process occurs in different locations in the host chromosomes, the viral cDNA can integrate in chromatin domain with different condensation states. In eukaryotic cells DNA is assembled with nucleosomes to form chromatin, which can assume at least two distinct functional forms: a condensed form (heterochromatin) that generally lacks transcriptional activity

and a looser, decondensed form (euchromatin) that provides the environment for DNA regulatory processes such as transcription. Integration in heterochromatic regions where transcription is repressed may lead to latent infection, whereas integration in decondensed chromatin areas may lead to transcriptionally active form of infection (Adams et al., 1994).

In the integrated provirus, the 5' LTR acts as the viral promoter; it contains several positive transcription factor binding sites (Fig. 3) even if, in the absence of the viral Tat protein, the binding of these factors is not sufficient to activate the transcription of viral genes. However, the presence of these promoter elements results in the correct positioning of RNA polymerase II at the site of initiation of transcription and to the assembly of the preinitiation complex. At this point transcription starts but the polymerase produces predominantly short, non-polyadenylated RNA that include a hairpin structure at the 5' end of the nascent viral RNA, named trans-activation-responsive region (TAR) (Fig. 3). Tat acts as a very powerful transcriptional activator of the integrated provirus by interacting with TAR and promoting the production of polyadenylated full-length RNA viral genomes (see below).

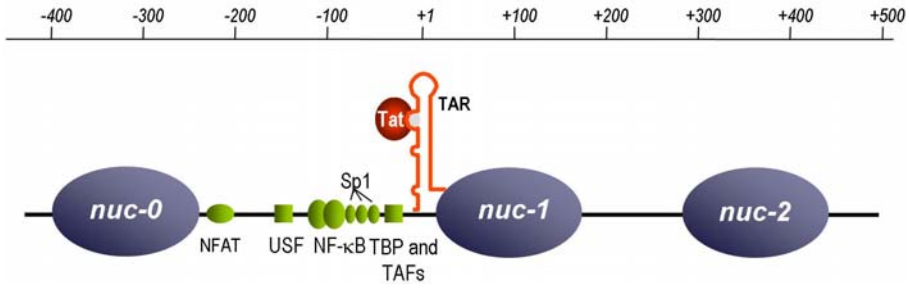


Fig. 3. Structural arrangement in chromatin of the HIV-1 promoter region.

Tat-activated transcription originates different transcripts derived by the splicing of the full-length viral genome. The first viral transcripts that appear after infection are completely spliced and are rapidly transported into the cytoplasm following the same pathway as cellular mRNA (Cullen, 1998). Since these first

transcripts are completely processed by splicing, they are the shortest viral mRNAs, and encode for Tat, Rev and Nef proteins. Incompletely spliced RNA are blocked in the nucleus by the cellular machinery that control the integrity of the splicing process; the single spliced and unspliced transcripts persist in the nucleus due to defective donor and acceptor splice sites and to the inhibitory effect of Rev on splicing (Luo et al., 1994; Powell et al., 1997). However, these transcripts are necessary for the production of new viral particles, as they encode for the Gag, Pol and Env proteins as well as they represent the viral genome. The translocation of these transcripts into the cytoplasm depends on the expression of the Rev protein (Pomerantz et al., 1992). Rev is able to shuttle between the nucleus and the cytoplasm and binds the viral transcripts through the interaction with an RNA stem-loop structure named Rev responsive element (RRE), located in the env gene (Fig. 4) (Malim et al., 1990). The binding between Rev and the RRE induce the multimerization of Rev that afterwards associates with the cellular CRM-1 and Ran proteins. Ran is a small GTP/GDP binding protein; Ran-GDP is found mainly in the cytoplasm whereas the GTP bound state is found in the nucleus. The Rev/CRM-1/Ran-GTP complex associated with viral RNA interacts with the proteins of the nuclear pore complex that allow nuclear export (Cullen, 1998). By this mechanism, Rev is able to translocate incompletely spliced transcripts into the cytoplasm leading to the synthesis of all viral proteins.

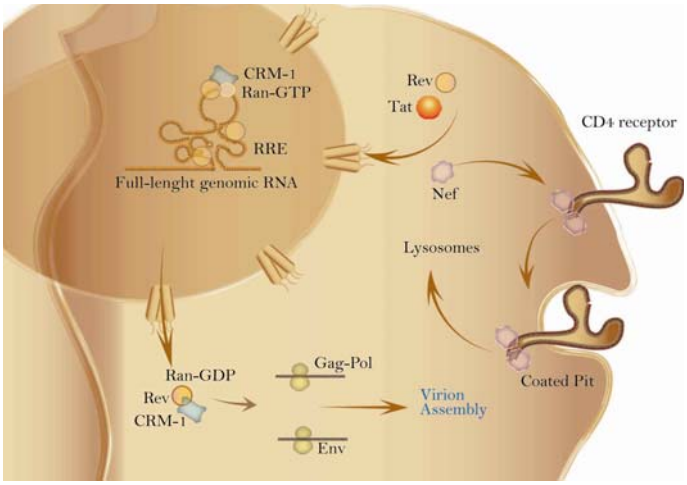


Fig. 4. Schematic representation of late step of viral infection. Highlighted are the role of Rev in viral RNA nuclear export and the Nef function in CD4 down-regulation.

Once translated, all the viral proteins necessary for virion assembly and RNA genomes are transported to the plasma membrane close to lipid raft membrane domains, where the building of new virions begins. The gp120/gp41 complex is transported via the Endoplasmic reticulum-Golgi pathway whereas the Gag-Pol polyproteins are targeted to the plasma membrane after the myristylation of Gag and the resulting attachment to the cytoplasmic side of the secretory vesicles (Fig. 5) (Gottlinger et al., 1989). The resulting virions bud from the plasma membrane but they are still incomplete, their maturation is finished by viral protease activity that first cleaves Gag-Pol and then, starting from the Gag and Pol separated precursors originates: the single core proteins, matrix and the viral enzymes. The proteolitic activity ends when the virion is already detached from the host plasma membrane and results in the formation of mature infectious viruses.

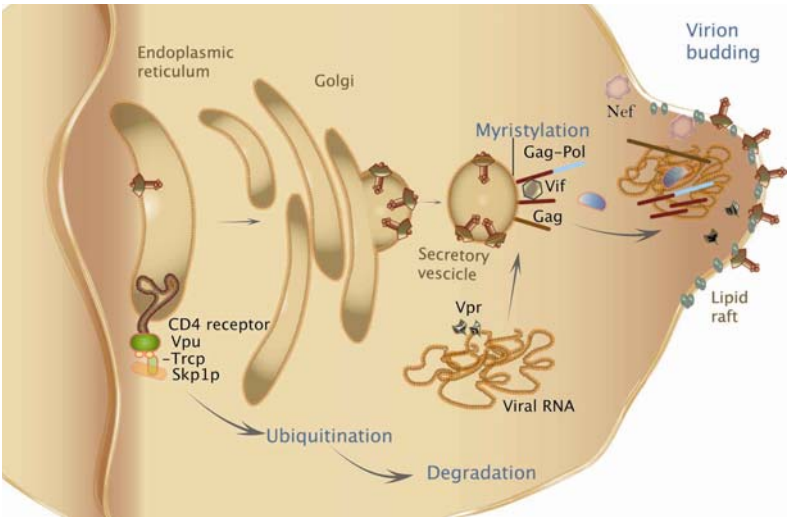


Fig. 5. Assembly of new virions on cell surface.

During the replication cycle, other auxiliary proteins of HIV-1 play a fundamental role in regulating the different steps of the intracellular viral pathway. Completely spliced transcripts encode for Nef that is targeted to the plasma membrane by myristylation of its N-terminus. The first studied activity of Nef is the down-regulation of CD4 receptor, a mechanism that is supposed to protect infected cells from the immune system and to facilitate the release of HIV-1 virions, even if the real advantage in removing CD4 from plasma membrane remains unclear. Nef promotes the endocytosis of CD4 by bridging the cytoplasmic tail of the receptor with the AP-2 adaptor complex that mediates the internalization of transmembrane proteins in clathrin-coated vesicle (Aiken et al., 1994). Internalization of CD4 through clathrin dependent endocytosis eventually leads to degradation of the receptor in lysosomes (Fig. 4).

In addition to CD4, Nef is able to promote the down-regulation of cell surface MHC I (major histocompatibility complex I) that presents viral epitopes to cytotoxic T lymphocytes (CTL). As a consequence, Nef impairs the CTL-mediated immune response that leads to the lysis of HIV-1 infected cells (Collins et al., 1998).

Nef is also packaged into virions with low efficiency; nevertheless viruses without Nef are still able to enter the cells but the proviral DNA synthesis is partially inhibited in infected cells. This event suggests a role for Nef in viral uncoating or reverse transcription, arguing for an additional mechanism mediated by Nef to enhance virion infectivity in a CD4-independent manner (Aiken and Trono, 1995).

The viral infectivity factor Vif is a 23-kDa protein that affect the infectivity but not the production of viral particles. This protein is encoded from singly spliced viral transcripts and its expression is therefore Rev-dependent. Once expressed by infected cells, this protein accumulates in the cytoplasm and is found in association with the plasma membrane through interactions with the C-terminal basic region.

Early studies showed that mutant viruses lacking Vif were less infectious than the wild type, although cell-to-cell transmission was only slightly lower than normal. For a long time, the biological activity of Vif has been cryptic except for

the enhancement of viral infectivity. It has been recently demonstrated that Vif promotes the degradation of APOBEC3G, a cellular factor able to be packaged into virions and to impair their infectivity (Sheehy et al., 2002).

Vpr is expressed at later stages of HIV-1 replication and is packaged in all HIV-1 virions by the p6 core protein. After viral entry, Vpr is incorporated inside the PIC and enhances HIV-1 replication in nondividing cells by mediating its nuclear import (Popov et al., 1998). In addition, Vpr induces cell cycle arrest in the G2 phase through the inhibition of $p34^{cdc2}$-cyclin B kinase activity (Re et al., 1995). As the LTR is more active in G2 phase of the cell cycle, viral transcription and virion production is increased in cells delayed in G2 by Vpr.

Vpu is a transmembrane protein unique to HIV-1. Likewise Nef activity, Vpu binds the cytoplasmic tail of CD4 and recruits the β-TrCP/Skp1p complex at the level of endoplasmic reticulum (Fig. 4). This event induce the ubiquitination and the subsequent proteasomal degradation of CD4 while Vpu is recycled (Margottin et al., 1998). In addition, Vpu enhances virus release by inducing the budding of virions from the plasma membrane and promoting the detachment of budding virions.

The Tat Protein of HIV-1

After infection, HIV-1, similar to most animal DNA viruses, requires the activity of the cellular transcriptional machinery to carry out transcription of viral genes. Synthesis of viral transcripts also depends on cellular transcription factors and co-activators that promote and regulate transcription.

The Tat proteins of HIV-1 and HIV-2 are powerful transcriptional activators of viral gene expression. HIV-1 Tat is a small polypeptide of 101 aa (with the exception of the widely studied HXB2 strain, in which a point mutation truncates the protein at 86 aa; (Jeang et al., 1999)) encoded by two exons, and is essential for the transcription of viral genes and for viral replication.

No crystal structure of the protein has been obtained to date; structural prediction and data from NMR spectroscopy (Bayer et al., 1995) indicate that the protein has a highly flexible structure and does not exhibit obvious secondary structure elements. On the basis of amino acid distribution in the sequence, and their conservation with homologous proteins from other lentiviruses, it is possible to identify five regions. In the first exon (aa 1-72) we find an N-terminal acidic domain (aa 1-21); a domain containing 7 cysteins (aa 22-37); a core region (aa 38-48); and a basic domain enriched in arginine and lysine amino acids, highly conserved among different strains (aa 49-57) (Fig. 6). The second exon starts at amino acid position 73 and has a relatively more variable sequence. The minimal domain capable of LTR transactivation is fully included in the first exon.

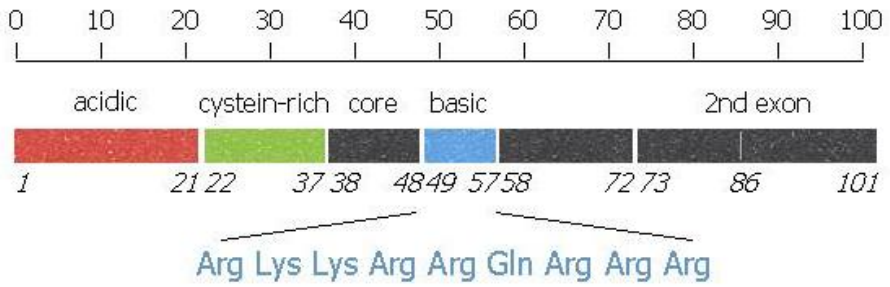


Fig. 6. Principal domains of HIV-1 Tat protein. The sequence of the basic domain is reported.

Tat-mediated transcriptional activation

After infection of susceptible cells, the HIV-1 genome integrates into the host cell DNA; independent of the integration site, the proviral DNA sequence is organized in a chromatin structure that exerts a repressive role on transcription; repression is overcome by a variety of extracellular stimuli that lead to cellular activation. Regulation of HIV-1 gene expression by the viral Tat transactivator is a critical step in the viral life cycle. In spite of continuous HIV-1 replication in all phases of AIDS disease, expression from the viral LTR is tightly regulated and shows a considerable correlation with the levels of host cell activation. In particular, expression of the Tat protein induces a striking activation of the LTR, making this regulatory sequence one of the most powerful promoters known in nature. Tat acts as a very powerful transcriptional activator of the integrated provirus by interacting with the cis-acting RNA element TAR, present at the 5' end of all viral mRNAs; nucleotides +1 to +60 (considering position +1 the start site of proviral DNA). The Tat:TAR interaction occurs between the basic domain of Tat and 3 nucleotides that form a bulge near the apex of the TAR stem (Fig. 3). The control of transcription is carried out through the concerted action of several cellular transcription factors that bind the LTR DNA. The function of Tat is to induce a chromatin modification at the LTR promoter and mediate the recruitment of elongation-competent RNA polymerase II.

Over the last few years, a number of cellular proteins have been reported to interact with Tat and to mediate or modulate its transcriptional activity. These include general transcription factors, among which TBP, TAFII250, TFIIB, TFIIH (Kashanchi et al., 1994; Parada and Roeder, 1996; Veschambre et al., 1997; Veschambre et al., 1995; Weissman et al., 1998); RNA polymerase II (Wu-Baer et al., 1995); transcription factor Sp1 (Jeang et al., 1993); the cyclin subunit of the positive transcription elongation factor complex (P-TEFb), cyclin T1 (Wei et al., 1998), and different transcriptional co-activators that possess histone acetyltransferase (HAT) activity.

In particular, through its interaction with TAR, Tat activates transcription from the HIV LTR by at least two different molecular mechanisms, (for a comprehensive review, see ref. (Marcello et al., 2001); Fig. 7).

The first mechanism involves the recruitment of cellular proteins with histone acetyltransferase (HAT) activity, which relieve the inhibition that chromatin conformation imposes onto the HIV promoter (Verdin, 1991; Verdin et al., 1993). The transcriptional latency of viral gene expression has been ascribed to nucleosomes that wrap the promoter region of the HIV-1 provirus. Independently of the site of integration, nulceosomes in the 5' LTR are exactly positioned with respect to cis-acting regulatory elements (Fig. 3); in the transcriptionally silent provirus, these nucleosomes define two nucleosome-free regions encompassing nucleotides -265 to -3 and +141 to +256 (Van Lint et al., 1996). These two regions are separated by a nucleosome named nuc-1 that is specifically and rapidly disrupted during transcriptional activation, leaving a large open chromatin region. The position of nuc-1 at the transcription start site and its destabilization during transcriptional activation indicates that chromatin plays a critical role in the suppression of HIV-1 transcription during latency and that nuc-1 disruption is required for transcriptional activation.

Complexes containing HATs facilitate transcriptional activation by modulating nucleosomal repression of specific promoters through acetylation of the N-terminal tails of histones, an event inducing destabilization of histone-DNA interactions. The HAT proteins responsible for the TAR-dependent Tat transactivation include the transcriptional co-activators p300 and the highly homologue cAMP-response element binding protein (CREB)-binding protein (CBP) (Marzio et al., 1998), the p300/CBP-associated factor (P/CAF) (Benkirane et al., 1998), the general control non-derepressible-5 (GCN5) factor (Col et al., 2001), the TIP60 protein (Kamine et al., 1996), and the general transcription factor TAFII250 (Weissman et al., 1998). Tat-recruited HATs acetylate histones in the LTR-proximal nucleosomes and activate viral gene expression (Lusic et al., 2003).

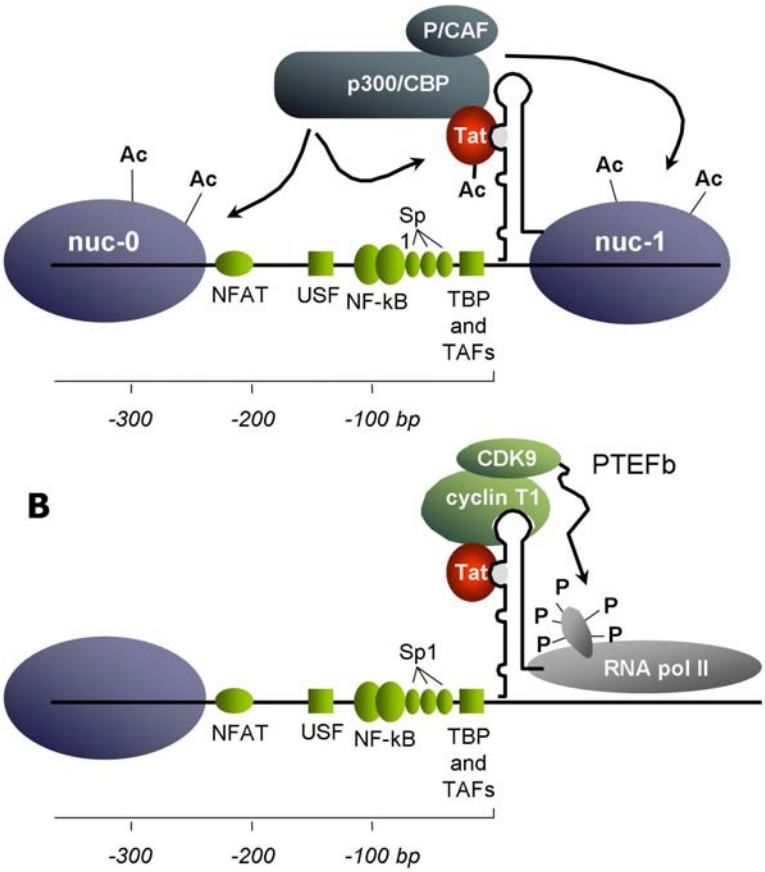


Fig. 7. A. Tat recruits histone acetyl-transferases that determine the acetylation of the N-terminal tails of histones 3 and 4. **B.** Binding of Tat to cyclin T1/CDK9 complex promotes the hyperphosphorylation of RNA polymerase II CTD.

The second mechanism of Tat-induced transcriptional activation is mediated by the interaction of the protein with human cyclin T1 (Wei et al., 1998), the cyclin subunit of CDK9 in the positive transcription elongation factor b (P-TEFb), a complex originally identified in *D. melanogaster* as a kinase required for the transcription of several genes (Marshall and Price, 1992)

Although the HIV-1 LTR contains DNA binding sites for several transcription factors (among which AP-1, COUP, Ets, LEF-1, NFAT, Rel/NFκB, Sp1, USF and

TFIID) (Fig. 3) in the absence of Tat, there is no viral gene expression and transcription from the HIV-1 promoter produces predominantly short, non-polyadenylated RNAs that include the TAR structure. Genomic footprinting experiments have demonstrated that most of these transcription factor binding sites are actually occupied by cellular proteins in both silent and activated HIV-1 infected cells (Demarchi et al., 1993). The inability to synthesize full-length viral RNA is caused by the low processivity of RNA polymerase II, which is overcome by P-TEFb. The P-TEFb complex is composed by one component of the cyclin T family and by the cyclin-dependent kinase Cdk9. The interaction between Tat and cyclin T1 increases the specificity and affinity binding of Tat to the TAR RNA, thus forming a ternary complex that brings onto the HIV promoter the kinase activity necessary for the transcriptional activation.

The Cdk9 subunit of P-TEFb hyperphosphorylates the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II. This modification turns the otherwise non-processive hypophosphorylated polymerase into a highly processive enzyme. In addition, Tat shifts the equilibrium in favor of CTD phosphorylation by interacting with and inhibiting a CTD phosphatase (Marshall et al., 1998).

P-TEFb kinase activity also results in the dissociation of the negative transcription elongation factor (N-TEF), which is associated with RNA polymerase II and impairs its transcriptional elongation activity (Fujinaga et al., 2004). N-TEF is composed by the DRB sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF); both factors are found on the HIV-1 LTR after the initiation of viral transcription (Ping and Rana, 2001). DSIF binds the hypo- but not the hyperphosphorylated form of the RNA polymerase II CTD whereas NELF contains an RNA recognition motif that binds the TAR structure. When Tat recruits P-TEFb on the viral promoter, Cdk9 phosphorylates DSIF, NELF and RNA polymerase II, removing N-TEF and its inhibitory effect on transcription from the transcription initiation site.

The action of Tat in transactivation of gene expression is not limited to the recruitment of cellular factors to viral promoter. It has been demonstrated that Tat can modify the kinase activity of Cdk9 (Zhou et al., 2000) and stimulate the

guanylyltransferase activity of the mRNA capping enzyme Mce1 (Chiu et al., 2002). The CTD of RNA polymerase II consists of 52 repeats of heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser. In the absence of Tat, Cdk9 phosphorylates the CTD at Ser 2. In the presence of Tat, Cdk9 changes its substrate specificity and phosphorylates the CTD at Ser 2 and Ser 5, thus promoting hyperphosphorylation of the polymerase.
The mammalian capping enzyme Mce1 only binds the phosphorylated CTD, and binding to the CTD phosphorylated at Ser 5 of the heptapeptide reinforces the guanylyltransferase activity of Mce1. In addition, Tat directly binds Mce1 with its C-terminal domain, resulting in a remarkable stimulation of the guanylyltransferase and triphosphatase activities and the recruitment of Mce1 to the transcription initiation site. Eventually, capping promotes subsequent splicing and polyadenylation of viral RNA and protects it from exonucleolytic decay.

In addition to promoting the association of HATs to the LTR promoter, Tat itself was found to be a substrate for acetylation by p300/CBP, PCAF and GCN5 (Bres et al., 2002; Col et al., 2001; Dorr et al., 2002; Kiernan et al., 1999; Mujtaba et al., 2002; Ott et al., 1999). Whereas p300/CBP and GCN5 acetylate lysines 50 and 51 within the RNA binding domain, PCAF acetylates lysine 28 in the activation domain, regulating the binding with the TAR structure and the cyclin T/CDK9 complex respectively; mutations in these lysines impair virus replication. Acetylation at lysine 28 impairs the binding of Tat to PCAF but strengthens its interaction with P-TEFb, while acetylation at lysine 50 promotes the dissociation of Tat from TAR RNA (Kiernan et al., 1999) and creates a new binding site for PCAF (Mujtaba et al., 2002). Starting from these observations, Brès and colleagues (Bres et al., 2002) proposed a model to explain the events triggered by Tat at the HIV-1 LTR (Fig. 8). Non-acetylated Tat first binds PCAF and following its acetylation at lysine 28, dissociates from PCAF and binds P-TEFb. The Tat/P-TEFb complex interacts with high affinity to TAR RNA, hyperphosphorylating the RNA polymerase II CTD. Subsequently, p300 acetylates Tat at lysine 50. This acetylation induces the Tat/P-TEFb dissociation from TAR and promotes the formation of a Tat/P-TEFb/PCAF complex that

remains associated with the elongation complex during transcriptional elongation. GCN5 also acetylates Tat on lysine 50 and lysine 51, leading to increased transactivation of the LTR (Col et al., 2001).

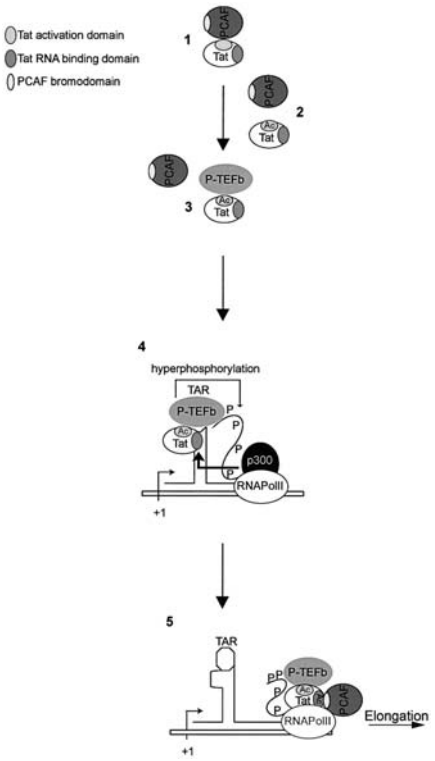


Fig. 8. Proposed model for the regulation of Tat transcriptional activity by p300 and PCAF. (1) PCAF interacts with Tat amino acids 20-40. (2) PCAF acetylates Tat at Lys28 and dissociates from Tat. (3) P-TEFb associates with Tat. (4) P-TEFb-Tat complex binds TAR RNA, and p300 acetylates Tat at Lys50. (5) Tat dissociates from TAR RNA and PCAF interacts with K50-acetylated TAR RNA-binding domain of Tat. Tat-P-TEFb-PCAF complex associates with the transcription elongation complex (Bres et al., 2002).

The Tat protein: beyond viral transcription

Besides its action on the regulation of HIV-1 gene expression, over fifteen years ago Frankel and Pabo (Frankel and Pabo, 1988) and Green and Lowenstein (Green and Loewenstein, 1988) first demonstrated that Tat also possesses the unusual property of entering the cells when present in the extracellular milieu. Since these early experiments were performed by assessing the capacity of the protein to transactivate an LTR-reporter gene cassette, their results also implied that not only the protein enters the cells, but also that it is transported to the nucleus in a transcriptionally active form. In a consistent manner, these authors also measured the amount of radioactive Tat present in different cellular fractions after exogenous delivery, and found that, after 6 hours, more than 80% of the internalized Tat was detectable in the nuclear compartment (Frankel and Pabo, 1988).

In addition to entering the cells when present in the extracellular environment, Tat is also secreted outside HIV-1-infected cells following a leaderless secretory pathway that does not involve the classical ER-Golgi-dependent exocytosis (Chang et al., 1997; Tyagi et al., 2001).

The finding that a short polypeptide of viral origin at the same time acts as a powerful transactivator of gene expression and also possesses the highly unusual property to traffic between cells raises several questions. A reasonable possibility is that HIV-1 has evolved this property in order to prime non-infected cells for their primary infection or to induce the generation of a tissue environment suitable for productive viral replication. In this respect, thanks also to the ability of Tat to induce the transcription of cellular genes, extracellular Tat has been reported to exert a number of pleiotropic activities when present in the extracellular environment. Extracellular Tat promotes the production of cytokines (Lotz et al., 1994; Nabell et al., 1994; Opalenik et al., 1995; Scala et al., 1994; Westendorp et al., 1994; Zauli et al., 1992) and cytokine receptors (Pocsik et al., 1992; Puri and Aggarwal, 1992; Purvis et al., 1992); modulates the survival, proliferation and migration of different cell types (Ensoli et al., 1990; Ensoli et al., 1993; Lafrenie et al., 1997; Zauli et al., 1993); exerts angiogenic activity *in*

vitro and *in vivo* (Albini et al., 1995; Albini et al., 1996a; Corallini et al., 1996); inhibits antigen-specific lymphocyte proliferation (Chirmule et al., 1995; Subramanyam et al., 1993; Viscidi et al., 1989). It is likely that some of the above mentioned effects of extracellular Tat could have important implications for the pathogenesis of HIV disease in an autocrine or paracrine fashion, with particular reference to the neuropathogenic role of HIV-1 infection in the central nervous system (Hofman et al., 1994; Nath et al., 1996; Philippon et al., 1994; Sabatier et al., 1991; Taylor et al., 1992; Weeks et al., 1995). Whether some of these pleiotropic activities are consequent to the interaction of Tat with cell surface receptors – including VEGFR-1 and -2 (Albini et al., 1998; Albini et al., 1996b; Mitola et al., 1997), integrins (Barillari et al., 1993) and chemokine receptors (Albini et al., 1998; Xiao et al., 2000) – and the consequent activation of intracellular signal transduction pathways, or might be due to the activation of transcription that follows Tat internalization (Demarchi et al., 1999) still remains to be understood. Of particular relevance appears the potential role of extracellular Tat in the pathogenesis of HIV-induced neurodegenerative disorders and of Kaposi sarcoma. In addition, Tat release might be involved in the induction of immunosuppression during the course of HIV disease. These possible functions of extracellular Tat are detailed in the following paragraphs.

Tat and neurodegenerative disorders

AIDS-related neurodegenerative disorders occur in approximately one-third of adults and half of the children afflicted with the disease. Productive infection in the central nervous system (CNS) occurs primarily in macrophages and resident microglia, but restricted infection also occurs in astrocytes. However, deterioration of brain tissue, eventually leading to dysfunction of cognition, dementia, or even paralysis occurs in the absence of infection of the neuronal tissue by HIV-1 and in the absence of other malignancies or opportunistic infections (Price et al., 1988). Such discrepancy between the severity of neurodegeneration and the lack of detectable HIV-1 within neurons has led to the hypothesis that viral products released from HIV-1-infected or HIV-1-

activated macrophages and glia cells might induce a pathologic process by altering the normal pattern of expression of cellular genes.

Transforming growth factor-β1 (TGF-β1) gene expression is enhanced in HIV-1-positive individuals (Kekow et al., 1990); it has been speculated that TGF-β1, a potent chemoattractant, may act by recruiting infected monocytes into the brain, thereby perpetuating and enhancing CNS dysfunction and deregulating expression of neurotoxic cytokines. Moreover, also non-infected monocyte infiltration of the CNS closely correlates with HIV-1-associated dementia probably because monocyte-derived toxins mediate nervous system damage. Tat released from HIV-1-infected cells can enter uninfected cells, in which it can modulate the expression of host genes. It has been demonstrated that expression of Tat upregulates transcription of the TGF-β1 (Rasty et al., 1996) and monocyte chemoattractant protein-1 (MCP-1) (Conant et al., 1998) genes both in cultured cells and in the mouse brain, suggesting that Tat-mediated stimulation of chemoattractant expression might be a key determinant of monocytic infiltration and subsequent neurotoxin release.

Extracellular Tat also up-regulates CCR5 chemokine receptor expression on human peripheral blood monocytes, an event that facilitates the migratory response and the transmigration of these cells across a tissue culture model of the human blood-brain barrier (Weiss et al., 1999). In addition, among the neurotoxins released by macrophages and astrocytes after HIV infection, the levels of tumor necrosis factor (TNF)-α were found to closely correlate with the severity of dementia. Therefore, the neurotoxic effects of Tat might be amplified by its ability to induce neuronal apoptosis through a mechanism that involves the activation of expression and the extracellular release of TNF-α from macrophages and astrocytes (Chen et al., 1997; New et al., 1998).

In order to better characterize the functional aberration induced by Tat in CNS, different groups have examined neuronal injury and spatial learning. They found that the intracerebroventricular injection of Tat leads to the attenuation of spatial learning accompanied by Long-Term Potentiation (LTP) suppression (Li et al., 2004). Of interest, the intra-hippocampal injection of Tat during this study did not lead to neuronal cell loss as previously described by other authors

(Sabatier et al., 1991), suggesting that Tat induces neuronal dysfunction before causing neuronal cell death.

Tat and Kaposi's sarcoma

Kaposi's sarcoma (KS) is an angioproliferative cancer that is very frequent and aggressive when associated to HIV-1 infection. All forms of KS show similar histological features, including the presence of spindle-shaped cells, vascular smooth cells, endothelial cells, fibroblasts, inflammatory cells and prominent vascularization. KS lesions are associated with infection by human herpesvirus 8 (HHV8) and are characterized by the presence of a complex network of cytokines (including interleukin (IL)-1, IL-6, gamma interferon (IFN-γ) and TNF-α), angiogenic growth factors (including basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor type A (VEGF-A) and VEGF-C), extracellular matrix components, and integrins (Fife and Bower, 1996; Nickoloff and Foreman, 1996).

The evidence that KS is much more frequent and aggressive during HIV infection suggests that the infection itself plays an important role in KS development and progression.

Tat is able to mimic extracellular proteins involved in adhesion, locomotion and growth of KS cells and endothelial cells, thus potentially enhancing angiogenesis and KS progression. However, unlike other angiogenic factors, Tat cannot induce primary endothelial cell response unless they are previously activated by inflammatory cytokines such as IFN-γ, TNF-α and IL-1β (Fiorelli et al., 1999) that induce the synthesis and release of bFGF. In order to promote cellular adhesion, Tat mimics the activity of matrix molecules including fibronectin and vitronectin by binding their cell surface integrin receptors. Tat binds integrins mainly with the Arg-Gly-Asp (RGD) domain, but its basic domain with its heparin binding activity is also involved in the adhesion process (Barillari et al., 1993).

In addition to cellular adhesion, integrins play a pivotal role in angiogenesis by inducing endothelial cell migration, inhibiting apoptosis and modulating endothelial cell response to angiogenic growth factors. The RGD domain of Tat induces endothelial cell locomotion likewise the chemotactic effect of the RGD

region of fibronectin and vitronectin, by promoting the expression and the activation of matrix metalloproteinase 2 (MMP-2) (Toschi et al., 2001) and focal adhesion kinases that are necessary for endothelial cell invasion of the extracellular matrix. In addition, the binding of Tat to integrins enhances the proliferative response to the angiogenic factor bFGF both *in vitro* and *in vivo* (Barillari et al., 1999).

In contrast, the basic domain of Tat induces endothelial cell proliferation through its heparan sulfate proteoglycans (HSPG) binding activity (see below). In particular, the binding of Tat to the HSPGs on one hand facilitates the interaction between RGD Tat and integrins. On the other hand, by competing for the same HSPG-binding sites, Tat retrieves extracellular matrix bound bFGF in a soluble form, thus promoting cell growth (Folkman et al., 1988). Indeed, similar to Tat, bFGF binds HSPG with its heparin-binding domain and remains attached to the extracellular matrix as a biologically active protein store that is released only when needed. Finally the basic domain of Tat can also bind VEGF receptor 2 and activate the corresponding signal transduction pathway leading to VEGF receptor 2 phosphorylation and endothelial cell proliferation (Mitola et al., 2000).

Tat and the immune response

Several studies on the neurodegenerative disorders occurring in HIV-infected patients indicate that Tat is an important mediator of HIV-mediated inflammatory responses. In addition to IFN-γ, TNF-α and IL-1β, Tat influences the expression of TNF-β, IL-2, IL-6, IL-8, IL-10, transforming growth factor (TGF) β, CXC-chemokine receptor 4, IL-2 receptors, IL-4 receptors, and TNFα-receptors.

HIV-1 infected individuals display symptoms of immune hyperactivation including lymphocyte proliferation, expression of T cell activation antigens and increased cytokine expression.

During the early stages of infection, Tat is very efficiently and selectively taken up by mature dendritic cells whereas immature dendritic cells and other antigen presenting cells internalize the protein 10 to 100 fold less efficiently (Fanales-Belasio et al., 2002). After uptake, Tat induces in these cells an increase of the

expression of the genes of the major histocompatibility complex (MHC) and of costimulatory molecules, as well as production of Th1 cytokines and β chemokines. Therefore, Tat promotes the antigen-presenting capacity of dendritic cells and increases T cell responses by reprogramming the expression levels of several dendritic cell genes that enhance the recruitment of T cells and macrophages (Izmailova et al., 2003). Since the chemokines induced by Tat recruit activated T cells and macrophages that are the ultimate cellular targets of HIV-1 infection, HIV can use dendritic cells as vehicles to infect them.
HIV, however, is not able to infect resting T cell and needs a pool of activated T cells in the host in order to propagate the infection. In addition to recruiting T lymphocytes, Tat up-regulates IL-2 production in infected cells at the transcriptional level in order to prime and activate bystander naive T cells for infection (Ott et al., 1997). IL-2 is the most important growth and differentiation factor of T lymphocytes. A mechanism by which Tat enhances the activity of the IL-2 promoter in stimulated T cells consists in the alteration of the composition of the set of transcription factors bound to the AP-1 site by replacing a repressor with an activator protein complex (Ehret et al., 2001).

The up-regulation of immunostimulatory cytokines and of molecules involved in monocytic chemotaxis is a clear evidence of the activating role of Tat on the immune system. However, several immunosuppressive functions have been attributed to exogenous Tat, which may account for the effect of immune depression in AIDS patients.
A few, and controversial reports, have suggested that extracellular Tat might induce apoptosis in peripheral blood lymphocytes, an event with a possible pathogenetic role in explaining the immunosuppression that accompanies the course of HIV disease (Li et al., 1995).
Impairment of natural killer (NK) cells activity is another possible factor contributing to the progressive immunosuppression in AIDS. The function of NK cells is depended on the transient increase in intracellular calcium concentration, and L-type calcium channels have been shown to be a molecular target of HIV-1 Tat in these cells (Zocchi et al., 1998). Extracellular calcium influx is also

responsible for dendritic cell activation and IL-12 production. Tat blocks calcium influx also in dendritic cells and it can therefore impair T lymphocyte priming and NK cell functions induced by activated dendritic cells (Poggi et al., 1998).

Another possible mechanism by which extracellular Tat might interfere with the cell-mediated immune response is by inducing the production of IL-10 by human peripheral blood monocytes through a protein kinase C-dependent pathway (Bennasser and Bahraoui, 2002). IL-10 is a highly immunosuppressive cytokine and production of IL-10 in infected patients is associated with HIV disease progression.

Finally, an elegant paper published about 15 years ago (and never confirmed later) has proposed that extracellular Tat specifically inhibits the antigen-specific T-cell activation response (Viscidi et al., 1989).

As a final comment, it should be emphasized that none of the above reported findings provides a clear-cut clue to the issue of whether and how Tat might be directly involved in the induction of immunosuppression, and that, at present, their conclusions remain, at best, suggestive.

Internalization of extracellular Tat and of other cell penetrating peptides

The capacity of Tat to enter the cells depends upon the integrity of the basic region of the protein, a 9-amino acid, arginine-rich sequence that also corresponds to the nuclear localization signal and to the TAR-binding domain of the protein (reviewed in refs. (Jeang et al., 1999; Marcello et al., 2001) Fig. 6). Work performed in different laboratories over the last few years has shown that short peptides corresponding to this amino acid stretch (Green and Loewenstein, 1988; Ignatovich et al., 2003; Vives et al., 1997), as well as other peptides rich in arginines (Suzuki et al., 2002; Wender et al., 2000) are rapidly internalized by the cells. A number of other protein and their peptide derivates have been found to possess similar cell internalization properties including the homeoprotein of *D. melanogaster* Antennapedia (Derossi et al., 1996) and the herpes virus tegument protein VP22 (Elliott and O'Hare, 1997).

Antennapedia is a transcription factor involved in the development of the fruit fly *D. melanogaster*. Similar to all other homeoproteins it contains an homeodomain, a 60 amino acid DNA binding domain which is composed of three α-helices and is involved in sequence-specific DNA binding. Surprisingly, the same domain has been also found to mediate the internalization of the protein inside the cells. In particular, the third helix of the homeodomain is sufficient to mediate transmembrane translocation (Derossi et al., 1996). Further studies have identified a 16 amino acids sequence derived from the third helix, termed penetratin which can be used as a cell penetrating peptide to deliver heterologous molecules into the cells (Derossi et al., 1998).
Biophysical studies on the internalization mechanism of penetratin suggested that this process is energy-independent and occurs through the formation of reverse micelles. A proposed model postulates that penetratin interacts electrostatically with cell surface phospholipids; its accumulation destabilizes the plasma membrane by inducing formation of inverted micelles (Prochiantz, 2000). Subsequently, these penetratin-containing micelles can cross the lipid bilayer and eventually release the peptide into the cytoplasm (Fig. 9).

However, the *in vivo* applications of the Antennapedia homeodomain for molecular delivery have been so far very limited, mainly due to the fact that this protein works well only with fusion proteins of less than 100 amino acids and that it looses its transduction activity when conjugated with DNA (Derossi et al., 1998).

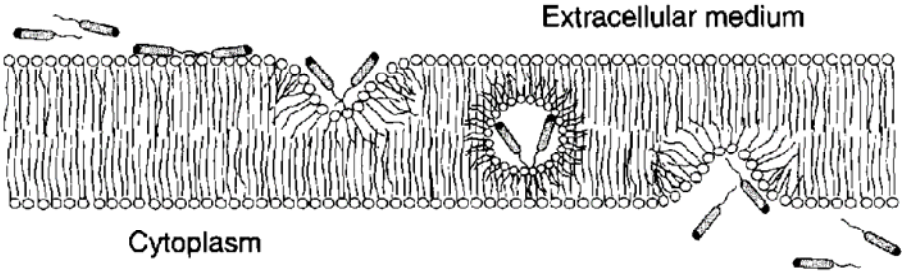


Fig. 9. A model for Antennapedia third helix internalization (Derossi et al., 1998).

The VP22 protein of Herpes simplex virus type 1 (HSV-1) is the major component of the viral tegument, located between the envelope and the capsid of the virion. Even though VP22 does not possess a conventional signal peptide, it is secreted from the cells in which it is produced and penetrates into neighboring cells, where it translocates into the nucleus.

A 40 amino acid domain in the C-terminus of VP22 is necessary for its trafficking activity; this domain has been shown to be able to carry viral RNA from infected to uninfected cells during the HSV-1 replication cycle (Sciortino et al., 2002). In this respect, however, it should be pointed out that the ultimate function of VP22 in viral infection has not been definitively established yet.

Since VP22 fused to heterologous proteins maintains its ability to translocate into the cells, it has been used to deliver molecules across the cell plasma membrane *in vitro* and *in vivo*. However, most of the experiments involving VP22 fusion proteins were performed with transfected producing cells, that require the expression of enough fusion protein to transduce surrounding cells. This approach is therefore disadvantageous in comparison to recombinant fusion protein production and purification, also because most of the protein expressed in producing cells remains entrapped in the nucleus.

A collection of novel human-derived peptides, denominated Vectocell®, has been recently identified with enhanced cell penetration characteristics (Avrameas et al., 1998). These peptides were selected from a pool of natural anti-DNA antibodies present in patients with autoimmune disease such as lupus erythematosus. Since the final target of these antibodies is cellular DNA or DNA-associated proteins, their pathogenetic role is explained by their capacity to enter the cells and be transported to the nucleus. In addition, Vectocell® peptides can ferry conjugated molecules of varying size into the cell, driving them to specific subcellular localizations.

Vectocell® peptides were developed and assessed for their capacity to transport a range of macromolecules across mammalian cell membranes. These Vectocell® peptides were derived either from known heparin binding proteins, or from anti-DNA auto-antibodies, or a chimera of both. The amino acid sequences and characteristics of a subset of the peptides are shown in Table 1 (de Coupade, Fittipaldi et al., 2005).

TABLE 1

Sequence of Vectocell® penetrating peptides

Vectocell® peptides	Sequence	Number of amino acids	% of basic amino acids	Source (NCB Accession #)	Intracellular localization of DPV-PO/IgG (HeLa cells)
DPV3	RKKRRRESRKKRRRES*	16	70	Superoxide Dismutase (AAH14418)	Cytoplasm
DPV6	GRPRESGKKRKRKRLKP*	17	50	Platelet Derived Growth Factor (PDGF) (AAA60045)	Cytoplasm
DPV7	GKRKKKGKLGKKRDP*	15	56	Epidermal-Like Growth Factor (AAC15470)	Cytoplasm
DPV7b	GKRKKKGKLGKKRPRSR*	17	61	Epidermal-Like Growth Factor (AAC15470)	Cytoplasm
DPV3/10	RKKRRRESRRARRSPRHL*	18	58	Superoxide dismutase and Intestinal Mucin	Cytoplasm
DPV10/6	SRRARRSPRESGKKRKRKR*	19	60	Intestinal Mucin and PDGF	Cytoplasm
DPV1047	*VKRGLKLRHVRPRVTRMDV	19	35	Apolipoprotein B (P04114) and anti DNA antibody (AAB26429)	Nucleus
DPV10	SRRARRSPRHLGSG*	14	33	Intestinal Mucin (AAA59164)	Nucleus
DPV15	LRRERQSRLRRERQSR*	16	47	CAP37 (1617124A)	Nucleus
DPV15b	*GAYDLRRRERQSRLRRRERQSR	22	43	CAP37 (1617124A)	Nucleus

The intracellular localization of DPV-PO and DPV-IgG conjugates were assessed as described in the text.

* indicates the position the cysteine residue that has been added for conjugation to the various molecules.

In an initial set of screening experiments, Vectocell® peptides, also termed as DPVs (Diatos Peptide Vectors), were chemically conjugated to two different reporter proteins, an enzymatically active peroxidase protein (PO, 40 kDa) or a full size immunoglobulin (anti-peroxidase antibody, anti-PO IgG, 150 kDa). The toxicity of the Vectocell® peptides was tested *in vitro* using mammalian cells as well as *in vivo* by the determination of the maximal tolerated dose (MTD). As shown in Table 2, the *in vitro* IC_{50} of the majority of the peptides was higher than the highest concentration tested (1 mM), with the exception of DPV3, DPV7b, DPV3/10 and DPV15, for which IC_{50} values were between 500 and 900 μM. The MTD was determined for each DPV tested after intravenous bolus injection as shown in Table 3. Death of the animals was the only parameter influencing the MTD value as no other signs were observed including any body weight loss, piloerection or diarrhea. DPV1047, DPV10, DPV15b and DPV7 were two to three fold less toxic than the Tat peptide. Since the ultimate use of these peptides would be by intravenous injection of therapeutic conjugates, the haemolytic activity of Vectocell® peptides was assessed. None of them caused haemolysis of human erythrocytes (1 hour incubation, 37°C at concentrations up to 1 mM) with the exception of DPV15b, for which the IC_{50} (concentration at which 50% haemolysis occurred) was approximately 500 μM. Overall, these data suggest that the cytotoxic and haemolytic effects exerted by these peptides occur at concentrations that are significantly higher than those required for *in vivo* applications.

TABLE 2

In vitro cytotoxicity of DPV-maleimide conjugates in mammalian cell lines

Vectocell® conjugate name	CHO-K1 *48h incubation*	HeLa *48h incubation*
DPV3-maleimide	~616 µM	~900 µM
DPV6-maleimide	>1000 µM	>1000 µM
DPV7-maleimide	>1000 µM	~1000 µM
DPV7b-maleimide	~650 µM	~850 µM
DPV3/10-maleimide	~600 µM	~675 µM
DPV10/6-maleimide	>1000 µM	>1000 µM
DPV1047-maleimide	>1000 µM	>1000 µM
DPV10-maleimide	>1000 µM	>1000 µM
DPV15b-maleimide	>1000 µM	>1000 µM
DPV15-maleimide	~516 µM	~833 µM
Tat-maleimide	>1000 µM	>1000 µM

Cells were incubated for 48 hours in the presence of peptide-maleimide conjugates before cell viability was estimated by a WST-1 test. IC_{50} (i.e. the conjugate concentration allowing 50% cell viability) was estimated by regression from the curves giving the percentage of viability as a function of the initial peptide concentration. Results are the mean of three independent experiments.

TABLE 3

In vivo cytotoxicity of DPV-maleimide conjugates

Vectocell® conjugate name	Maximum tolerated dose (MTD, µmol/kg)
DPV3-maleimide	9-12
DPV6-maleimide	12-15
DPV7-maleimide	≥ 18
DPV7b-maleimide	< 9
DPV3/10-maleimide	< 9
DPV10/6-maleimide	< 9
DPV1047-maleimide	> 21
DPV10-maleimide	> 21
DPV15b-maleimide	> 21
DPV15-maleimide	< 9
Tat-maleimide	7-9

Vectocell® peptides were administered by bolus i. v. injection in the lateral tail vein for each group (three mice/group). Body weight and mortality were evaluated every two or three days during at least 7 days following the injection.

All cell penetrating peptides share no sequence similarity one to each other, besides being generally enriched in arginines and lysines. This only similarity, and the observation that their internalization also occurs at low temperature, in the absence of energy and only takes a few minutes from their addition to the cell culture supernatant, have led some authors to postulate that the mechanism of cell entry has to be receptor-independent and might imply the direct translocation of these molecules across the plasma membrane (Prochiantz, 1999; Suzuki et al., 2002; Vives et al., 1997; Vives et al., 2003). This clearly appears not to be the case for the Tat basic domain when fused to large protein cargos. In a previous paper with E. Vives and collaborators, it was observed that, when cells were simultaneously incubated with a short peptide from the Tat basic domain and a large fusion protein encompassing the same domain, only the former entered the cells at 4°C in the absence of energy (Silhol et al., 2002) (Fig. 10). Moreover, the addition of an excess of the Tat peptide could not compete for the entry of the Tat fusion protein, further suggesting the existence of a different internalization pathway for the larger proteins. These clear differences have to be taken into account when evaluating several results presented in recent literature using short peptides, which vary drastically according to chosen experimental settings. In addition, the outcome of some of the peptide studies are blurred by a recent observation that challenged the conclusion that peptide penetration might occur at low-temperature and fast kinetics by showing that this might derive from artifacts in the experimental procedures for fixation, or removal, of membrane-bound peptides (Richard et al., 2003).

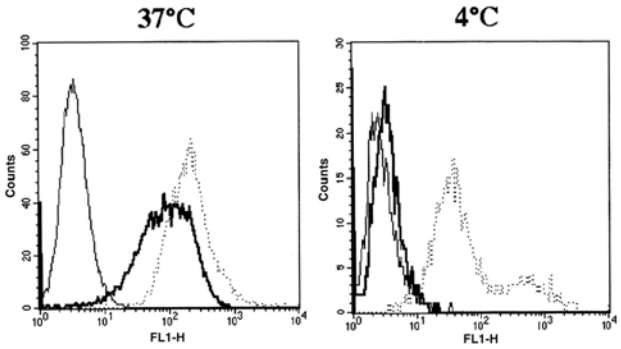


Fig. 10. Cells were incubated during 4 h with Tat-EGFP (solid lines) or with fluorescein-labeled Tat transduction domain (dotted lines) at 37 °C (left panel) or at 4 °C (right panel). Uptake was monitored by flow cytometry analysis (Silhol et al., 2002).

It was already suggested, in the course of early experiments on the capacity of Tat to be internalized from the extracellular compartment, that this process might occur through adsorptive endocytosis (Mann and Frankel, 1991). This conclusion was mainly based on the observations that the 72 (first exon) or 86 (full-length HXB-2 clone) protein binds non specifically to the cell surface, with $>1x10^7$ sites per cell, and that its uptake is temperature and energy-dependent (Mann and Frankel, 1991). The nature of the cell surface receptor and the mechanism of internalization have remained elusive until few years ago. Work performed by several investigators has shown that Tat binds heparin, and that heparin/Tat interaction involves the basic domain of Tat (Hakansson and Caffrey, 2003; Hakansson et al., 2001; Mann and Frankel, 1991; Rusnati et al., 1997a; Rusnati et al., 1999; Rusnati et al., 1998; Watson et al., 1999; Ziegler and Seelig, 2004). Heparin is a close structural homologue of the heparan sulfate (HS) glycosaminoglycan (GAG), a major constituent of cell surface and extracellular matrix proteoglycans (reviewed in refs. (Esko, 1991; Ruoslahti, 1989; Yanagishita and Hascall, 1992)), thus suggesting that membrane-bound HS proteoglycans (HSPG) might be involved in the Tat uptake and internalization process. Direct proof for the requirement of HSPGs came from the observation that hamster cell lines, genetically defective in different biochemical steps of

HSPG production, fail to internalize exogenous Tat (Fig. 11) (Tyagi et al., 2001). The identification of HSPGs as the cell surface receptors for Tat internalization is consistent with the notion that Tat is able to enter into a wide variety of human, rodent and simian cell lines, indicating that it utilizes a ubiquitous cell surface molecule for cell entry, as well as with the observation that extracellular Tat enters most of the exposed cells. A common peptidic motif for heparin/HS binding consists of a region rich in basic amino acids flanked by hydrophobic residues (Esko, 1991; Ruoslahti, 1989; Yanagishita and Hascall, 1992). The basic domain of Tat (aa 49-57) conforms to these characteristics.

Consistently, mutation of the arginines in this domain or its occupancy by polysulfonated compounds prevents heparin binding and cell internalization of recombinant Tat, respectively (Rusnati et al., 1998).

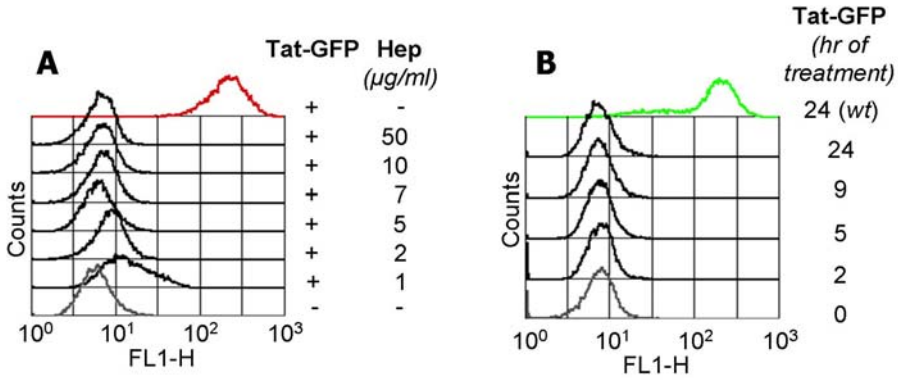


Fig. 11. A. Cells were incubated with Tat-EGFP in the presence of the indicated concentrations of heparin (Hep). **B.** pgs A-745 mutant cells defective in HSPG production were incubated with Tat-EGFP for different time periods. Cells were finally analyzed by flow cytometry (Tyagi et al., 2001).

One critical issue is to assess the specificity of Tat binding to heparin/HS. A large set of experimental data indicates that this interaction, not different from other macromolecules, is not only determined by ionic interactions (Lyon and Gallagher, 1994; Maccarana et al., 1993; Spillmann et al., 1998) but also requires specific structure recognition. In fact, other small basic proteins (having isolectric points and sizes comparable to those of Tat) such as histone H1 and

bFGF, while binding to heparan sulfate, cannot enter the cells through this interaction nor mediate protein transduction (Lappi et al., 1994; Sosnowski et al., 1996). Additionally, other proteins with the same characteristics such as cytochrome c do not even bind with high affinity to heparan sulfates (Albini et al., 1996b). Tat/HS binding affinity is proportional to the size of heparin oligosaccharides, with at least six saccharide residues being required for this interaction to occur (Rusnati et al., 1997b; Rusnati et al., 1999). Finally, both biochemical and genetic results indicate that a proper sulfation of the oligosaccharide chains is required for Tat binding and internalization (Rusnati et al., 1998; Tyagi et al., 2001).

Taking all of these considerations together, it may be concluded that the interaction between Tat and heparin/HS is specified by both charge and structure, and is determined by size, saccharide composition, and extent and distribution of sulfation of the GAG backbone.

Tat as a vehicle for transcellular transduction of proteins

Given the potency of the Tat basic domain in mediating the cellular uptake of small and large macromolecular cargos, it is not surprising that, in the last few years, a large number of laboratories have exploited this system as a tool for transcellular transduction in cultured cells. Most of these applications are based on the fusion of the protein transduction domain of Tat to the protein of interest, either at the N-terminus or at the C-terminus, followed by the addition of the recombinant fusion protein to the culture medium of the cells of interest. Most notably, fusion or conjugation to Tat has been shown not only to mediate cell internalization of heterologous proteins, but also of larger molecules or particles, including magnetic nanoparticles, phages and retroviral vectors, liposomes, and plasmid DNA, with variable efficiency. Some of these practical applications are reported below.

One use of the Tat protein transduction domain was the inhibition of growth and invasion of renal tumors. Renal cell carcinoma is particularly resistant to all chemotherapeutic and radiation therapy and surgical tumor excision is the only option to cure affected patients. Renal tumor is associated with several

mutations in pVHL, a tumor suppressor protein that down-regulates different hypoxia-inducible genes such as VPF/VEGF and hypoxia inducible factor-1α. To evaluate the effects of pVHL in tumor development, the β-domain of pVHL (amino acid region 104-123) was fused to the Tat protein transduction domain and the corresponding recombinant protein was delivered to cancer cells (Datta et al., 2001). These authors found that the fusion protein was sufficient to inhibit renal cell carcinoma proliferation and invasion in tissue culture and to partially arrest tumor growth and invasion of a renal tumor implanted on the dorsal flank of nude mice.

Another application was the inhibition of NF-κB activity to prevent inflammation. In normal conditions, IκB, the specific inhibitor of NF-κB, binds this transcription factor and retains it in the cytoplasm, by blocking its translocation into the nucleus. When cells are activated, the IκB protein is phosphorylated, polyubiquitinated and subsequently degraded by the 26S proteasome. IκB and its non-ubiquitinable mutants have been largely used to inhibit inflammation in experimental systems. In order to inhibit NF-κB activation, the basic domain of Tat was fused to a non degradable mutant of IκB (Kabouridis et al., 2002). Delivery of the fusion protein into a human leukemic T cell line resulted in the inhibition of NF-κB mediated transcription. This approach might find application in a number of other pathological conditions in which inflammation is known to play a detrimental role.

Tat-derived fusion proteins have also been used as growth factors to induce stem cells proliferation *in vitro*. The understanding of cellular mechanisms that supports self-renewal is of crucial importance to maintain *in vitro* proliferation of stem cells. Human homeobox B4 (HOXB4) is one of the factors that can stimulate self-renewal of hematopoietic stem cells; the use of a soluble Tat-HOXB4 fusion protein was found to induce cultured hematopoietic stem cell expansion (Krosl et al., 2003). These expanded cells were shown to retain full *in vivo* differentiation and proliferation potential.

Dendritic cells are powerful antigen presenting cells that may be manipulated in order to stimulate an immune response against cancer, by specifically inducing the presentation of tumor marker epitopes. One possibility is the genetic

modification of these cells, but practical and ethic issues have limited the use of this approach in patients. An alternative is the direct transduction of the target protein without changing the genetic information of the cells; in this respect, it is also important to notice that protein instead of genetic delivery is more convenient and efficient. Different attempts to use this approach have shown that Tat is able to carry inside dendritic cells tumor-associated antigens that are processed and presented to $CD4^+$ T-cells. In addition, immunization with dendritic cells treated with the Tat fusion proteins resulted in complete protective immunity as well as in tumor regression in a cancer model of adoptive tumorigenesis in mice (Shibagaki and Udey, 2002; Wang et al., 2002).

Besides heterologous protein, Tat also mediates cellular internalization of large molecules, such as plasmid DNA. Macromolecule delivery occurs after chemical conjugation to the Tat transduction domain as well by the simple electrostatic interaction between Tat and polyaninic molecules (Sandgren et al., 2002) and is mediated by an endocytic process (Ignatovich et al., 2003). Intravenous injection of Tat-DNA in mice has also been shown to result in the expression of the transferred genes. The use of Tat to translocate DNA into the cells is therefore a valid alternative to the use of viral vectors.

Tat has been used also to increase the efficiency of viral gene transfer. One example is the improvement of retroviral and adenoviral delivery using the protein transduction domain of Tat, which interacts in solution with the viral surface and facilitate the interaction with the host cell plasma membrane (Gratton et al., 2003). In addition, the basic domain of Tat displayed on the surface of recombinant λ phages greatly improved the transfer into mammalian cells of marker genes encapsulated in the phage particles (Eguchi et al., 2001).

A further mechanism to carry DNA, drugs or other large molecules into the cell is the use of liposomes. Torchilin and coworkers found that multiple Tat transduction domains attached to the liposome surface increase the efficiency of liposome load delivery (Torchilin et al., 2001). This system may have significant implications for local drug administration.

In addition to macromolecules, Tat is even able carry magnetic nanoparticles into the cells (Lewin et al., 2000). This approach has potential applications in cell

tracking by magnetic resonance high resolution imaging as well as magnetic separation of specific molecules *in vitro* and *in vivo.*

Besides the applications in cultured cells, Tat-mediated transcellular protein transduction also occurs in living animals, as originally suggested by the observation that the intraperitoneal injection of a >120 kDa-β-galactosidase protein carrying the Tat basic domain is followed by the translocation of the fusion protein into the cells of all tissues in mice, including the brain (Schwarze et al., 1999). This observation has opened the way to a number of possible therapeutic applications of Tat-mediated transcellular protein transduction *in vivo.*

One of the applications that has been considered is the prevention of massive cell death in the brain and in the heart following ischemic damage. After acute cerebral ischemia, a large part of neuronal death is due to the activation of an active apoptotic process. In neurons, the Bcl-xL protein plays a critical role in preventing the apoptosis that occurs during brain development or results from diverse pathologic stimuli, including cerebral ischemia. A fusion protein between the Tat transduction domain and Bcl-xL, when administered intraperitoneally, could be visualized in the brain after a few hours from injection, and was found to determine a significant decrease in the infarcted area in a dose dependent-manner in the mouse and gerbil models of cerebral ischemia (Asoh et al., 2002; Cao et al., 2002). In isolated perfused hearts, the intracoronary delivery of the apoptosis repressor with caspase recruitment domain (ARC), fused to the Tat transduction domain, was found to reduce the extent of the infarcted area after ischemia/reperfusion (Gustafsson et al., 2002). In a pig model of acute myocardial infarction, the intracoronary infusion of a delta-PKC inhibitor conjugated to the Tat transduction domain decreased infarct size, improved cardiac function and reduced apoptosis (Inagaki et al., 2003).

Tat not only enters the cells when present in the extracellular environment, but also exits the cells through a still poorly defined, non-canonical, Golgi-independent pathway of release (Chang et al., 1997; Tyagi et al., 2001). Thus, the delivery of genes encoding for Tat fusion proteins *in viv*o permits the release

of these proteins outside of the expressed cells, thus allowing an extension of the desired effect. An example of this application is the increase of the distribution of the lysosomal enzyme beta-glucuronidase, the protein deficient in the disease mucopolysaccharidosis VII, when expressed as a fusion gene to the Tat transduction domain using viral vectors delivered intravenously or by direct brain injection in mice (Xia et al., 2001).

Another potentially interesting application of the delivery of genes encoding Tat fusion proteins is for cancer gene therapy. In this field, one major limitation is the inefficiency of the currently available gene transfer vectors to deliver the therapeutic genes into all the transformed cells. For example, this limitation has been the major reason for the failure of one of the largest gene therapy clinical trails, which was aimed at treating glioblastoma by transferring the gene for the herpes simplex virus enzyme thymidine kinase (TK), which induces cytotoxicity to the cancer cells when the patients are treated with the nucleotide analog gancyclovir (Crumpacker, 1996). An appealing possibility in this field is to extend the bystander effect of the enzyme also to non-transduced cells by fusing TK to the Tat transduction domain: this approach has been shown to significantly increase the cytotoxic activity of TK beyond the actually expressing cells (Tasciotti et al., 2003).

Extracellular protein internalization through endocytosis

Whereas small molecules can cross the plasma membrane through protein channels and pumps, macromolecules are internalized into endocytic vesicles derived from the plasma membrane. Endocytosis consists in the formation of transport vesicles containing particles and fluids; these vesicles originate from the invagination of the plasma membrane, which behaves as a dynamic structure to segregate the cytoplasm from the extracellular environment.

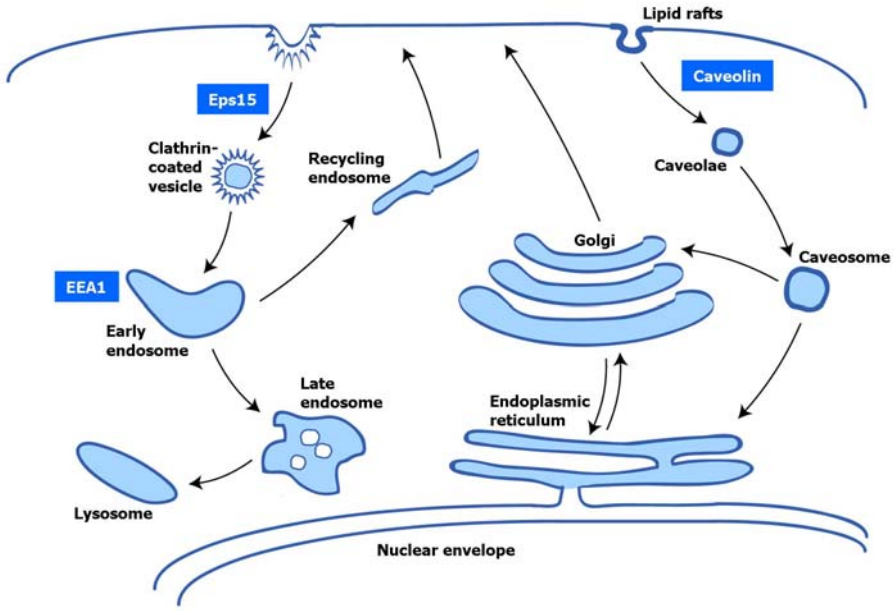


Fig. 12. The figure shows the major pathways of endocytosis and some of the proteins involved in the internalization process.

The best-characterized endocytic pathway is clathrin-dependent endocytosis, which starts on the plasma membrane with the formation of clathrin-coated invaginations that pinch off to make up clathrin-coated vesicles (Schmid, 1997) (Fig. 12). Clathrin is involved in several receptor-mediated endocytic pathways, such as the uptake of the iron-binding protein transferrin, which, after interaction with the transferrin receptor, triggers the internalization of the ligand-receptor complex. After formation of the clathrin coated-pits, the vesicles shed their coat and the endosomes reach the sorting compartment that sorts protein in either early or recycling endosomes. Early endosomes develop in late endosomes and then in lysosomes as a result of a gradual vesicle acidification. In contrast, recycling endosomes carry their load back to the plasma membrane in order to re-use these molecules.

Less defined are the non-classical, clathrin-independent pathways, among which caveolae-mediated endocytosis (Fig. 12). Caveolae are flask-shaped, small (50-70 nm) invaginations characterized by a special lipid composition that is distinct from the bulk of the plasma membrane; they constitute a subclass of detergent-resistant membrane domains enriched in cholesterol and sphingolipids, named lipid rafts (Anderson, 1998). Caveolin, a cholesterol binding protein, is a critical component of caveolae: cells lacking caveolae, such as lymphocytes, produce flask-shaped invaginations only upon expression of this single protein (Fra et al., 1995). Caveolae can detach from plasma membrane as small vesicle and reach pre-existing larger endosomes named caveosomes (Pelkmans et al., 2001). Caveosomes are stationary structures distributed throughout the cytoplasm, they are enriched in caveolin but they are not directly connected to the extracellular space.

Caveolae are involved in signal transduction, cholesterol homeostasis and the intracellular transport of lipid raft-associated molecules, whereas proteins internalized by the clathrin pathway, such as transferrin, are excluded from these membrane domains (Simons and Toomre, 2000). Several bacterial toxins, including cholera toxin (Nichols and Lippincott-Schwartz, 2001), and some viruses, including SV40 (Pelkmans et al., 2001), make use of caveolar endocytosis to enter the cells. In this way, they escape lysosome degradation that results from the clathrin pathway.

Chapter 2

Materials and methods

MATERIALS AND METHODS

Antibodies, fluorescent markers and reagents. Antibodies against early endosome antigen 1 (EEA1), caveolin-1 and GM-130 were from Transduction Laboratories (Lexington, KY). Polyclonal antibody against GFP was from Clontech (Palo Alto, CA). Monoclonal antibody against horseradish peroxidase was generated in the Diatos laboratory (Paris, France) and used at 25 μg/ml. TRITC-conjugated affinipure donkey anti-mouse was from Jackson ImmunoResearch (West Grove, PA). Secondary Alexa Fluor 568 goat anti-mouse antibody, TRITC-labeled transferrin, Alexa Fluor 594-labeled cholera toxin subunit B, TRITC-labeled M_r 10,000 dextran, and Lysotracker Red were all from Molecular Probes Inc (Eugene, OR). EZ-Link maleimide activated horseradish peroxidase was from Pierce (Woburn, MA). The protease inhibitor cocktail was from Roche (Strasbourg, France). Streptolysin O (SLO) was supplied by Dr. Heinz-Georg W. Meyer - Institut fuer Medizinische Mikrobiologie und Hygiene - Johannes Gutenberg-Universitaet Mainz - Hochhaus Augustusplatz - 55101 Mainz – Germany. SLO was dissolved at 1 mg/ml in ICT/DTT buffer (78 mM KCl, 4 mM MgCl2, 8.4 mM CaCl2, 10 mM EGTA, 1 mM DTT, 50 mM Hepes pH 7.2) and stored at -80°C. The colorimetric assay for the determination of N-acetyl-β-D-glucosaminidase and the cytotoxicity detection kit used to quantify the lactate dehydrogenase (LDH) activity were from Roche (Basel, Switzerland) and were both used according to the manufacturer's instructions.
All peptides were synthesized and purified by Neosystem (Strasbourg, France) with a terminal cysteine residue at their N or C-terminal position to allow chemical conjugation.
All other reagents were from Sigma unless otherwise specified.

Cell cultures and transfections. HL3T1 cells (a HeLa cell derivative stably transfected with a silent LTR-CAT cassette), a kind gift of B. Felber (Felber and Pavlakis, 1988) and HeLa and Cos-1 cells (obtained together with other cell lines from the American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium. Jurkat T-cells were cultured in RPMI 1640.

HCT116 were cultivated in Mc Coy's 5a medium. CHO-K1 and PgsA-745 were maintained in Kaighn's modification of Ham's F12 medium. K562 cells were maintained in Iscove's modified DMEM. All cell culture media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamicin, with the exception of Iscove's medium supplemented with 4 mM L-glutamine and 20% FCS.

Transfections were performed using ProFection mammalian transfection system (Promega, Madison, WI). The plasmid expressing the mutant Eps15 Δ 95/295 fused to EGFP is a kind gift of A. Dautry-Varsat (Benmerah et al., 1999).

Peptide conjugation. A cysteine residue was added to either the C- or N-terminus of all peptides to allow conjugation of the peptide to various macromolecules. To prepare PO-peptide conjugates, 1 mg of EZ-Link™ maleimide activated horseradish peroxidase (Pierce) at a concentration of 5 mg/ml in 0.5 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.0 was conjugated with 125 μmol of peptide (solution at 5 mM in 0.5 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7), for 45 min at room temperature. Free peptide was then eliminated by ultrafiltration through a membrane with a molecular cut off of 10,000 Da (Vivascience, Chartres, France). PO activity was determined by a classical ELISA test using Ophenylenediamine dihydrochloride (OPD) as a substrate of the enzyme. By comparing the activity of the conjugates to a standard curve of free peroxidase activity, the concentration of the conjugate solutions were determined, and adjusted to 1 mg/ml in 0.15 M NaCl. Peptide-PO conjugates were stored at -20°C. To prepare tetramethylrhodamine (TMR)-peptide conjugates, 200 μl of a 50 mM solution of TMR-maleimide in dimethylformamide (DMF) were mixed with 700 μl of a peptide solution (10 mM in DMF). After a brief vortex and incubation for 2 hours at room temperature in the dark followed by dichloromethane extraction, the aqueous phase was freeze dried and stored lyophilized. To prepare maleimide-peptide conjugates, 500 μl of a 100 mM solution of maleimide in dimethylformamide (DMF) were mixed with 500 μl of a peptide solution (10 mM in DMF). After a brief vortex and incubation for 3 hours at room temperature in the dark, followed by dichloromethane

extraction, the aqueous phase was freeze dried and stored lyophilized.
GST-Tat86 was conjugated with Cy3.5 Reactive Dye Pack (Amersham Bioscience, Inc., Piscataway, NJ), according to manufacturer's instructions.

Recombinant fusion proteins. The plasmid (derived from the pGEX-2T vector, Amersham Bioscience, Inc.) expressing the glutathione S-transferase (GST)-Tat11-EGFP fusion protein was obtained by replacing the Tat86-coding region in the GST-Tat86-EGFP plasmid (Tyagi et al., 2001) with the sequence encoding for amino acids 48–58 in the basic domain of Tat using the BamH I and Hind III restriction sites. These sequences were obtained by the annealing of the following oligonucleotides that present protruding single strand DNA corresponding to BamH I and Hind III sites:

5'*GATCC*ATGTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA*A*3'
5'*AGCTT*TCTTCGTCGCTGTCTCCGCTTCTTCCTGCCATACAT*G*3'

The same was done for the plasmids expressing the GST-DPVs-EGFP fusion proteins, using the following oligos:

DPV3
5'*GATCC*CGTAAAAAGCGTCGTCGAGAAAGCCGTAAGAAACGTCGACGTGAAAGC*A*3'
5'*AGCTT*GCTTTCACGTCGACGTTTCTTACGGCTTTCTCGACGACGCTTTTTACG*G*3'

DPV15b
5'*GATCC*GGTGCGTATGATCTGCGTCGTCGAGAACGTCAGAGCCGTCTGCGTCGACGT
GAAAGACAGAGCAGA*A*3'
5'*AGCTT*TCTGCTCTGTCTTTCACGTCGACGCAGACGGCTCTGACGTTCTCGACGACGC
AGATCATACGCACC*G*3'

DPV1047
5'*GATCC*GTTAAACGTGGACTGAAACTTCGTCATGTTCGTCCGCGTGTGACCCGTGATG
TG*A*3'

5'*AGCTT*CACATCACGGGTCACACGCGGACGAACATGACGAAGTTTCAGTCCACGTTTA
AC*G*3'

The recombinant GST fusion proteins were produced and purified from BL21 bacteria transformed with the respective plasmids. Bacterial cultures were grown in terrific broth + ampicillin and protein production was induced with IPTG 0.5 mM for 3 hours at 30°C with OD_{600} between 0.6 and 0.8. Bacteria were then resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% glycerol, 2 mM dithiothreitol) and sonicated by 4 pulses of 30 sec each. Bacterial lysates were mixed with a 50% (vol/vol) slurry of glutathione cross-linked agarose beads and the GST-fusion proteins were allowed to bind the beads at 4°C on a rotating wheel for 1 hour. The suspension was then loaded on an empty plastic column (Bio-Rad, Richmond, CA), letting the unbound proteins pass through, and the beads were washed with 400 beds volumes of lysis buffer. The fusion proteins were eluted in the elution buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% glycerol, 2 mM dithiothreitol) containing 20 mM free glutathione. The purity and integrity of the proteins were routinely checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Fluorescence microscopy. For treatment with the different fluorescent molecules, HeLa and Cos-1 cells were grown on chamber slides (Labtech International, Woodside, UK) to about 60% confluence; Jurkat cells were treated in suspension cultures. After a 30-min incubation in serum-free Dulbecco's modified Eagle's medium, fresh, serum-free medium supplemented with Hepes 25 mM (pH 7.2) and containing the different fluorescent molecules was added. Final concentrations were 2 μg/ml recombinant EGFP fusion proteins, 10 μg/ml transferrin-TRITC, 10 μg/ml cholera toxin B-subunit, 50 nM Lysotracker Red, and 0.1 mg/ml dextran-TRITC. At different time points, cells were washed twice with PBS and fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. Cells were subsequently washed and incubated with glycine 100 mM in PBS for 5 min and mounted in Vectashield mounting medium (Vector

Laboratories Inc., Burlingame, CA). Lysotracker Red was added 30 min before fixation. For immuno-staining, fixed cells were washed twice with 0.1% Triton X-100 in PBS for 10 min and incubated with antibodies in PBS supplemented with 0.15% glycine and 0.5% bovine serum albumin. Images were obtained using a TCS-SP laser-scanning confocal microscope (Leica Microsystems, Mannheim, Germany). For live cell recording, cells plated on 3-cm glass bottom dishes were placed Plexiglas chamber and maintained at 37°C and 5% CO_2 throughout the experiment.

Treatment with Triton X-100. Cells were incubated with EGFP fusion proteins together with labeled transferrin for 1 hour, washed, and left in ice-cold 1% Triton X-100 in PBS for 20 min. Cells were then washed once with with 2% paraformaldehyde in PBS and fixed as above.

Drug treatments. Cells were pretreated with the different drugs (5 μM cytochalasin D, 20 μM nocodazole, 20 μM taxol, 10 μM brefeldin A, 10 μg/ml heparin, 5 mM methyl-β-cyclodextrin) for 30 min in serum-free Dulbecco's modified Eagle's medium, and then recombinant proteins in fresh serum-free medium containing the same inhibitors were added. Cells were then processed at different time points (1 hour for fluorescence microscopy and 8 hours for flow cytometry and competitive reverse transcriptase-PCR analysis).

Flow cytometry. Cells were plated in 6-well plates to about 60% confluence and incubated with either recombinant EGFP fusion proteins, or TRITC-labeled transferrin for the time points indicated. Cells were then washed twice with PBS, trypsinized, again washed with PBS, washed with 2 M NaCl to completely take off surface-bound proteins, again washed twice with PBS, and finally, analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences). A total of 10,000 cells per sample were considered.

Competitive PCR. The day before treatment, $2.5x10^5$ HL3T1 cells were plated on 6-cm-diameter dishes. Cells were incubated with recombinant Tat86-EGFP

protein (or Tat11-EGFP control; 2 μg/ml). After incubation, cells were harvested, and RNA was isolated using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). After treatment with DNase I amplification grade (Invitrogen), 1 μg of RNA was reverse-transcribed with SuperScript™ II RNase H reverse transcriptase and random hexamers (Invitrogen) according to the manufacturer's instructions. Competitive PCR was performed by mixing the cDNA obtained in previous step with a fixed amount of a competitor DNA molecule containing the primers for amplification of the β-actin RNA (BA1 and BA4) and of the CAT RNA (CATupper and CATlower) (Demarchi et al., 1996). PCR amplification was performed with 1/25 and 1/250 of the cDNA for the CAT and β-actin amplifications, respectively. For both primers, the PCR profiles were: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec for 30 cycles of amplification. Amplification products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified with ImageJ software.

Retroviral vector production. Phoenix packaging cells containing the gag-pol genes were co-transfected with a Moloney murine leukemia virus based retroviral vector (pLXSM, Clontech, Palo Alto, CA) expressing EGFP and a plasmid expressing the envelope vesicular stomatitis virus glycoprotein (VSV-G), with the addition of either a plasmid coding for the Tat86 protein or an empty vector, in order to produce retroviral vectors in the presence or absence of Tat. Six μg of each plasmid were used for all transfections. Supernatants from transfected cells were collected 40 hours after transfection and viral titer was quantified by measuring reverse transcriptase (RT) activity in the cell culture. Transduction efficiency was measured by adding the retrovirus-producing cells' supernatants to HeLa cells, which had been plated in 12-well plates the day before to about 60% confluence. Cells were incubated for 8 hours with the viral vector, washed, incubated in fresh medium for further 48 hours and analyzed by flow cytometry. In order to calculate RT activity, 10 μl supernatant were mixed with 0.1 A_{260} Units/ml poly(A)X$(dT)_{15}$ (Roche Diagnostics) and 10 μCi/ml [^{32}P]dTTP (Amersham Bioscience) dissolved in 25 μl RT buffer (60 mM Tris-HCl

pH 8.0, 75 mM KCl, 5 mM MgCl2, 0,1% Triton X-100, 1 mM EDTA, 40 mM DTT). The reaction was incubated for 2 hours at 37°C, then 15 µl were spotted onto a DEAE filter (DE81 paper), washed 3 times in 2X SSC, once in ethanol, dried and quantified using a Cyclone Phosphorimager (Packard Instruments Co.).

Quantitative analysis of the internalized material. Peptide-PO conjugates were diluted in the appropriate culture medium. Cells were then rinsed in PBS three times and detached from the culture slides using trypsin-EDTA. Cells were counted, washed in PBS and then lysed in lysis buffer (0.1 M Tris pH 8.0, 0.5% (v/v) NP40, 4°C), in the presence of protease inhibitors. The peroxidase present in the cells was quantified using OPD. For the quantification of the peptide-TMR conjugates, after dilution of the conjugates in culture medium and incubation with the cells, cells were rinsed in PBS three times and detached from the culture slides by trypsin-EDTA. Cells were counted, washed in PBS and then lysed in RIPA buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 1% (v/v) NP40, 1% (v/v) DOC, 2 mM EDTA, 4°C) in the presence of protease inhibitors. The quantity of internalized TMR was determined by measuring the levels of fluorescence emitted by the cell lysates after comparison to a standard curve.

Visualization of the internalized material. Visualization of the internalized peptide-PO conjugates was performed using a peroxidase substrate. Briefly, after the internalization experiment, cells were rinsed three times in PBS before fixation in cold ethanol for 15 min at -20°C. Cells were then rinsed in PBS at room temperature before addition of peroxidase substrate (di-aminobenzidine, one tablet in 10 ml H_20 + 330 µl H_2O_2 3%). Cells were rinsed three times in PBS to stop the reaction and kept in H_2O; observations were performed using a Leica DMR-HC microscope and photographed with a Nikon DXM 1200 camera. Images were further analyzed using the Lucia 4.8 software.

Determination of the peptide conjugate released into the cytosol. HeLa cells were seeded on 2 well glass slides (Labtech) $1.5x10^5$ cells/ml. Penetration studies were undertaken 24 hours after seeding. All internalization experiments

were performed in three independent experiments each in duplicate. Briefly, internalization of the conjugates took place at 37°C - 5% CO_2 in complete culture medium for 2 hours. DPV-IgG, DPV-PO and DPV-TMR were incubated at an initial concentration of 75 μg/ml, 25 μg/ml and 60 μg/ml, respectively. Cells were then rinsed twice in PBS, trypsinised and then counted. Cells were then incubated with 1 μg/ml Streptolysin O (SLO) in PBS for 10 min at 37°C, briefly centrifuged and the supernatant (fraction A) was kept on ice. PBS (400 μl) was then added to the cells followed by incubation for 30 min at 37°C. Cells were then centrifuged, and the supernatant (fraction B) was kept on ice. The pellet was then lysed in RIPA buffer containing protease inhibitors. All three fractions (A + B + pellet) were tested for the presence of the following enzymes: N-acetyl-β-D-glucosaminidase (NabGase), a lysosomal enzyme that should not be present in the cytosolic fractions if the lysosomes have not been damaged by the SLO treatment and Lactose dehydrogenase (LDH), a cytosolic enzyme that should only be present in fractions A and B, and allows to quantify the purity of cytosol that was recovered during the experiment. The reported molecules were then quantified in the three fractions and the proportion of each conjugate in the cytosol was calculated.

Generation of the LoxP-EGFP reporter cell line and Cre fusion proteins.

A DNA fragment comprising the 5'loxP1 site (5' ATGATAACTTCGTATAATGTATGCTATACTAATGGAG-NdeI-3'), the HSV-1 Thymidine Kinase (TK) gene (obtained by PCR amplification), the 3' loxP2 site (5' Nde I-TAGTCGACGCGTATAACTTCGTATAATGCTACGAAGTTATCAGGCCTGCACCCG 3'-Hind III) and the *GFP* cDNA (obtained by PCR amplification) was obtained by PCR amplification and inserted into the pcDNA3 eukaryotic expression vector, also expressing the neoR gene. The resulting vector (named LTLG) was transfected into HEK293 cells. The transfected cells were selected by resistance to G418, and tested for Cre-mediated LoxP recombination by transfection of a Cre-expression vector.

To obtain plasmids expressing the Cre recombinase carrying an N-terminal extension corresponding to the different Vectocell® peptides and to the HIV-1

Tat transduction domain, the Cre gene was PCR amplified using primer pairs carrying Hind III and EcoR I tails and cloned into the GST-DPVs-EGFP or the GST-Tat-EGFP plasmids to replace the EGFP portion. Recombinant protein were expressed and purified as above mentioned.

Chapter 3

Results

RESULTS

PART I – INTERNALIZATION OF HIV-1 TAT FUSION PROTEINS

Tat fusion proteins

Besides its fundamental role in the control of HIV-1 gene expression, over fifteen years ago it has been first demonstrated that Tat also possesses the unusual property of entering the cells and translocating to the nucleus when present in the extracellular environment (Frankel and Pabo, 1988; Green and Loewenstein, 1988; Mann and Frankel, 1991). This unusual characteristic depends upon the integrity of the basic region of the protein, a 9-amino acid, arginine-rich sequence that also corresponds to the nuclear localization signal and the TAR-binding domain of the protein (Jeang et al., 1999; Marcello et al., 2001).

One possible method to study the internalization of a protein into the cells is to purify the protein of interest fused with the green fluorescent protein (GFP, derived from the jellyfish *Aequorea victoria*), and analyze its fluorescence by flow cytometry and confocal microscopy. With this system we can also study the ability of full-length Tat or its basic domain to deliver heterologous proteins into the cells.

In order to follow the internalization of the full-length Tat protein or just its basic domain into the cells and to study the molecular pathway involved in the internalization of extracellular Tat, I obtained two recombinant proteins, each one containing the glutathione S-transferase (GST) protein at the N-terminus, the enhanced variant of the green fluorescent protein (EGFP) at the C-terminus and, in between, either the 86-aa form of Tat or only its 11 amino acid transduction domain (GST-Tat86-EGFP and GST-Tat11-EGFP respectively; hereafter named Tat86-EGFP and Tat11-EGFP). All constructs derived from the pGEX-2T plasmid (Fig. 13). The recombinant proteins were first expressed in bacteria and then purified exploiting the affinity of GST for glutathione, as described in Materials and methods. To check the quality and quantify the concentration of the purified GST-fusion proteins, the eluted products were loaded on SDS-polyacrylamide gel followed by Coomassie staining (Fig. 13).

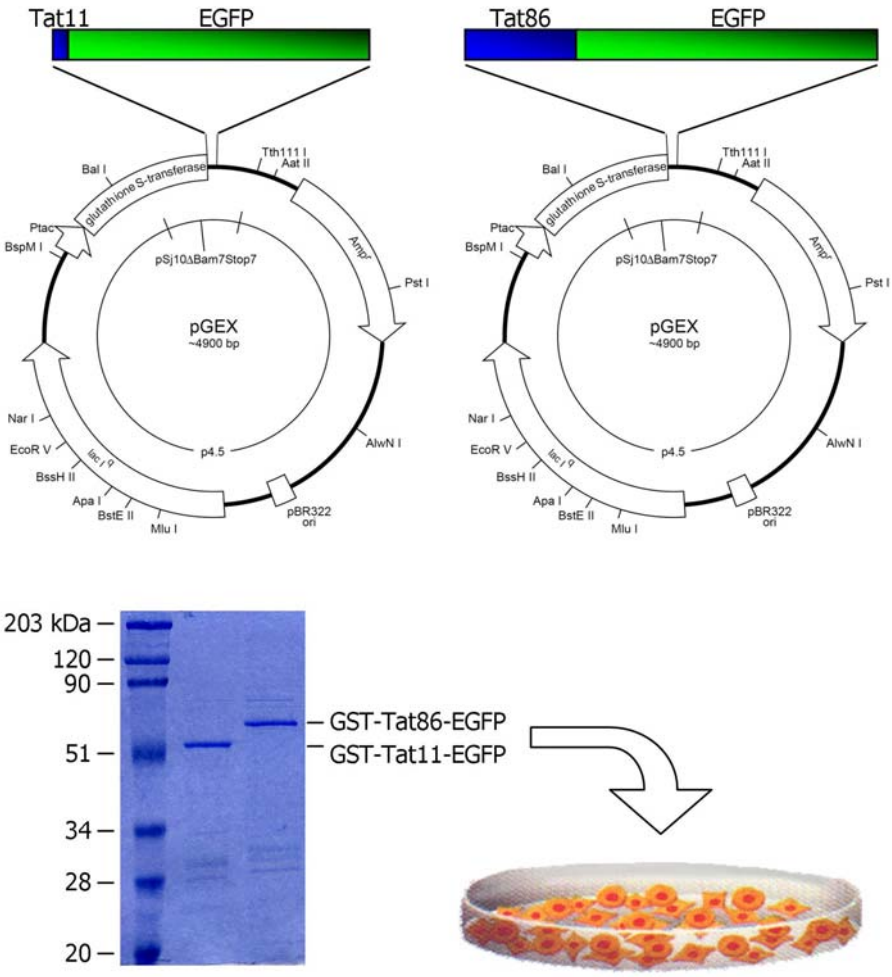


Fig. 13. The plasmids expressing the Tat11 and Tat86 recombinant proteins are schematically drown. The Tat11-EGFP and Tat86-EGFP sequences were cloned using the BamH I and EcoR I restriction sites of the pGEX-2T plasmid. The recombinant proteins were purified using the affinity of GST for glutathione and then loaded on a 10% SDS-polyacrylamide gel and visualized by Coomassie blu staining after electrophoresis. In the course of this work, fusion proteins were added in the cell culture medium to follow their internalization.

Kinetics of Tat internalization

The laboratory has previously shown that Tat86-EGFP is efficiently internalized by different cell types when added to the extracellular medium of these cells (Tyagi et al., 2001). In order to characterize the mechanism of cell internalization, Tat11-EGFP and Tat86-EGFP were added to the cell culture medium of human HeLa epithelial cells at a concentration of 2 μg/ml. Cells were also incubated with TRITC-labeled transferrin, a protein rapidly internalized through receptor-mediated endocytosis. Cells were then harvested after treatment with trypsin to remove any protein adsorbed to the cell surface, and cellular fluorescence was quantitatively assessed at different time points by flow cytometry.

The kinetics of cellular internalization of the two Tat fusions is shown comparatively in Fig. 14, together with the kinetics of internalization of recombinant TRITC-labeled transferrin. Analysis of the flow cytometry profiles and of the mean cellular fluorescence values clearly indicates that the kinetics of internalization of transferrin and of Tat-fusion proteins is remarkably different. Transferrin is internalized rapidly, with >80% of maximum fluorescence already obtained after 15 min incubation. In contrast, internalization of both the Tat86 and Tat11 proteins is slower and progresses in the first 10 hours, after which it reaches a plateau, which persists for at least up to 20 hours. There was no difference noticed between Tat86-EGFP and Tat11-EGFP.

A microscopic analysis of cells treated with the three proteins (inset in the graphs of Fig. 14) indicated that fluorescence was localized to discrete compartments in the cytoplasm, suggestive of internalization by an endocytic process. This observation is consistent with a number of reports, from our as well as from other laboratories, that showed that the internalization of large protein cargos fused or conjugated to Tat is an energy-dependent process, that it does not occur at 4°C, and that is sensitive to drugs blocking endocytosis (Silhol et al., 2002) – see also Fig. 28 and 32. However the localization of Tat and transferrin are different (Fig. 14); Tat containing vesicles are spread throughout the cytoplasm, whereas transferrin accumulates in a perinuclear

region corresponding to the recycling compartment, where transferrin and its receptor are sorted to return onto the plasma membrane.

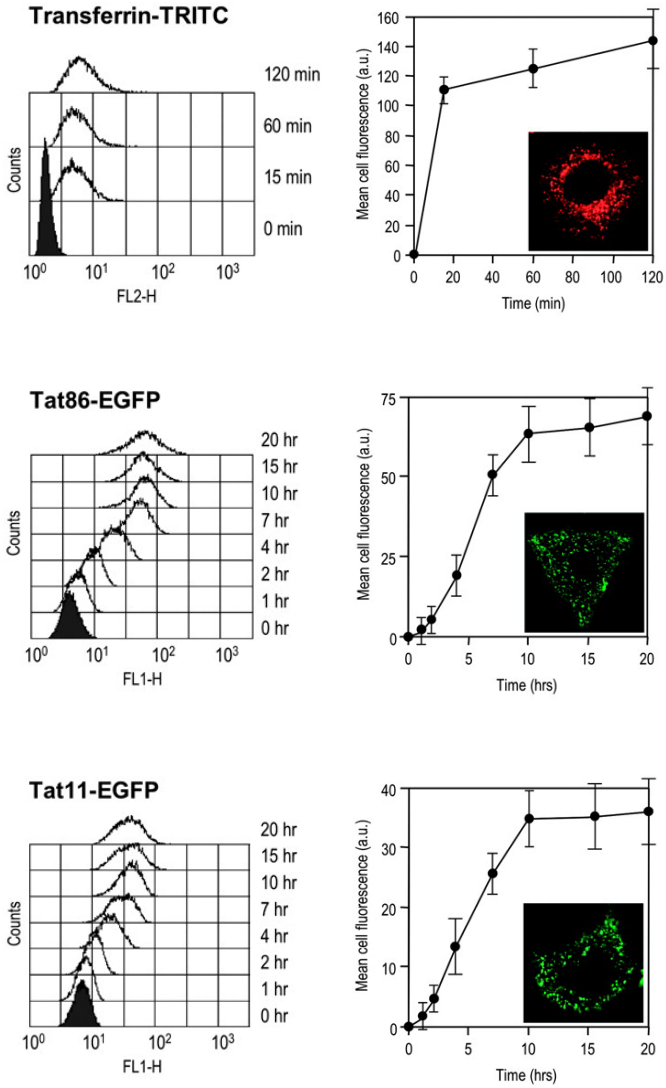


Fig. 14. HeLa cells were incubated with transferrin-TRITC, Tat86-EGFP and Tat11-EGFP for different periods of time, as indicated, and then cell fluorescence was analysed by flow cytometry. The filled peaks report fluorescence of cells incubated without recombinant proteins. Graphs on the right panels show mean fluorescence of HeLa cells analyzed by flow cytometry after incubation with the recombinant proteins. Experiments were performed in triplicate and the S.D. is

reported. Images in the insets show cells incubated for 1 hour with the respective fluorescent proteins.

Tat is internalized through endocytosis

The cellular internalization of Tat by endocytosis was further corroborated by the observations that both extracellular Tat11-EGFP and Tat86-EGFP were found to be internalized in discrete vesicles when mixed together with labeled dextran 10,000 MW, a marker of the fluid phase endocytosis, before addition to the cell medium (shown in Fig. 15 for Tat11-EGFP). In this experiment cells were incubated with either Tat11- or Tat86-EGFP together with TRITC-labeled dextran 10,000 MW, then fixed and analyzed by confocal microscopy. After 1 hour incubation, some of the red vesicles (i.e. endosomes) contain both Tat and dextran. This is a clear indication that Tat is internalized through an endocytic pathway.

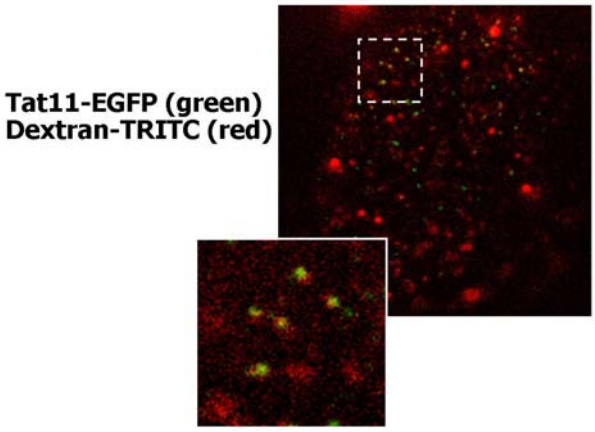


Fig. 15. HeLa cells were incubated with Tat11-EGFP and TRITC-labeled dextran for 1 hour. The magnified area shows some endosomes containing both Tat and dextran.

Tat is not internalized in clathrin-coated endosomes

Which is the endocytic pathway involved in Tat internalization? Transferrin is known to be internalized from clathrin-coated invaginations on the plasma membrane that detach to form clathrin-coated vesicles (Schmid, 1997). Maturation and trafficking of a vast proportion of these endosomes eventually lead to their fusion with cell lysosomes (Fig. 16). Transferrin is internalized after the binding to its receptor that induce the formation of clathrin-coated pits on the plasma membrane. Transferrin-containing endosome are then translocated into the early endosome compartment which sorts transferrin and its receptor to recycling endosomes that in turn translocate them back onto the plasma membrane.

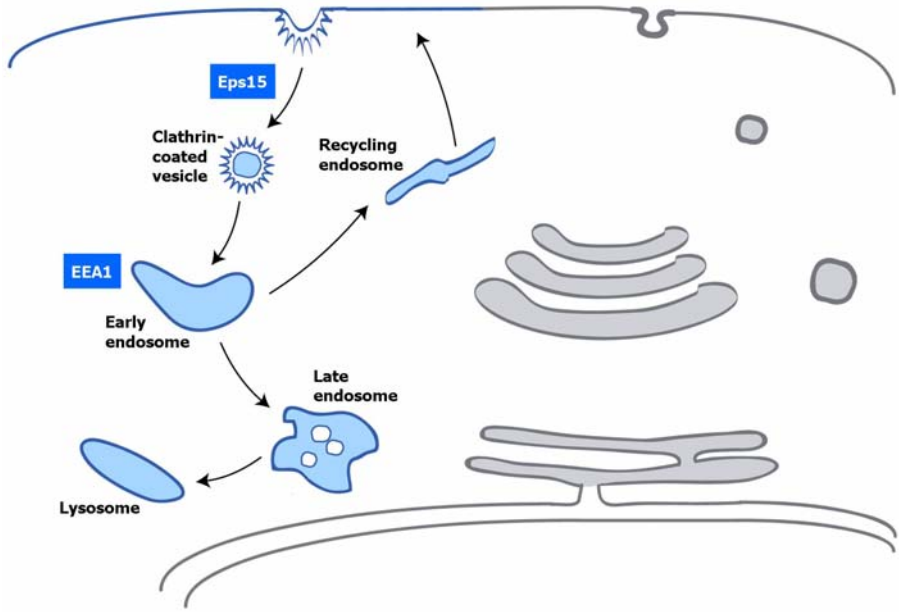


Fig. 16. The figure shows the different steps involved in clathrin-mediated endocytosis.

Therefore, I decided to follow the fate of Tat and transferrin inside the cells and to assess whether Tat is internalized by clathrin-mediated endocytosis. Either Tat11-EGFP or Tat86-GFP were mixed to TRITC-labeled transferrin and the two proteins were added to the culture medium of HeLa cells. At different time points cells were fixed and analyzed by confocal microscopy (shown for Tat11 in Fig. 17). In agreement with the internalization kinetics, 10 min after the addition of the two recombinants proteins, transferrin was clearly detected inside the cell and transferrin-containing endosomes were dispersed all through the cytoplasm, enriched in the perinuclear region. In contrast, during the first minutes of incubation, Tat endosomes were still localized at the cell periphery, consistent with the slower internalization kinetics, and only after 1 hour it was possible to clearly visualize Tat endosomes inside the cytoplasm. Additionally, both proteins appeared in discrete intracellular endosomes at different time points after treatment; however these compartments were completely distinguishable. The finding that transferrin and Tat did not display any colocalization in endosomes is a direct evidence that these two proteins follow two different pathways and suggests that Tat is not internalized through clathrin-mediated endocytosis.

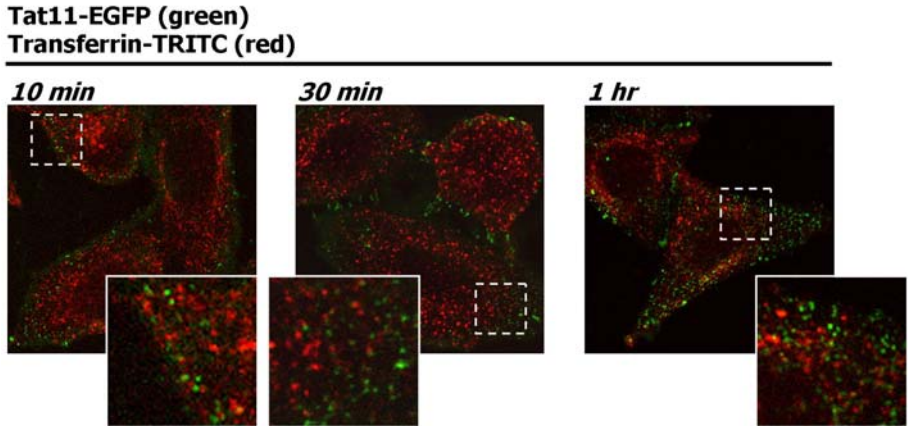


Fig. 17. HeLa cells were incubated with Tat11-EGFP and transferrin-TRITC for 10 min, 30 min and 1 hour. The two recombinant proteins are internalized in distinct endosomal structures inside the cells.

One of the cellular proteins involved in clathrin-mediated endocytosis is the AP-2-associated protein Eps15. This protein works at early stages of the clathrin pathway and it is necessary for the constitution of clathrin-coated endosomes. A dominant mutant of Eps15 generated by the deletion of 200 amino acids in the N-terminal domain (Eps15 Δ 95/295) has been characterized (Benmerah et al., 1999). When expressed inside the cells, this mutant blocks the formation of clathrin-coated vesicles and impairs clathrin-mediated endocytosis.

To verify whether the clathrin pathway is necessary for Tat entry, HeLa cells were transfected with the dominant mutant of Eps15 fused to EGFP (mut-Eps15-EGFP), in order to visualize the cells in which clathrin-mediated endocytosis was blocked (Fig. 18). Transfected cells were then incubated with either TRITC-labeled transferrin or GST-Tat86 conjugated with the red fluorophore Cy3.5 (Tat-Cy3.5). Since mut-Eps15-EGFP expressing cells do not form clathrin-coated endosomes, transferrin internalization was impaired in cells that express the dominant mutant protein, whereas the remaining cells were still able to internalize the recombinant protein. In contrast, Tat was internalized even in cells where clathrin-mediated endocytosis was blocked. This represents a strong evidence that Tat internalization does not require the formation of clathrin-coated endosomes.

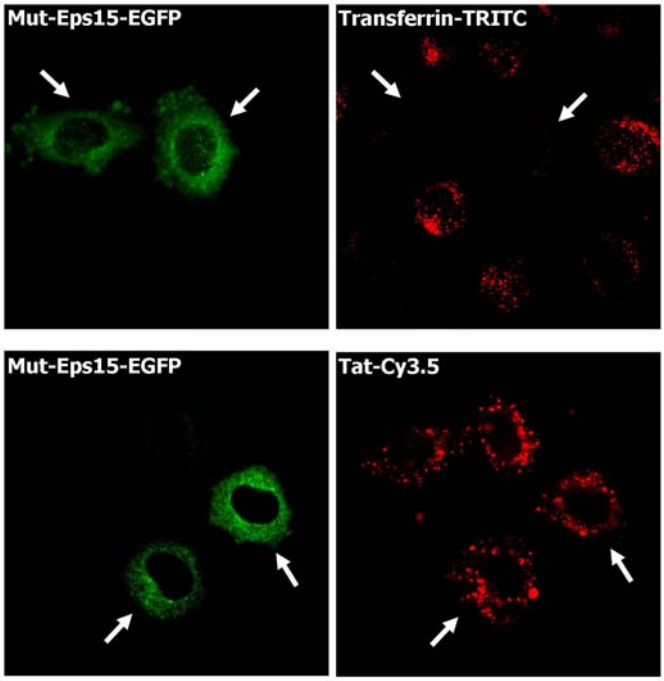


Fig. 18. Hela cells were first transfected with a plasmid expressing the mut-Eps15-EGFP protein and then incubated with either transferrin-TRITC (upper panels) or Tat-Cy3.5 recombinant proteins (lower panels) for 1 hour. In these experiments, a Cy3.5 conjugated GST-Tat86 instead of Tat-EGFP was used, in order to discriminate from the green fluorescence of the Eps15 mutant protein.

After their formation, clathrin-coated vesicles reach early endosomes (Fig. 16), characterized by the presence of the early endosome antigen 1 protein (EEA1), which specifically localizes in this compartment.

To investigate whether Tat reaches early endosomes after its internalization, EEA1 was used as a marker of these vesicles. Cells were incubated with either Tat11- or Tat86-EGFP, then fixed and early endosomes stained with an antibody against the EEA1 protein. As shown in Fig. 19, Tat was not found in early endosomes, which remain distinct from Tat-containing vesicles. Since early endosome formation is a crucial step in clathrin-mediated endocytosis, the involvement of clathrin-coated endosomes in Tat internalization was further ruled out.

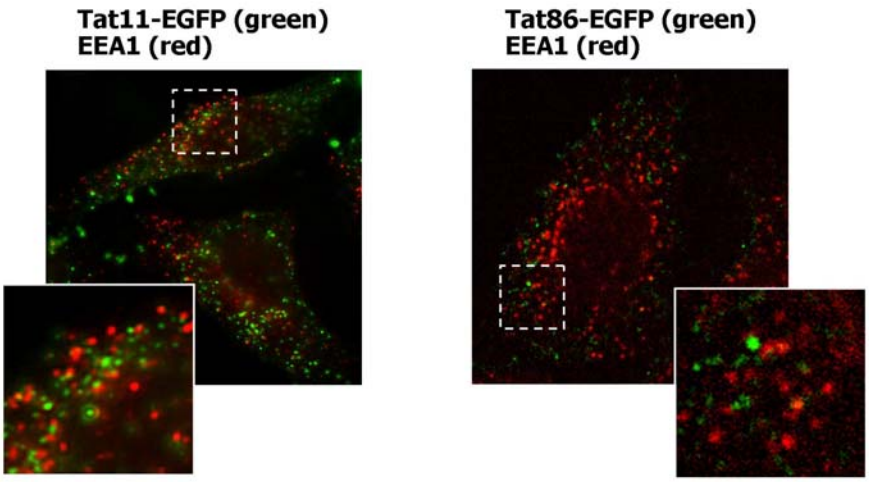


Fig. 19. HeLa cells were incubated with Tat11- or Tat86-EGFP , fixed and stained with an anti-EEA1 antibody.

Endocytosis of clathrin-coated vesicles is known to involve continuous endosome maturation and the eventual fusion with the cell lysosomes. This pathway starts at the level of early endosomes, where internalized molecules can be sorted into late endosomes that in turn lead to lysosomes after progressive vesicle acidification (Fig. 16). It is possible to stain lysosomes using a specific marker termed lysotracker red, a red-fluorescent dye which stains acidic compartment in live cells and is well retained after fixation. Therefore, I tested the possible co-localization of internalized fluorescent Tat with lysosomes; HeLa cells were incubated with Tat11- or Tat86-EGFP and after lysotracker treatment, cells were fixed at different time points. Fig. 20 shows confocal images of these cells; at all analyzed time points, even after 6 hours, Tat11-EGFP and lysosome fluorescence appeared in vesicles that were clearly distinct. Results obtained with the Tat86-EGFP protein were superimposable. This means that, after its internalization, Tat does not reach the late endosome/lysosome pathway.

Taken together, all these results clearly indicate that Tat is not internalized by clathrin-mediated endocytosis.

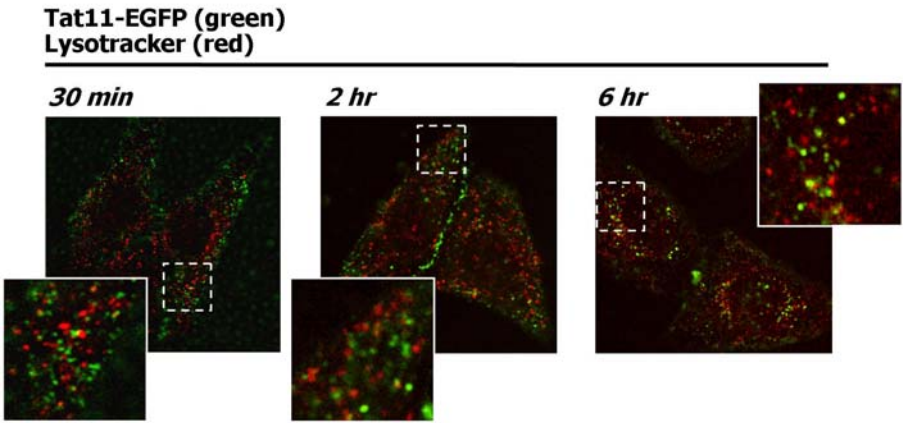


Fig. 20. HeLa cells were incubated with Tat11-EGFP and then lysosomes were stained with lysotracker red before fixation.

Tat endocytosis occurs from cell membrane lipid rafts

At this point, I decided to focus on caveolar endocytosis, in order to study the possible involvement of this alternative pathway in Tat entry. The caveolar endocytosis pathway consists in the formation of flask-shaped invaginations on the plasma membrane named caveolae, that can pinch off as small fast endosomes (Fig. 21). These vesicles may then reach and fuse with caveosomes, larger pre-existing structures that function as a stationary store for molecules internalized in the caveolae. Even if caveosomes do not display any appreciable movement inside the cytoplasm, they can exchange proteins with Golgi apparatus and the endoplasmic reticulum.

The image in Fig. 21 shows the magnification of the cytoplasm of a living HeLa cell incubated with Tat-Cy3.5. It is possible to identify two types of endosomes: small and large endosomes such as the ones indicated by the upper and lower arrow, respectively. Additionally, time-lapse imaging of these cells showed that smaller endosomes move very fast inside the cytoplasm, whereas larger ones remains stationary, consistent with the conclusion that the former are small caveolae while the latter are large, unmoving caveosomes.

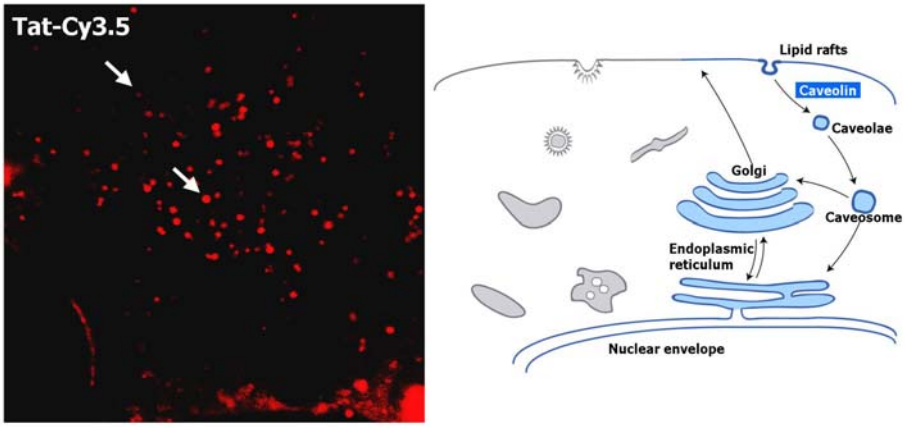


Fig. 21. Confocal image of a living HeLa cell incubated with Tat-Cy3.5; the upper and lower arrows indicate small and large Tat-Cy3.5-containing endosomes, respectively. The right panel reports the compartments that characterize caveolar endocytosis.

If Tat is internalized by caveolar endocytosis, Tat containing endosomes are also expected to have transmembrane caveolin on their surface (Nichols and Lippincott-Schwartz, 2001). To verify this possibility, Cos-1 cells - which are known to express high levels of caveolin – were incubated with Tat11- or Tat86-EGFP for 1 hour, then fixed and stained with an antibody against the caveolin-1 protein. Confocal images of these cells showed that Tat containing endosomes were also positive for caveolin-1.

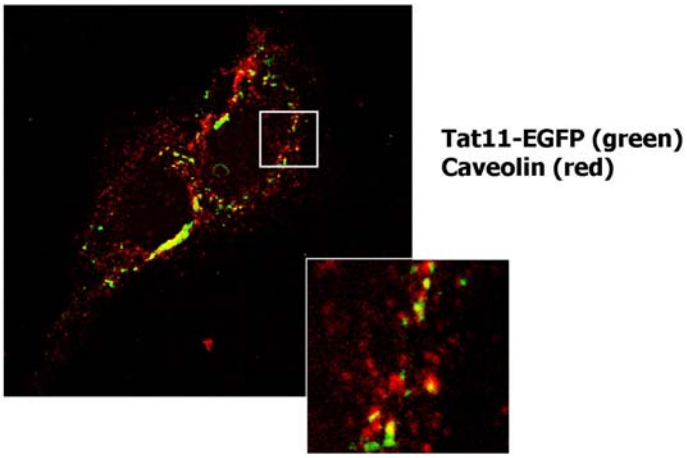


Fig. 22. Cos-1 cells were incubated with Tat11-EGFP for 2 hours, fixed and stained with an antibody against caveolin-1. In some of these endosomes it is possible to visualize the Tat protein in the central part, whereas caveolin-1 surrounds the vesicle. Same results were obtained with Tat86-EGFP.

One of the molecules that exploit caveolar endocytosis to enter the cells is cholera toxin. Similar to other microbial toxins (Falnes and Sandvig, 2000), the active fraction of cholera toxin is the one internalized by the caveolar pathway (Orlandi and Fishman, 1998). Cholera toxin exploits clathrin-independent endocytosis to avoid protein degradation in lysosomes. To assess whether this might be the case also for Tat, the co-localization with the pentameric B-subunit of cholera toxin was tested. The B-subunit is the nontoxic portion of cholera toxin, which mediates the internalization of the active A-subunit; as a consequence, it is possible to use the B-subunit as a marker of caveolar endocytosis.

HeLa cells were incubated with either Tat11- or Tat86-EGFP together with Alexa594-conjugated B-subunit of cholera toxin. As shown in Fig. 23, more than 80% of both Tat11-EGFP- and Tat86-EGFP-endosomes also contained cholera toxin. This means that Tat and cholera toxin follow the same internalization pathway and represents further evidence that Tat enter the cells through caveolar endocytosis.

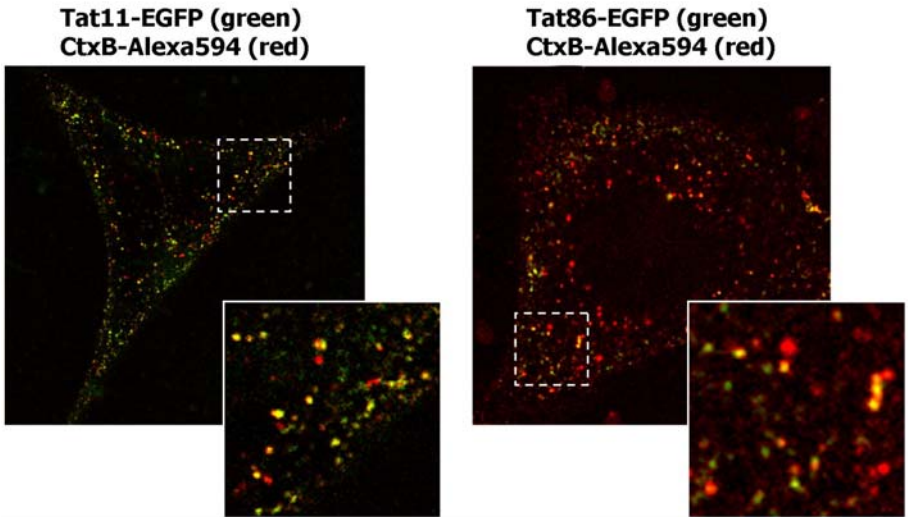


Fig. 23. HeLa cells were incubated with Tat11-EGFP or Tat86-EGFP and Alexa 594-conjugated B subunit of cholera toxin (CtxB-Alexa594) for 1 hour. Note the extensive colocalization of the recombinant proteins in endocytic vesicles.

As an additional indication that Tat uptake involves caveolar endocytosis, internalization of Tat, transferrin, and the B subunit of cholera toxin was comparatively analyzed in living cells by time-lapse confocal microscopy. Tat86-EGFP was mixed to either transferrin-TRITC or to Alexa 594-cholera toxin B and added to the culture medium of HeLa cells. After 30 min, fluorescence of endosomes was visualized at 10-second intervals. Fig. 24 shows one confocal plane for each experiment, with images taken for a total of 40 seconds. In cells exposed to Tat and transferrin, the localization of the endosomes containing Tat appeared unchanged during this time frame, in sharp contrast with the relative mobility of transferrin-containing endosomes. In cells treated with Tat and cholera toxin B, endosomes containing both proteins again were relatively immobile, while the localization of a subset of endosomes only containing cholera toxin B was changing over time – internalization of cholera toxin B is known to occur, in part, also in clathrin-coated endosomes (Orlandi and Fishman, 1998). These results are in agreement with the notion that the velocity

at which caveolar endocytosis proceeds is remarkably slower than that of clathrin-dependent endocytosis (Wacker et al., 1997).

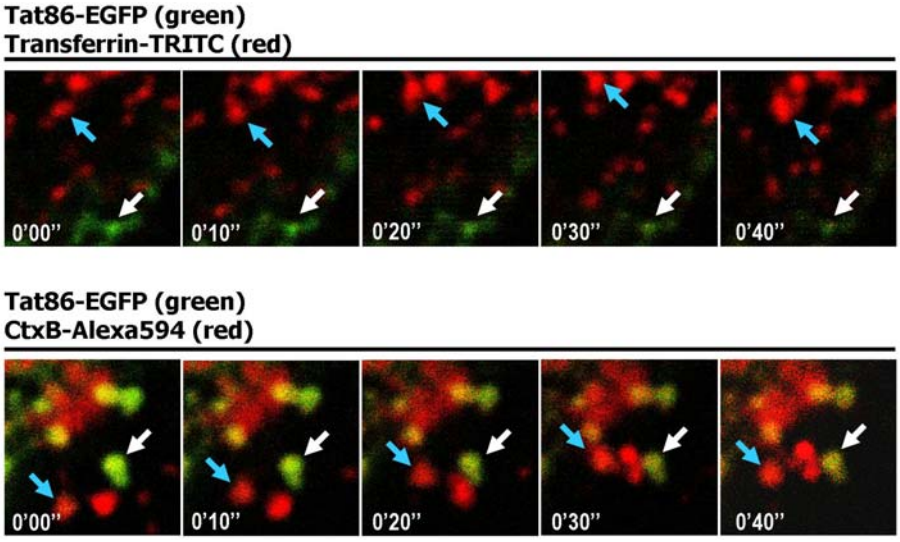


Fig. 24. The figure shows the time-lapse imaging of fluorescence co-localization of Tat86-EGFP with transferrin-TRITC (upper panel) or with CtxB-Alexa 594 (lower panel). Confocal frames were taken every 10 seconds. The Tat86-EGFP-positive vesicles (in green) and the Tat86/EGFP-CtxB-Alexa 594-double positive vesicles (in yellow) did not show any appreciable movement during the 40 sec time frame of investigation (representative endosomes are indicated by white arrows in the two panels). In contrast, transferrin-TRITC endosomes (in red in upper panel) and a subset of cholera toxin-positive endosomes (in red in lower panel) were remarkably mobile (representative endosomes are indicated by cyan arrows in both panels).

As already described in the introduction, caveolar endocytosis emanates from cell membrane lipid rafts, which are operationally defined by their insolubility in nonionic detergents such as Triton X-100 (Henley et al., 1998; Parton et al., 1994; Simons and Ikonen, 1997). Thus, the effect of Triton X-100 was tested on cells that had previously (1 hour earlier) internalized either Tat11- or Tat86-EGFP together with transferrin. Before Triton X-100 treatment, both Tat and transferrin were clearly detected inside the cytoplasm. In contrast, after treatment, the detergent completely solubilized the transferrin endosomes, while

those containing either Tat11-EGFP or Tat86-EGFP remained unaffected (Fig. 25). This provides clear evidence that the Tat containing endosomes originate from cell membrane lipid rafts.

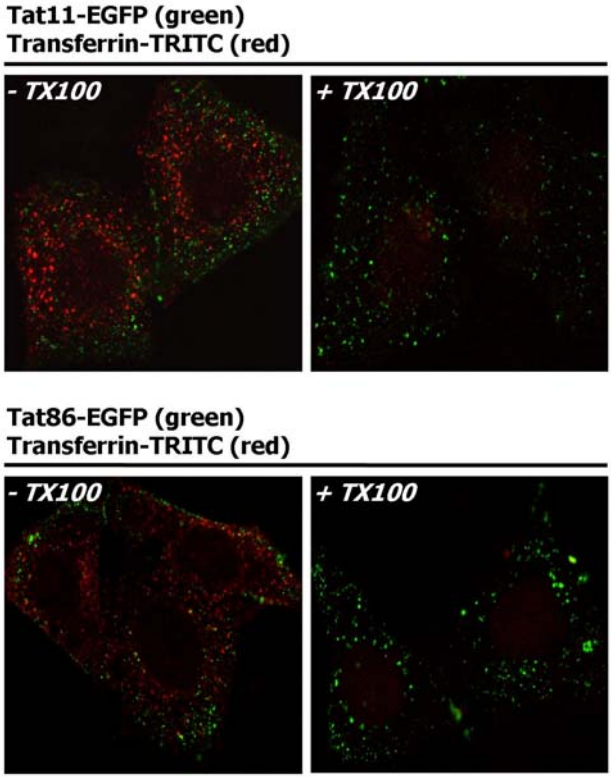


Fig. 25. Triton X-100 extraction of HeLa cells after 1 hour incubation with Tat11-EGFP (upper panel) or Tat86-EGFP (lower panel) and transferrin-TRITC. Tat11 and Tat86 recombinant proteins were found in detergent-resistant structures, whereas transferrin endosomes were disrupted by Triton X-100 extraction.

Since cholesterol is a primary component of lipid rafts, cholesterol depletion of plasma membrane impairs the formation of lipid rafts. To further corroborate the conclusion that the internalization of extracellular Tat occurs from cell membrane lipid rafts, cells were treated with methyl-β-cyclodextrin (MβCD), a drug that extracts cholesterol from cell membranes, thus disrupting lipid rafts. After MβCD

treatment, cells were incubated with Tat11-EGFP and Tat86-EGFP, then fixed and imaged by confocal microscopy. As shown in Fig. 26, in untreated cells, Tat containing endosomes were detected in the cytoplasm, while in cells treated with MβCD, endocytosis of Tat was clearly impaired and no internalized fluorescent endosomes were evident. However, a peculiar fluorescence pattern was visualized marking the plasma membrane, suggesting that Tat, in these conditions, accumulates on the cell surface, probably through its interaction with cell surface heparan sulfate proteoglycans. In contrast, internalization of transferrin was not affected by cholesterol depletion. The inhibition of Tat entry by cholesterol extraction is an additional evidence that Tat internalization occurs via the lipid raft pathway.

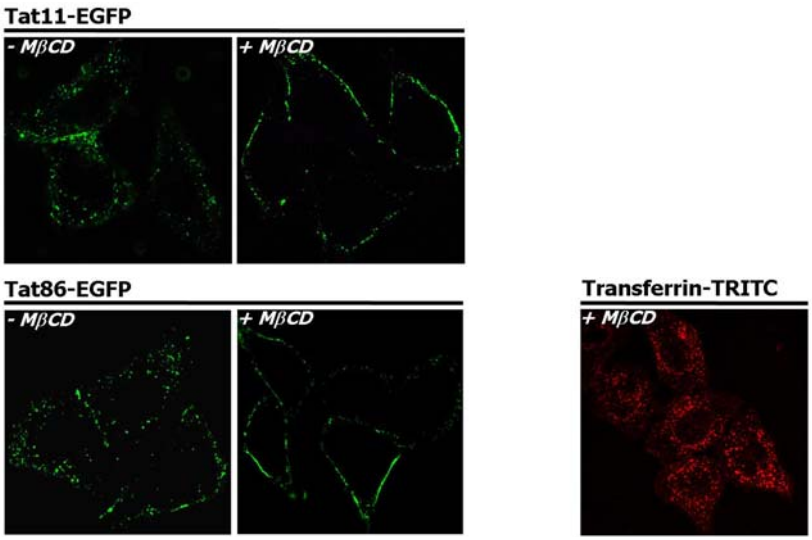


Fig. 26. Cells were pre-treated with methyl-β-cyclodextrin (MβCD) for 30 min and then Tat11-EGFP (upper panel) or Tat86-EGFP (lower panel) was added. The internalization of recombinant Tat was blocked by cholesterol depletion whereas transferrin uptake was not affected.

To better characterize Tat internalization, HeLa cells were treated with different drugs that affect different steps of endocytosis. Cells were then incubated with Tat11-EGFP or Tat86-GFP and qualitatively analyzed by confocal microscopy.

Fig. 27 shows representative cell images for each of these treatments. Of interest is the pattern of cell fluorescence in cells treated with cytochalasin D, a drug causing depolymerization of cell microfilaments, displaying the formation of large Tat-EGFP clusters at the cell periphery. Since the actin cytoskeleton is connected with caveolae, this evidence further supports the caveolar endocytosis of Tat.

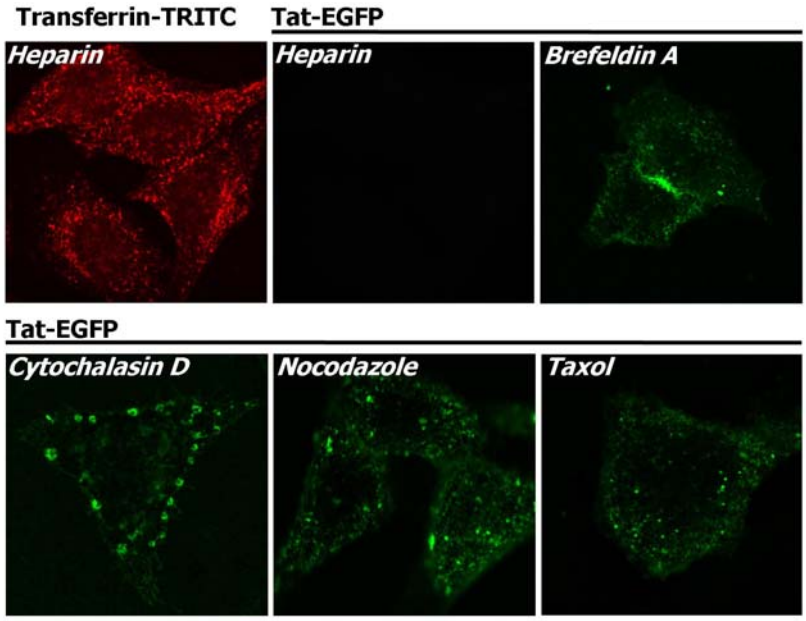


Fig. 27. Subcellular localization of Tat86-EGFP in HeLa cells after drug treatment. Note that heparin treatment did not block internalization of transferrin.

The internalization of fluorescent Tat in the presence of different cell treatments was also quantitatively addressed by flow cytometry and compared to the internalization of labeled transferrin. As shown in Fig. 28, Tat uptake in HeLa cells was blocked at 4°C (as expected for an active endocytic process) and by treatment with heparin (a soluble competitor of cell membrane-associated HSPGs), consistent with previous findings (Tyagi et al., 2001). The former

treatment also inhibited the internalization of transferrin, as expected. Two drugs that affect cellular microtubules (taxol, which stabilizes microtubules, and nocodazole, which disrupts polymerized microtubules) had no apparent effect on either Tat or transferrin internalization. Notably, cell treatment with MβCD drastically reduced Tat uptake, thus indicating that the fluorescence apparent at the cell periphery in cells treated with this drug (Fig. 26) was due to protein interacting with cell surface HSPGs which eventually were not internalized and were removed by trypsin treatment before flow cytometry. Interestingly, cytochalasin D significantly impaired Tat (but not transferrin) internalization, a finding which is consistent with the involvement of microfilaments in caveolar trafficking (Stahlhut and van Deurs, 2000). Brefeldin A, which impairs Golgi trafficking, had no apparent effect on Tat uptake. All the data shown in Fig. 27 and 28 were obtained by using Tat86-EGFP but superimposable results were also observed with Tat11-EGFP.

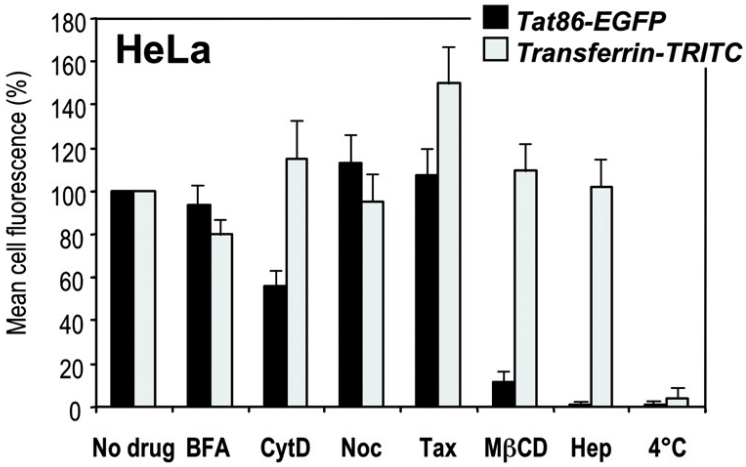


Fig. 28. Flow cytometry analysis of Tat86-EGFP uptake in HeLa cells after drug treatment. Treatment with cytochalasin D (CytD), methyl-β-cyclodextrin (MβCD) and heparin (Hep) as well as incubation at 4°C impaired Tat entry, whereas treatment with nocodazole (Noc) or taxol (Tax) did not affect Tat uptake. Flow cytometry analysis of transferrin-TRITC entry after drug treatments is also shown. The histogram shows mean and S.D. of three independent experiments for each drug treatment, shown as percent of mean fluorescence over untreated control.

Finally, it was also tested whether the observed pathway of extracellular Tat internalization also holds true for $CD4^+$ T-cells expressing the CXCR4 chemokine receptor, which, besides acting as a co-receptor for HIV-1 infection, has also been shown to be a biologically relevant receptor for extracellular Tat (Ghezzi et al., 2000; Xiao et al., 2000). As assessed by flow cytometric analysis (Fig. 29), both Tat86-EGFP and Tat11-EGFP readily entered $CD4^+$ $CXCR4^+$ Jurkat T-lymphocytes, with internalization being inhibited by treatment with soluble heparin or at 4°C. Similar to HeLa cells, treatment with MβCD significantly blocked Tat uptake also in these cells, again indicating the involvement of lipid rafts in the internalization process.

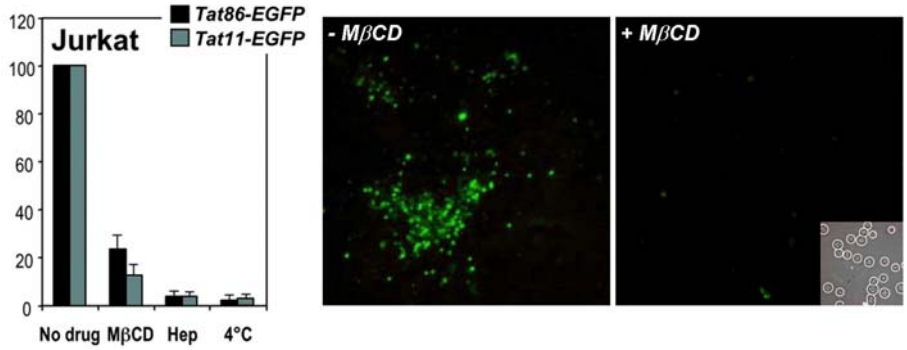


Fig. 29. Flow cytometry analysis of Tat86-EGFP and Tat11-EGFP uptake in Jurkat cells (left panel). The histogram shows the mean and S.D. of three independent experiments for each treatment, reported as percent of mean fluorescence over untreated control. The images represent Jurkat cells incubated with or without methyl-β-cyclodextrin (MβCD) and Tat86-EGFP for 2 hours; cells were then spinned down and analyzed by fluorescence microscopy. Inset in the right picture shows the phase contrast image of the cells treated with MβCD.

Caveolar endocytosis leads to the translocation of functional Tat into the cell nucleus

The laboratory has previously demonstrated that the Tat86-EGFP fusion protein, when exogenously added to the cell culture medium, is internalized and translocated into the nucleus (Tyagi et al., 2001), where it retains its full transcriptional activity on the HIV-1 LTR. Thus, to detect the nuclear translocation of Tat, I measured the transactivation of a viral LTR integrated in the genome of HL3T1 cells, a cell clone derived from HeLa cells stably transfected with a silent HIV-1 LTR-CAT construct (Felber and Pavlakis, 1988). By using these cells, it is possible to analyze the presence of Tat in the nucleus by measuring the amount of CAT gene expression. In the absence of Tat there is no CAT gene transcription, whereas, when Tat translocates into the nucleus, it activates the LTR promoter and induces the synthesis of the CAT mRNA. In order to quantify the CAT transcripts after the addition of exogenous Tat86-EGFP in the culture medium, total cellular RNA was extracted and reverse transcribed into cDNA (Fig. 30). Subsequently, CAT cDNA was quantified by competitive PCR, which is based on the co-amplification of the cellular cDNA with a multicompetitor DNA bearing the primer recognition sequences for the CAT transcript and for the reference cellular β-actin mRNA. β-actin quantification is necessary to measure the amount of total cDNA loaded in the PCR reaction.

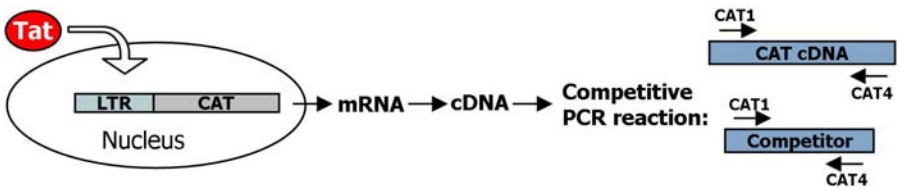
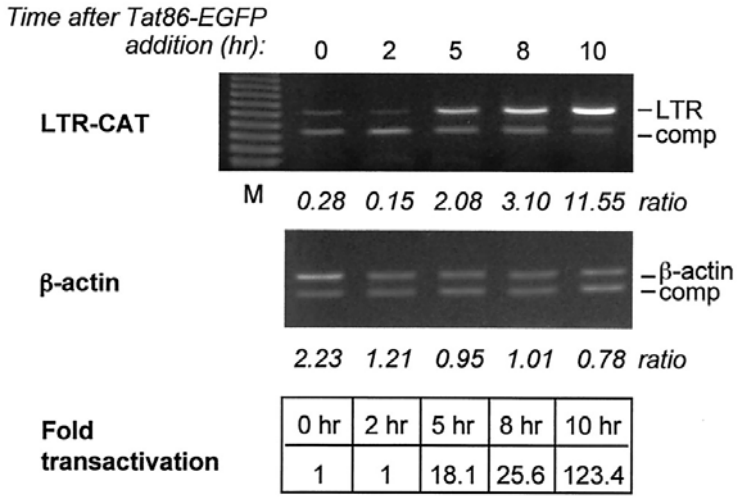


Fig. 30. Translocation of Tat into the nucleus induces expression of the CAT reporter gene from the HIV-1 LTR promoter. CAT transcripts are then quantified by competitive RT-PCR.

This system was used to verify that the fraction of protein that was internalized through caveolar endocytosis from lipid rafts was indeed the same one that was eventually translocated to the nucleus and was active in transactivation. A representative competitive PCR experiment is shown in Fig. 31. Treatment of HL3T1 cells with Tat86-EGFP for different time periods determined a remarkable increase of LTR-driven transcription. The levels of reporter gene transcripts were already 18 times higher after 5 hours from the addition of the recombinant protein to the medium, and reached over 100 times after 10 hours.



0 hr	2 hr	5 hr	8 hr	10 hr
1	1	18.1	25.6	123.4

Fig. 31. Tat86-EGFP was added to the cell culture medium of HL3T1 cells (bearing a silently integrated LTR-CAT construct) and, at the indicated time points, the transcripts of the LTR-CAT reporter gene were quantified by competitive PCR. Cellular RNA was extracted, reverse transcribed, mixed to a competitor DNA bearing primer recognition sequences for the CAT gene and for the cellular β-actin reference gene, and PCR amplified. The two figures show representative competitive PCR amplifications for the two cDNA species (comp: competitor). On the bottom side of each lane, the ratio between the amounts of the two amplification products is reported. The table reports the levels of Tat-induced LTR transactivation at the different time points.

To study which process is involved in Tat nuclear translocation, HL3T1 cells were subsequently treated with the same drugs as in Fig. 28. Consistent with the fluorescent protein internalization data, when cells were treated with MβCD, heparin, or were kept at 4°C during internalization, transactivation of the LTR was almost completely abolished (Fig. 32). In contrast, treatment with nocodazole had no effect. Notably, brefeldin A, which had no apparent effect on fluorescent protein internalization, caused a remarkable decrease in the levels of transactivation. As expected, no transactivation was detected in control cells incubated with Tat11-EGFP.

These observations clearly indicate that the same drugs or treatments impairing caveolar endocytosis from lipid rafts also block transactivation by exogenously added Tat, thus indicating that this route of internalization eventually leads to the presence of transcriptionally active protein in the cell nucleus.

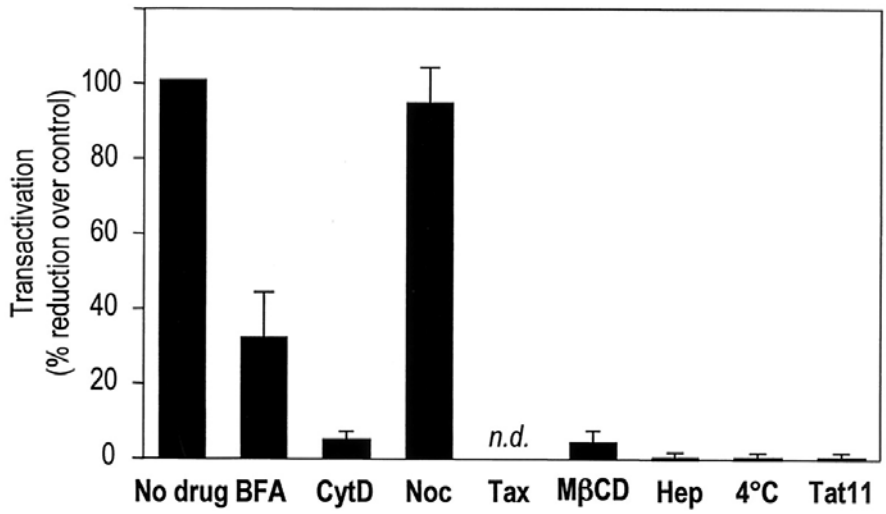


Fig. 32. The histogram shows the effects of drug treatments on the Tat86-EGFP transactivation activity. The effects were calculated as percent of changes in fold transactivation for each experiment; shown are mean and S.D. of three independent experiments for each treatment. *n.d.*: not done.

Role of Tat in viral infectivity

Recent findings have suggested that Tat is displayed on the envelope of HIV-1 virions (Marchio et al., 2004). Tat is indeed released outside HIV-1-infected cells following a leaderless secretory pathway that does not involve the classical ER-Golgi-dependent exocytosis (Chang et al., 1997; Tyagi et al., 2001). Once outside the cell, Tat interacts with HSPGs on the cell surface and accumulates on cell membrane lipid rafts. An additional event that supports the presence of Tat on viral envelope is that the intracellular Gag polyprotein associates with lipid rafts (Ono and Freed, 2001) and virion budding occurs through these membrane microdomains, producing viruses with cholesterol-rich membranes (Campbell et al., 2001; Nguyen and Hildreth, 2000).

The presence of Tat on the viral surface might have an effect on HIV infectivity by facilitating virus adsorption with its HSPG binding activity. If this is the case, a virus with Tat on its surface should be more infectious than one without Tat. To address this hypothesis two different Moloney-based retroviral vectors were produced by co-expressing or not the Tat protein in the producing packaging cell line, and the amount of produced virus was measured by assessing the associated reverse-transcriptase activity. A set of preliminary experiments was performed to test the relative infectivity of viruses with or without Tat on their surface; target HeLa cells were infected and the efficiency of transduction was quantified by measuring the EGFP fluorescence through flow cytometry. These data are shown in Fig. 33. The left panel reports the measurement of reverse transcriptase activity in the viral supernatants, obtained by measuring the incorporation of [^{32}P]dTTP in a DNA template spotted on a paper filter. According to these calculations, the same amount of virus was used for the subsequent target cell infection. The results obtained (right panel) indicate that the virus produced in the presence of Tat was almost three times more infective than the virus obtained without Tat.

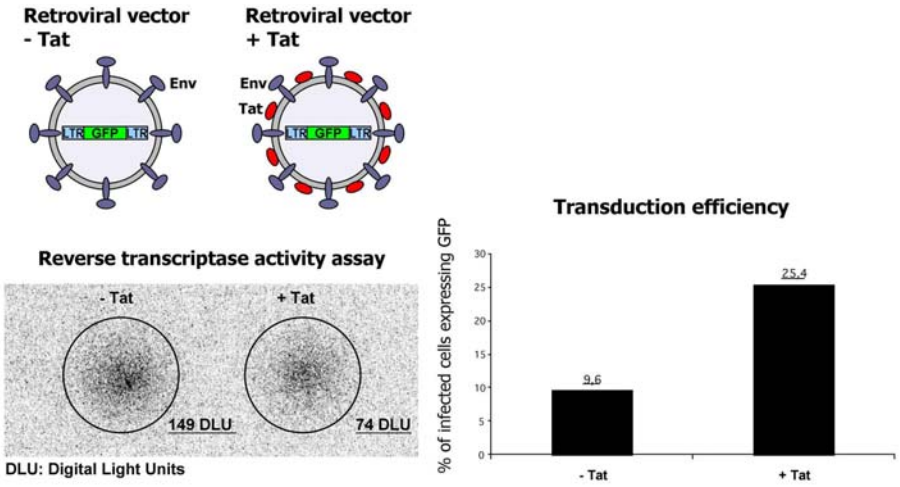


Fig. 33. Two different recombinant viruses, with or without Tat on their surface, were produced and the respective reverse transcriptase activity quantified (left panel). To measure viral infectivity, HeLa cells were transduced with equal amounts of the two different viruses and EGFP expression was quantified by flow cytometry.

PART II – INTERNALIZATION OF CELL PENETRATING PEPTIDES

Vectocell® peptide internalization

As described in the intruduction, Vectocell® peptides were developed for their capacity to transport a range of macromolecules across mammalian cell membranes. On the basis of the cellular localization of the DPV-PO and DPV-IgG conjugates in the HeLa cells (see introduction, Table 1), two sub-groups of peptides could be distinguished. DPV1047, DPV10, DPV15 and DPV15b transported both reporter proteins to the nucleus, whereas the six remaining DPVs resulted in cytoplasmic localization of the reporter proteins. The heparin-binding proteins-derived DPV3 and DPV15b peptides were selected for subsequent studies, being representative of peptides driving cytoplasmic and nuclear localization respectively. DPV1047, a peptide partially derived from an anti-DNA auto-antibody was also selected for further study because of both its distinct origin and its low basic amino acid content. In addition to PO and anti-PO IgG, these three peptides were conjugated to two other reporter molecules of dissimilar sizes and properties: the small fluorochrome tetramethylrhodamine (TMR, 386 Da) and the large auto-fluorescent protein EGFP (26 kDa). As a control for all experiments, a cysteine-TMR (or PO or IgG) conjugate or the GST-EGFP fusion protein was included, to check for nonspecific internalization.

All experiments were performed after determination of the dose-response curve for each of the conjugates, in order to allow selection of non-saturating concentrations. The uptake of Vectocell® peptides was quantified after cell trypsinization to remove cell surface-bound DPV conjugate (Richard et al., 2003).

The three analyzed peptides (DPV3, DPV15b and DPV1047) internalized all four reporter molecules in a variety of fixed mammalian cell lines (both adherent and nonadherent) including epithelial (HeLa, HCT116), myeloid (HL-60), erythroid (K562), lymphoid (Molt4) fibroblast cells (NIH3T3) and primary hepatocyte cultures. The efficiency of internalization was variable, as a function of both the reporter molecule and the cell line. A representative set of data is shown in Fig. 34, in which the internalization of DPV3, DPV15b and DPV1047 in HeLa and

HCT116 cells is compared to that of the Tat11 peptide, conjugated to the same reporter molecules. Despite variability in the quantity of reporter molecule uptake between different cell types, DPV3 always showed the greatest internalization efficiency of the three DPV peptides, with uptake being greater than that of Tat11. DPV15b displayed a lower level of internalization, but was more efficient than DPV1047 (Fig. 34 A and B). This observation suggests that the internalization level of penetrating peptides is dependent on their cationic nature (71%, 73%, 43% and 35% for DPV3, Tat11, DPV15b and DPV1047, respectively) as already suggested (Vives et al., 2003). The relative internalization efficiencies of the three different DPVs were maintained when fused to EGFP (Fig. 34 C). Interestingly, rather different results were obtained when the peptides were conjugated to the small TMR molecule. As shown in Fig. 34 D, the DPV3-TMR, DPV15b-TMR and Tat11-TMR conjugates were internalized in comparable quantities, whilst DPV1047-TMR, was internalized at levels only slightly higher than the negative control (Cysteine-TMR). The simple mixing of the DPVs with any of the reporter molecules without conjugation did not result in internalization, thus confirming previous reports that covalent conjugation is required for peptide driven internalization (Morris et al., 2001).

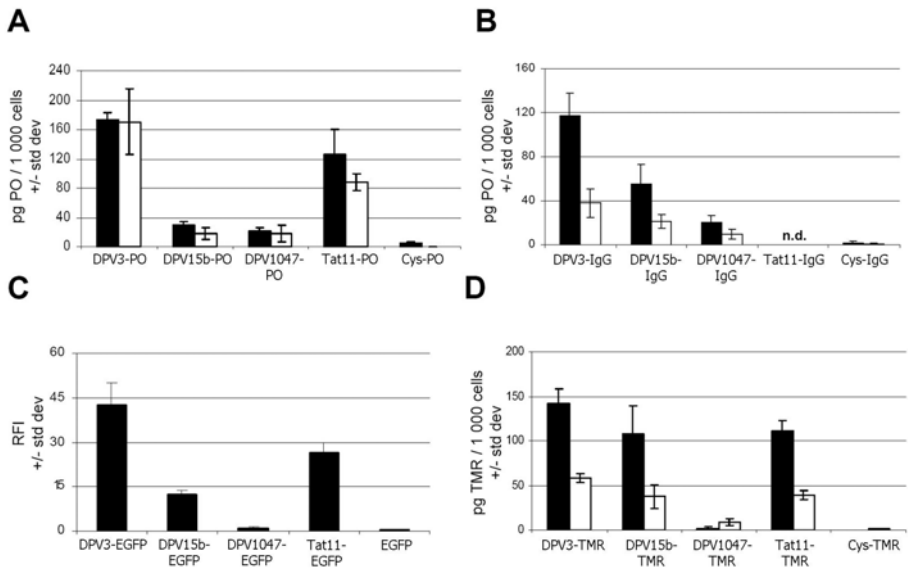


Fig. 34. HeLa cells (black bars) or HCT116 cells (white bars) were incubated at 37°C with DPV conjugates for 4 hours (PO and IgG conjugates; panels A and B respectively), 8 hours (EGFP recombinant conjugates; panel C) or 2 hours (TMR conjugates; panel D). A higher level of internalization is observed for HeLa cells compared to HCT116. Internalization is dependent on both the conjugated molecule (PO, IgG, TMR and EGFP) and on the cell line. All the results reported in the graphs are mean values and S.D. from three independent experiments each performed in duplicate; n.d.: not done.

Subcellular localization of Vectocell® peptides

Assessment of the cellular localization of the Vectocell® in the cells was made either by enzymatic activity and histochemical localization for PO and anti-PO IgG, direct microscopic visualisation for TMR and EGFP in HeLa cells. A number of research groups have shown that fixation artefacts can arise when studying the cellular localization of highly charged peptides (Lundberg et al., 2003; Pichon et al., 1999); for this reason the intracellular localization of Vectocell® peptides was assessed in both fixed and live cells. As shown in Fig. 35, using colorimetric detection of the intracellular PO activity, subcellular localization was dependent on the choice of the peptide conjugate. DPV3-PO was mainly detected in the cytoplasm, whereas both DPV15b-PO and DPV1047-PO were principally located in the nuclei. These experiments were confirmed using an anti-PO antibody (Fig. 35 column B) and confocal microscopy. Vectocell® conjugated to anti-PO immunoglobulin were internalized to the same intracellular locations as the DPV-PO conjugates (i.e. the nucleus for DPV15b and DPV1047, and the cytoplasm for DPV3), as shown in Fig. 35 columns C and D. When Vectocell® peptides were conjugated to the small TMR molecule, fluorescence was observed to be mainly cytoplasmic (Fig. 35 column E). The internalization of the DPV-EGFP fusion proteins was highly efficient for both DPV3 and DPV15b, leading to a strong punctuate pattern in the cytoplasm of HeLa cells (Fig. 35 column F). No nuclear staining was visible for DPV15b-EGFP. This was also the case for the Tat-EGFP conjugates, although Tat peptide contains a basic region responsible for its nuclear import (Trehin and Merkle, 2004). This observation confirms that, due to its folding characteristics, EGFP is not a suitable reporter to assess the final nuclear destination of internalized proteins.

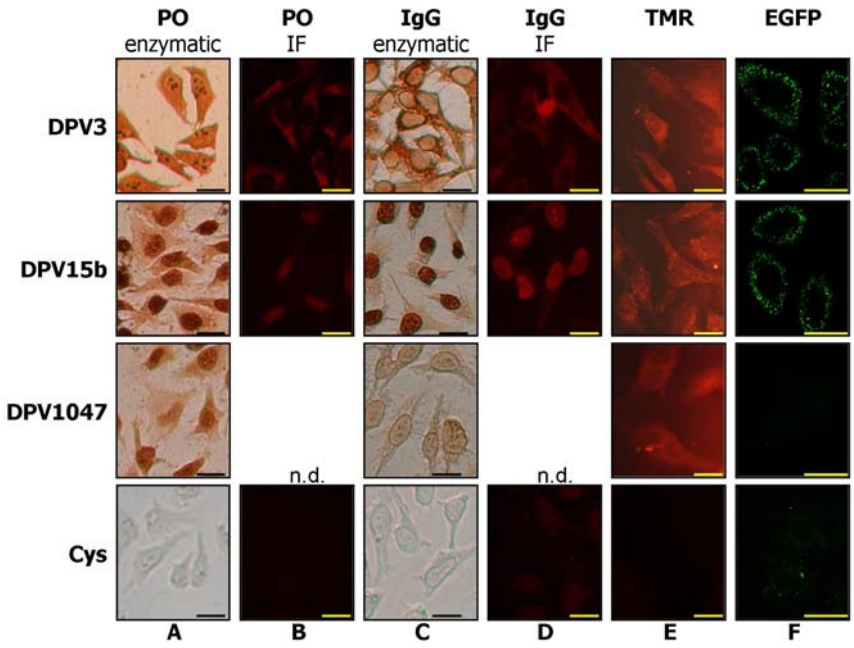


Fig. 35. HeLa cells were incubated with the different conjugates for 2 to 4 hours, before fixation (A-E) or direct observation of live cells (F). A: cells were incubated with di-aminobenzidine for the visualization of active PO. B and D: peroxidase or antiperoxidase IgG, respectively, were detected by indirect immunofluorescence. C: cells were incubated with PO, then with di-aminobenzidine for the visualization of anti-PO immunoglobulin. E and F: TMR and EGFP were directly visualized. n.d.: not done. Bar scale = 10 μm.

For many therapeutic applications, the internalized conjugate is required to be freely available in the cytoplasm. This was assessed using Streptolysin O (SLO), which allows the selective permeabilization of the plasma membrane of the cells without disruption of intracellular organelles (Palmer, 2001; Walev et al., 2001; Walev et al., 2002). After incubation of HeLa cells with DPV3 conjugated to PO, IgG and TMR, followed by elimination of surface bound material, the proportion of intracellular material that was free in the cytoplasm was quantified. As shown in Fig. 36, for each of the three reporter molecules tested, 11 to 35% of the internalized conjugates were freely available in the cytosol. In this experiment,

the integrity of the intracellular organelles after SLO treatment was verified by analysing the levels of the cytosolic protein LDH and the lysosomal protein NabGase. More than 85% of the total LDH was recovered in the cytosol for each experiment, whereas, the contamination of the cytosol by the lysosomal enzyme never exceeded 18% of its total activity.

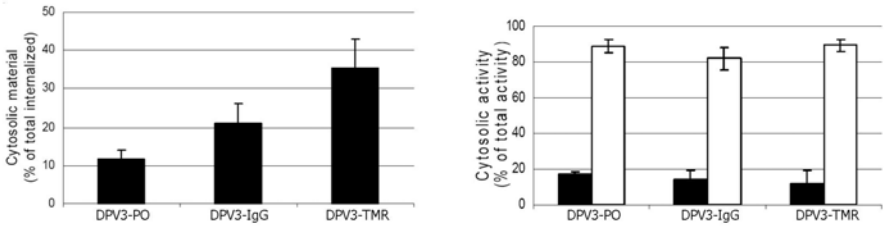


Fig. 36. After internalization of PO, IgG or TMR conjugated to DPV3, cells were lysed using streptolysin O, allowing separation of the cytosolic material from the membranes and intracellular organelles. Left panel, percentage of internalized material that was recovered in the cytosolic fraction. Right panel, the cytosolic enzyme LDH (white bars) and the lysosomal enzyme NabGase (black bars) were quantified in each experiment in order to verify the integrity of the fractions. All the reported values are means and S.D. of three to six independent experiments.

In order to obtain an independent conformation of DPV-mediated internalization of functional macromolecules in living cells, and to verify their different subcellular localizations, Vectocell® peptides were fused to Cre recombinase and tested for their efficiency to drive genetic recombination of a LoxP cassette. For this purpose a cell line was obtained containing an integrated construct in which the EGFP gene was separated from its promoter by an intervening cassette flanked by two LoxP sites (Fig. 37). After Cre mediated recombination (driven by the internalized DPV-Cre conjugate), the cassette is excised and EGFP is expressed. Thus the quantitative analysis of cell fluorescence provides a tool to assess cellular internalization, protein functionality and nuclear targeting of the DPV-Cre conjugates in living cells.

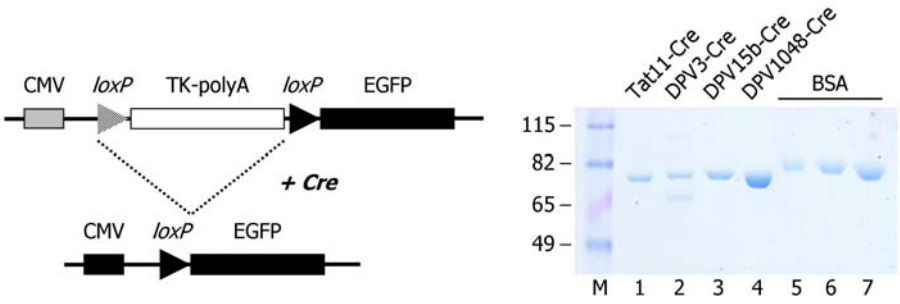


Fig. 37. Schematic representation of the integrated LoxP-EGFP construct, before and after Cre-mediated recombination. The right panel shows a Coomassie-stained gel showing the recombinant proteins containing Cre and the indicated DPV peptides or the Tat11 peptide, as indicated, after purification as fusions with GST (lanes 1-4; 10 μl of each eluate per lane). Lane M: protein ladder marker; lanes 5-7: 1.0, 2.5, 5.0 μg BSA respectively.

DPVs and Tat peptide were conjugated to the N-terminus of the recombinant Cre protein and were purified from bacterial lysates as fusions to GST (>80% purification, as estimated by Coomassie gel staining; Fig. 37) and added to the supernatant of HEK293 cells carrying the integrated LoxP-EGFP reporter construct. Using flow cytometry analysis DPV3-Cre and DPV15b-Cre, as well as Tat-Cre, were shown (72 hours after treatment) to induce LoxP recombination in a dose-dependent manner (as shown for DPV15b-Cre in Fig. 38). However, the levels of recombination induced by treatment with DPV1047-Cre were very low, most probably due to the modest internalization of this Vectocell® peptide in HEK293 cells. Fig. 38 shows the efficiency of LoxP recombination induced by treatment with the three proteins after normalization according to their relative efficiency of cell penetration. Interestingly, DPV15b was found to be the most active in driving the recombination process, a result that is consistent with the preferential nuclear localization of this peptide.

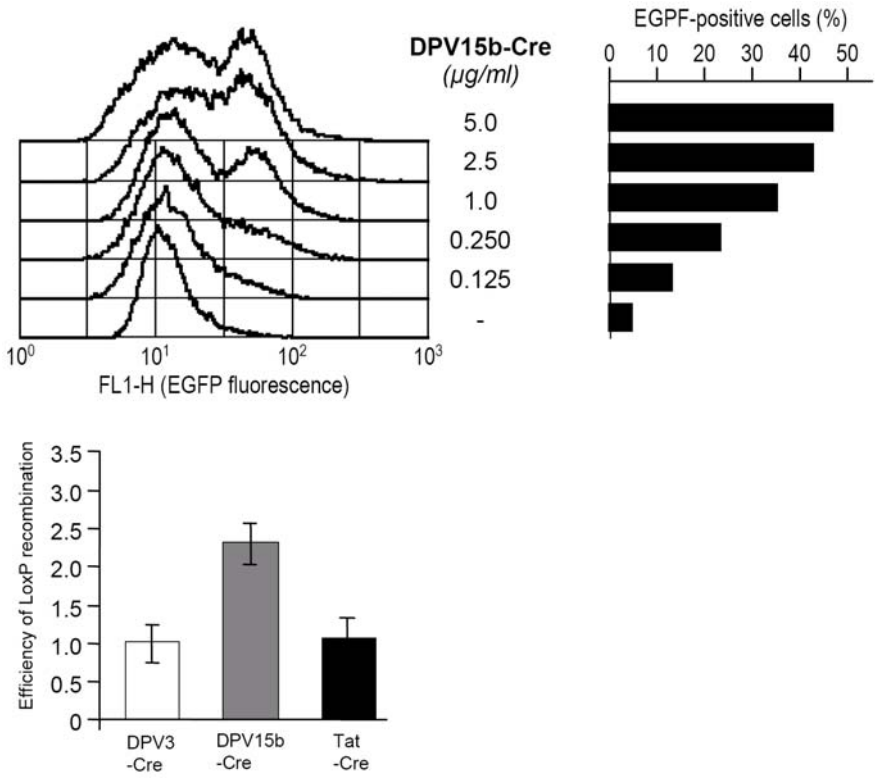


Fig. 38. Flow cytometry profiles showing the EGFP fluorescence of cells stably transfected with a Lox-TK-Lox-EGFP construct after treatment with recombinant DPV15b-Cre. The indicated amounts of protein were added to the cell culture supernatant; after 4 hours, the cells were extensively washed; fluorescence was analyzed after additional 72 hours. The graph on the right side shows the percentage of EGFP-positive cells at each protein dose. The bottom histogram shows the efficiency of LoxP recombination after DPV or Tat11 treatment, obtained by dividing the number of EGFP positive cells (after substraction of the background) by the efficiency of internalization of each peptide relative to DPV3. The latter parameter was measured by treating the same cells with recombinant EGFP fused to the different DPVs or to Tat11. Data represent mean values and S.D. of at least three independent experiments.

Internalization mechanism of Vectocell® peptides

The kinetics of cellular internalization of various reporter molecules conjugated to DPV3 is shown in Fig. 39. Large molecules such as the DPV3-PO conjugate and the GST-DPV3-EGFP recombinant protein both displayed maximum

internalization levels after several hours of incubation with HeLa cells. In contrast DPV3-TMR conjugate reached maximum internalization after only 15 to 30 min of incubation. This observation clearly suggests that the kinetics of cellular uptake depend upon the size of the conjugated molecules as has been reported for Tat11 conjugates (Silhol et al., 2002).

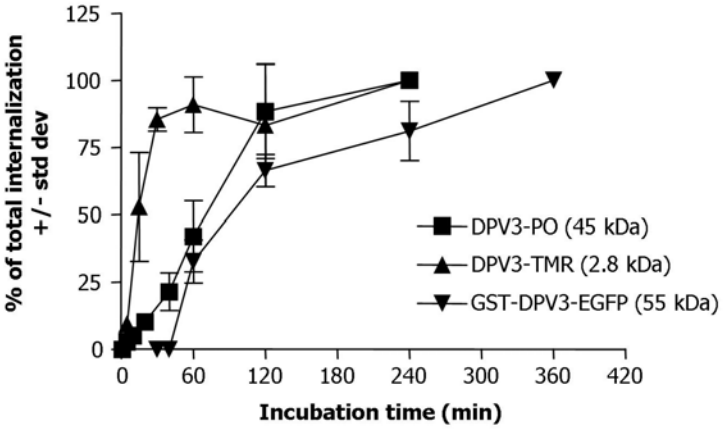


Fig. 39. HeLa cells were incubated in the presence of DPV3-PO (25 μg/ml), DPV3-TMR (20 μM) or the recombinant protein DPV3-EGFP (2 μg/ml) for the indicated period of time, before trypsin treatment, lysis of the cells, and quantification of the internalized material in the appropriate measurement unit.

To obtain further insight into the mechanism of cell entry, the energy dependent nature of the internalization process was assessed by monitoring its temperature dependence. The results presented in Fig. 40 A show that internalization of PO by both Vectocell® peptides and Tat11 in HeLa cells is completely inhibited at 4°C, an observation that is consistent with an energy-dependent endocytosis process. In sharp contrast, as shown in Fig. 40 B, when the experiments were performed with the much smaller DPV-TMR conjugates (~2.5 kDa) internalization still occurred at 4°C although the efficiency was decreased (45%, 27% and 10% internalization for DPV3-TMR, Tat11-TMR and DPV15b-TMR respectively) indicating an energy-independent process. Internalization of the DPV-TMR conjugates at 4°C most likely occurs by diffusion through the plasma

membrane as has been suggested for both Tat and several other cell penetrating peptides (Hallbrink et al., 2001; Lindgren et al., 2000; Pooga et al., 1998). This conclusion is also in agreement with the rapid kinetics of internalization of the TMR conjugates shown in Fig. 39.

In order to assess whether binding to glycosaminoglycans (GAGs) is necessary for Vectocell® internalization, quantitative experiments were performed in CHO-K1 cells and the daughter cell line PgsA-745 that is devoid of the xylosyltransferase enzyme and consequently does not produce detectable levels of proteoglycans including GAGs (Esko et al., 1985; Rostand and Esko, 1997). As shown in Fig. 40 C and D, the internalization of the DPV3 and DPV15b conjugates (PO and TMR) was impaired by approximately 80% in PgsA-745 cells as compared to their wild type counterpart demonstrating the importance of HSPGs in the internalization of these peptides. The observation that the internalization of all conjugates was also inhibited by heparin is consistent with this conclusion.

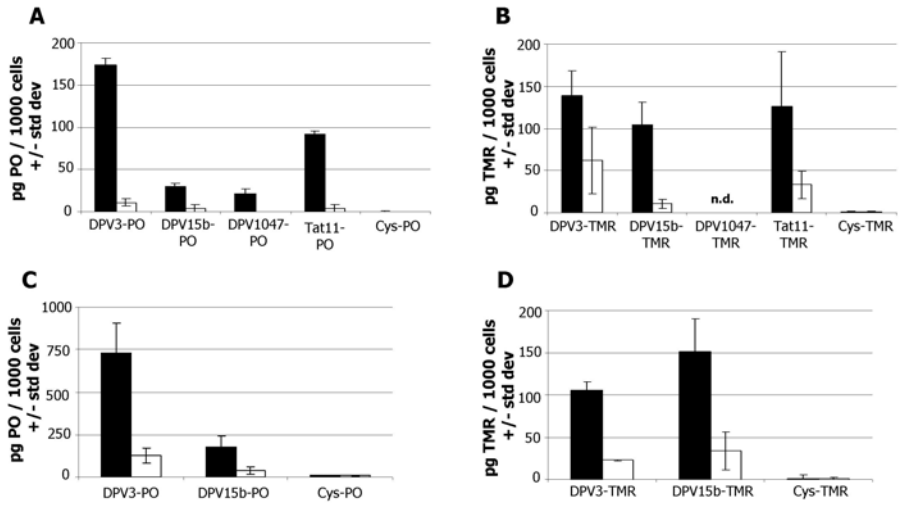


Fig. 40. A and B, HeLa cells were incubated with DPV/Tat-PO conjugates for 4 hours at a concentration of 25 μg/ml or DPV/Tat11-TMR conjugates for 2 hours at a concentration of 20 μM, at either 37°C (black bars) or 4°C (white bars). Intracellular PO or TMR were then quantified after trypsin treatment and lysis of the cells. C and D, CHO-K1 cells (black bars) or PgsA-745 cells (white bars) were incubated for 4 hours in the presence of the indicated conjugates at the same

concentrations, before trypsin treatment and lysis of the cells. PO or TMR were then quantified as described. All graphs display mean values and S.D. from 3 independent experiments each performed in duplicate.

The pathways of DPV3 and DPV15b internalization were further characterized using recombinant GST-DPV-EGFP fusion proteins. Whilst these proteins are not suitable to determine the final sub-cellular DPV destination (see Fig. 35), they are still excellent tools for visualising the early steps of the internalization process. Recombinant DPV-EGFP fusion proteins were mixed with either labeled-transferrin or the B subunit of cholera toxin before treatment of HeLa cells. As shown in Fig. 41 A and B, the recombinant DPV-EGFP proteins were found to co-localize with cholera toxin B but not with transferrin in the intracellular vesicles, suggesting that internalization is dependent on the caveolar pathway.

Since the endocytic pathway leading to cholera toxin internalization originates from cell membrane lipid rafts, the sensitivity of DPV containing endosomes to Triton X-100 treatment was tested. As shown in Fig. 42 A, treatment with Triton X-100 completely solubilized the endosomes containing transferrin-TRITC whilst leaving the DPV-EGFP and cholera toxin B containing vesicles unaffected. To further confirm the involvement of caveolar endocytosis, HeLa cells were treated with the cholesterol-binding agent MβCD, which disrupts lipid rafts and impairs internalization via this pathway. As shown in Fig. 42 B, following cell treatment with MβCD, the cellular internalization of both DPV3-EGFP and DPV15b-EGFP containing endosomes was blocked at the level of the cell surface, in a similar manner to that observed for cholera toxin and Tat, following MβCD treatment.

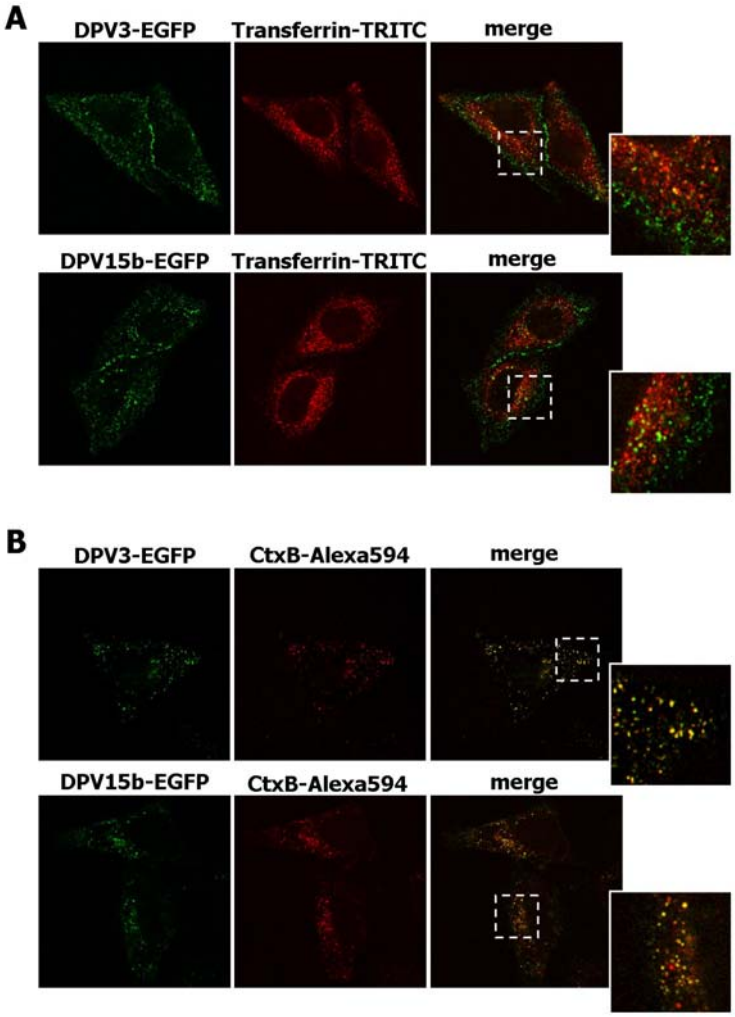


Fig. 41. A, HeLa cells were incubated with DPV3-EGFP (upper panel) or DPV15b-EGFP (lower panel) and transferrin-TRITC for 1 hour, followed by fixation and visualization of the internalized proteins by confocal microscopy. B, HeLa cells were incubated for 1 hour with either DPV3-EGFP (upper panel) or DPV15b-EGFP (lower panel) and the B subunit of cholera toxin (CtxB)-Alexa 594.

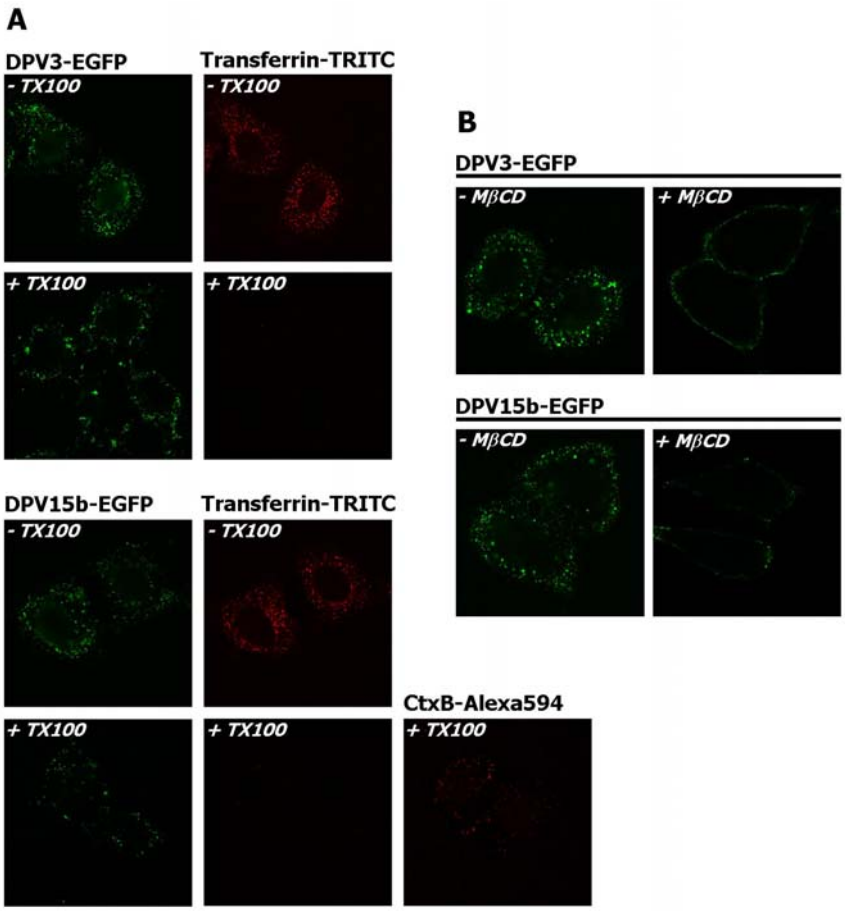


Fig. 42. A, HeLa cells were incubated with DPV3-EGFP or DPV15b-EGFP (upper and lower panels, respectively) and with transferrin-TRITC. After 1 hour incubation, cells were treated with Triton X-100, which was found to selectively solubilize transferrin containing endosomes but not the endosomes containing the Vectocell® peptides. Endosomes containing the CtxB protein (bottom right panel) were equally unsensitive to Triton X-100 solubilization. B, HeLa cells were treated with methyl-β-cyclodextrin (MβCD; panels on the right side) for 30 min before treatment with DPV3-EGFP or DPV15b-EGFP.

DPV1047 originates from the fusion of the heparin-binding domain of apolipoprotein B to the complementary determining region 3 (CDR3) of an anti-DNA antibody. As shown in Fig. 34 and 35, low levels of internalization were observed in attached cells for this Vectocell® peptide compared to the other internalizing peptides. In contrast, as shown in Fig. 43 A and B, internalization was high in suspension cells (K562), in which both DPV3 and Tat11 were largely ineffective. The molecular mechanism of DPV1047 internalization was therefore characterized further in K562 cells. The process was found to be dependent on temperature and to be inhibited by heparin with a dose-response curve not dissimilar to that of Tat entry in other cell types (the concentration of heparin at which 50% of entry occurred was 6.4 μg/ml; Fig. 43 C) (Tyagi et al., 2001). This result most probably indicates that the positive charges of DPV1047 are required for internalization. However, one peculiarity of K562 cells is that they only display very low levels of HSPGs at their cell surface (Zhang et al., 2001), thus rendering the possibility that internalization involves HSPG binding unlikely (this observation also accounts for the low efficiency of both DPV3 and Tat11 in these cells). Finally, the effect of cell incubation with poly-L-Lysine, which masks the negative charges on the cell surface, was investigated. As shown in Fig. 43 D, the uptake of DPV1047 was only partially inhibited even when using high concentrations of the polymer. Taken together, these results indicate that DPV1047 has a distinct mechanism of internalization from other commonly known cell penetrating peptides since it is internalized by an energy dependent mechanism that is independent of HSPGs expressed at the cell surface.

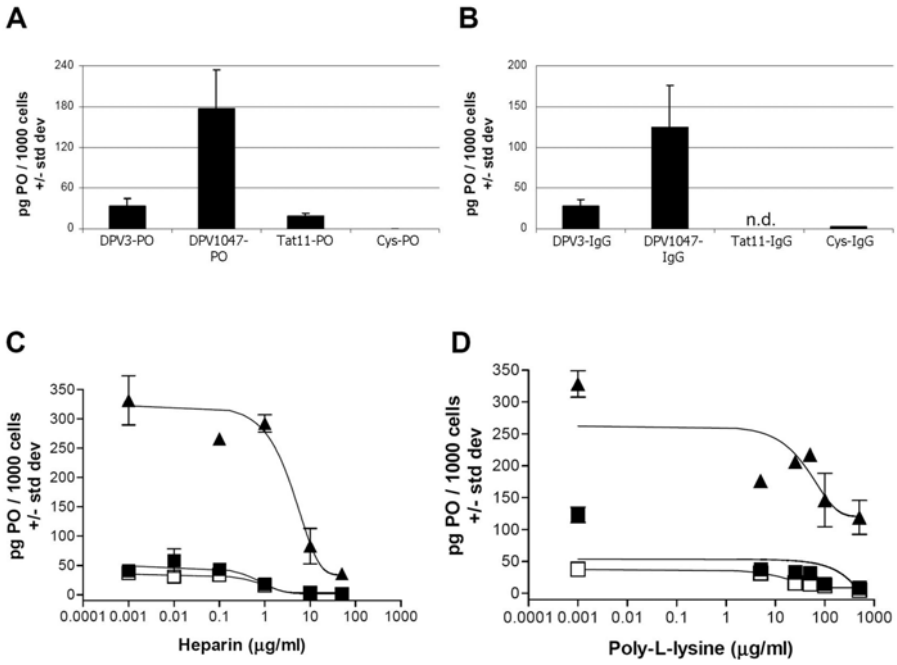


Fig. 43. A and B, K562 cells were incubated for 4 hours with DPV/Tat-PO (A) or DPV-IgG (B) conjugates. Intracellular material was quantified following trypsin treatment and lysis of the cells. Data show mean values and S.D. from 3 independent experiments each performed in duplicate. C and D: DPV3-PO (■), DPV1047-PO (▲) and Tat-PO (□) were incubated at 25 µg/ml in the presence of increasing concentrations of heparin (C) or poly-L-Lysine (D). Values are from 1 representative experiment performed in duplicate out of 3.

Chapter 4

Discussion

DISCUSSION

Due to its hydrophobic nature, the cell plasma membrane acts as a selective barrier that physiologically precludes the passage of hydrophilic proteins. This barrier also poses a limit to the use of pharmacologically active macromolecules that are not actively translocated inside the cells. For some time it has been recognized that several toxins of bacterial and plant origin are capable of translocating across cellular membranes to exert their effects in the cytosol or in the nucleus. In most cases, the exploitation of these proteins for the intracellular delivery of other, heterologous proteins has been hampered by their toxicity, large size and relatively low efficiency. Therefore, the appreciation that a very short amino acid stretch from the basic domain of the HIV-1 Tat could confer translocating capacity even to very large protein cargos has been met with great enthusiasm and has permitted the development of a number of interesting applications *in vitro* and *in vivo*.

Mechanism of Tat internalization

The work described in this thesis indicates that the cell uptake of large protein cargos fused to both full-length Tat or to its 11 amino acid transduction domain occurs through a temperature-dependent endocytic pathway that originates from cell membrane lipid rafts and follows caveolar endocytosis (Fittipaldi et al., 2003). This conclusion is supported by the following observations:

i) The internalization of Tat requires energy (since it does not occur at 4°C) and is slow when compared to transferrin: the amount of internalized Tat reaches a plateau only at several hours after addition to the cell culture supernatant, while the uptake of transferrin is complete in few minutes.

ii) Tat co-localizes in endosomes with the B subunit of cholera toxin and with caveolin, but not with transferrin or other markers of the clathrin-mediated endocytosis, such as the early endosome antigen-1 (EEA-1); furthermore, the

internalized Tat is never found in association with markers of the lysosome compartment.

iii) The endosomes containing Tat are resistant to the nonionic detergent Triton X-100, an observation indicating that their membrane emanates from lipid raft domains.

iv) The depletion of cholesterol, an essential component of lipid rafts, with methyl-β-cyclodextrin, disrupts lipid rafts and impairs Tat entry and its nuclear translocation.

v) The slow movement of the Tat endosomes is dependent on the integrity of the actin cytoskeleton, an observation that is consistent with the finding that caveolae are connected with the actin cytoskeleton (Stahlhut and van Deurs, 2000) and that cytochalasin D – a drug that disrupts the actin filaments – determines the clustering of caveolin and Tat on the cell surface (Thomsen et al., 2002); in contrast, microtubule disruption has no effect on Tat uptake.

Taken together, all the above mentioned results data clearly indicate that the internalization of Tat fusion proteins occurs through a process of caveolar endocytosis that emanates from cell membrane lipid rafts, and not by clathrin-mediated endocytosis (reviewed in (Fittipaldi and Giacca, 2005)).

While caveolar endocytosis undoubtedly appears to be the route of internalization of large particles fused to Tat (also including a recombinant λ phage particle displaying the Tat transduction domain on its surface (Eguchi et al., 2001)), much less clear is whether small peptides encompassing the Tat transduction domain might also enter the cells by alternative pathways. Evidence has been provided to show that short basic peptides - including the Tat basic domain - can directly cross cell membranes by a mechanism that is insensitive to typical endocytosis and metabolic inhibitors (Suzuki et al., 2002; Vives et al., 1997). When the Tat86-EGFP protein was compared to a fluorescent peptide encompassing the Tat basic domain by simultaneously adding the two molecules to the cell culture medium, it was observed that the peptide entered the cells at 4°C while the larger recombinant protein did not (Silhol et al., 2002). However, recent experimental evidence seems to indicate that the notion that the Tat

basic domain can directly cross the cell membrane might be the consequence of artifacts due to post-fixation diffusion or poor removal of extracellularly bound protein (Richard et al., 2003). Thus, an active endocytosis process might also be required for the cellular uptake of short, polybasic peptides, as also originally proposed by Mann and Frankel (Mann and Frankel, 1991). The identification of caveolar endocytosis as the cellular entry pathway of Tat fusion proteins will now permit this issue to be readdressed more specifically. Finally, it is also worth mentioning that other mechanism of internalization, such as macropinocytosis, might also operate in specific cell types besides caveolar endocytosis (Wadia et al., 2004).

It was recently observed that Tat internalization requires binding of the protein to cell surface HSPGs, since the uptake process does not occur in cells selectively impaired in HSPG biosynthesis and can be abolished by cell treatment with heparinase III or by competition with soluble, extracellular heparin (Rusnati et al., 1999; Tyagi et al., 2001). What might the connection between HSPGs, acting as membrane receptors for extracellular Tat and caveolar endocytosis be? One of the two families of cell surface HSPGs are the glypicans, which lack a membrane-spanning domain and are anchored to the external surface of the plasma membrane via glycosylphosphatidylinositol (GPI) (David, 1993). Indeed, GPI-anchored proteins are highly enriched in lipid rafts on the cell surface (Muniz and Riezman, 2000; Nichols et al., 2001). In addition, glypican-1 is known to be internalized and recycled via the Golgi (Fransson et al., 1995), with the internalization process occurring in caveolin-1-containing endosomes (Cheng et al., 2002). Thus, a testable prediction is that the internalization of Tat might occur following its interaction with the sugar moiety of this HSPG.

In this respect, it is of interest noting that the internalization process in $CD4^+$ T-lymphocytes expressing CXCR4 – a chemokine receptor that specifically binds extracellular Tat (Ghezzi et al., 2000; Xiao et al., 2000) and mainly resides outside of lipid rafts (Kozak et al., 2002; Nguyen and Taub, 2002; Popik et al., 2002) – is also severely inhibited by cholesterol depletion, indistinguishable from CXCR4-negative cells. This observation indicates that either this receptor is not

significantly involved in Tat internalization, or that its recruitment to lipid rafts is anyhow required for the internalization process to occur, similar to HIV-1 entry.

Intracellular fate of internalized Tat

Several extracellular macromolecules, including bacterial toxins and viruses, are internalized via caveolar endocytosis. Different studies have demonstrated that the dynamics of this pathway are relatively slow when compared to clathrin-dependent endocytosis (Thomsen et al., 2002; Wacker et al., 1997). This conclusion is in perfect agreement with the observation made in living cells that Tat-containing endosomes are relatively immobile as compared to transferrin endosomes when analyzed in a ten-second time scale. Consistent with these conclusions, complementary biophysical work performed on living, unfixed cells has shown that caveosomes containing an average of ~300 molecules of the Tat86-EGFP molecule proceed from the cell membrane toward the perinuclear region with an average velocity of 3 μm/h, and that this translocation process requires the integrity of the actin cytoskeleton (Ferrari et al., 2003). As shown in Fig. 27, treatment with cytochalasin D determined the aggregation of Tat-EGFP endosomes in large clusters at the cell periphery. This observation is in perfect agreement with the notion that caveolae are connected with the actin cytoskeleton (Stahlhut and van Deurs, 2000) and that treatment with cytochalasin D leads to clustering of caveolae connected to the plasma membrane (Fujimoto et al., 1995; Thomsen et al., 2002). In contrast, disruption of microtubules had no effect on Tat-EGFP entry.

Extracellular Tat is internalized by the cells and, eventually, it ends up in the nucleus in a transcriptionally active form. One formal possibility existed that the pathway leading to caveolar endocytosis of the protein might be different from the one leading to the presence of the protein in the nucleus. This is clearly not the case, since all the drugs and treatments that inhibited lipid raft endocytosis also impaired the transcriptional activity of the protein. This observation excludes that there is an alternative pathway for extracellular Tat uptake. In

addition, it also implies that the internalized Tat eventually has to egress the endosomes in order to be transported to the nucleus.

The exit from the endocytic compartment is a molecular process that is likely to require translocation through a cellular membrane. In this respect, it is worth noting that, in the experiments with the Tat fusions reported, at no time EGFP fluorescence was observed in the nucleus. However, the protein was transactivating the LTR and could be evidenced by staining with an anti-GFP antibody (Fig. 44). Most probably, this observation implies that the Tat-EGFP fusion proteins unfold during membrane translocation, as commonly occurs in this process; once unfolded, an intrinsic property of GFP is to re-fold in an optically active form only at very low efficiency (Sacchetti et al., 2001). This consideration also implies that the use of the EGFP reporter tag as the sole method by which to study the release of Tat from the cells (another process possibly requiring crossing of a cell membrane) - as recently described (Leifert et al., 2002) - might possibly bring to misleading conclusions.

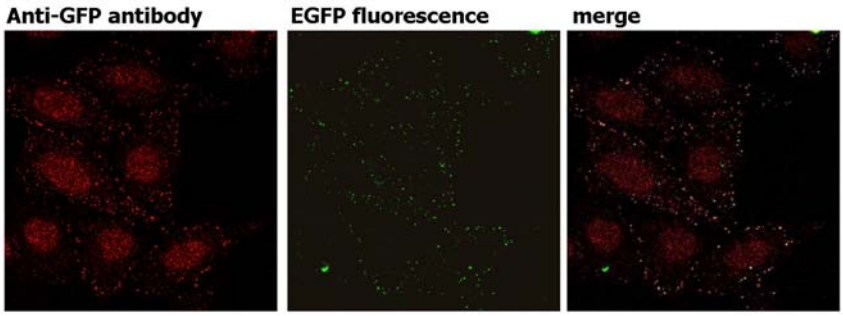


Fig. 44. HeLa cells were treated with 1 μg/ml GST-Tat86-EGFP. After 8 hours, cells were fixed, reacted with anti-GFP antibody followed by a secondary antibody labeled with TRITC, and visualized for EGFP and TRITC fluorescence. The antibody stains EGFP both in endosomes and in the nucleus; EGFP fluorescence is only visible in endosomes.

Bacterial and plant toxins such as cholera toxin and ricin translocate to the cytosol after retrograde transport through the Golgi apparatus and the endoplasmic reticulum (Majoul et al., 1996; Rapak et al., 1997); consistent with these findings, disruption of Golgi trafficking with brefeldin A (Fig. 45) inhibits toxin action (Orlandi et al., 1993; Simpson et al., 1996). Of possible interest in this respect, is the observation that treatment with brefeldin A did not affect entry of the Tat-EGFP recombinant protein into the cells, as observed by flow cytometry, while it did impair its nuclear translocation, as shown by the LTR transactivation data. This observation indirectly implies that the Golgi transport might be required for this process. Despite this suggestion, I have so far failed to visualize Tat in the Golgi or in the endoplasmic reticulum, possibly indicating that the translocation process only occurs in a minority of the internalized molecules, as is also the case for ricin (Rapak et al., 1997). Experiments aimed at identifying specific molecular modification of proteins transiting through the Golgi will directly permit to address this issue.

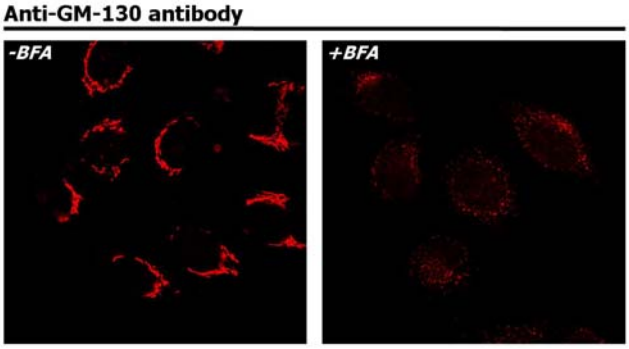


Fig. 45. To stain Golgi apparatus, HeLa cells were incubated with an antibody against GM-130 protein. Note the disruption of Golgi after brefeldin A (BFA) treatment.

What is the role of extracellular Tat?

Tat is released by infected cells and might play a crucial role in several pathological processes which contribute to nonimmune and immune dysfunction during HIV infection and AIDS, including disregulation of angiogenesis, neurodegeneration and impairment of the immune response. In addition Tat trafficking may also lead to association of Tat on the external surface of HIV envelope (Marchio et al., 2004).

The presence of Tat on the viral surface might have an effect on HIV infectivity and as a consequence, a virus with Tat on its surface should be more infectious than one without Tat. The preliminary data shown in Fig. 33 indicate that the virus produced in the presence of Tat is almost three times more infectious than the virus obtained without Tat. Although I cannot rule out at the moment that Tat might act at different steps in the packaging cells, these results are nevertheless suggestive of a direct role of Tat in increasing virion infectivity. In this respect it is worth mentioning that these retroviral vectors were pseudotyped with the G protein of the vesicular stomatitis virus (VSV-G), which *per se* mediates a highly effective infection of the target cells. Therefore, it would not be surprising if the presence of Tat might be even more relevant in conditions in which other envelope proteins are used.

Should the evidence that Tat might act at the level of virion infectivity be confirmed, this would reinforce its relevance as a novel target for antiviral therapy. In addition, the expression of Tat in the packaging cells might also be exploited to improve viral vector transduction efficiency in gene therapy applications with both onco- and lenti-viruses.

Another intriguing possibility is that the intercellular trafficking properties of Tat might confer still unexplored properties to the HIV virions. Indeed, even if this possibility has not been formally addressed so far, the envelope that surrounds the HIV-1 virions budding out of the infected cells most likely contains cell-derived HSPGs binding to extracellular Tat. What could be the role of this virion-associated Tat? During the physiological events encompassing HIV-1 infection, among the remarkable properties of the virus is its ability to cross the mucosal

and the blood-brain barriers. The former event takes place during primary infection; infection of the brain is one of the still unexplained hallmarks of the disease, occurring at the clinical or sub-clinical level in the majority of patients (Resnick et al., 1988). Indeed, HIV-1 is able to pass through brain microvascular cells using the same pathway as cholera toxin does, which depends on the integrity of lipid rafts, without disruption of the tight junctions and in the absence of productive infection of endothelial cells (Liu et al., 2002). This process resembles transcytosis, the characteristic epithelial transcellular vesicular pathway that occurs through caveolar endocytosis. In a consistent manner, as early as 30 minutes after apical contact, primary virus isolates generated from primary peripheral blood leukocytes from HIV-infected patients were shown to cross an epithelial cell line barrier using transcytosis (Bomsel, 1997).

Taken these considerations together, a possible explanation of the biological function of Tat release is its involvement in HIV-1 transcytosis through the caveloar pathway. In HIV-1-infected cells, Tat is secreted outside the cells following a leaderless secretory pathway that does not involve the classical ER-Golgi-dependent exocytosis and results in the accumulation of Tat on extracellular and cell-bound HSPGs in lipid rafts microdomains. Since the virions that bud from these cells contain Tat on their surface, they can enter a lipid rafts-dependent pathway of caveolar transcytosis, which allows them to cross the epithelial and blood-brain barriers. An indirect support to this model is given by the recent observation that different cell penetrating peptides are able to pass across a Caco-2 epithelial cell monolayer and that this passage is indeed mediated by a transcellular pathway (Lindgren et al., 2004).

Common features of cell penetrating peptides

In the second part of the Results chapter, I described the internalization property of a novel family of cell penetrating peptides, named Vectocell® (or DPVs). These Vectocell® peptides were demonstrated to internalize a variety of molecules in both adherent and suspension cell lines. They have the ability to

efficiently mediate the internalization of molecules from as little as a few Daltons, up to 200 kDa. Moreover, the use of different Vectocell® peptides allows delivery of molecules to either the cell nucleus or cytoplasm. These novel human-derived peptides have low *in vivo* toxicity profiles consistent with their potential use as therapeutic delivery systems, unlike existing carrier peptides (Trehin and Merkle, 2004).

The experiments described here show that the mechanism of internalization of Vectocell® peptides (with the noticeable exception of DPV1047), similar to Tat require their interaction with cell surface heparan sulphate proteoglycans. This conclusion is supported by two observations. Firstly, cell penetration is inhibited by soluble heparin; this occurs due to the heparin sequestering Vectocell® peptides and thus blocking internalization. Secondly, cells defective in HSPG synthesis fail to internalize the peptides. As is the case for receptor-mediated internalization, the mechanism of uptake of the cell surface Vectocell® peptides-HSPG complexes requires an active endocytosis mechanism. Additionally, the internalization of Vectocell® peptides (except for DPV1047) can be inhibited by the use of poly-L-lysine, which masks the negative charge on the surface of the cell. This observation reinforces the notion that binding of these Vectocell® peptides to HSPGs is due to an electrostatic interaction rather than specific receptor recognition.

Following release from the vesicle compartment, Vectocell® peptides allow delivery to both the cytosol (for example DPV3) and the nucleus (for example DPV15b), although the route by which these peptides escape the endosomal compartment is still obscure. In this respect, Fischer and coworkers have recently proposed that the retrograde transport pathway through the Golgi apparatus might be involved in the translocation of several cell penetrating peptides (Fischer et al., 2004) as well as some bacterial and plant toxins. Previous studies on the intracellular localization of peptide-transported molecules have been misleading due to artefactual effects of the fixation methods used (Lundberg and Johansson, 2001; Lundberg and Johansson, 2002; Richard et al., 2003). In the present study erroneous artefacts were eliminated by assessing the cellular location of peptide conjugates in living cells by either using the EGFP

fluorescent protein or quantifying a nuclear specific recombination event (Cre-Lox). The capacity to deliver the conjugate either to the nucleus or to the cytosol of the cell is dependent on the peptide and entails molecular mechanisms that have not been yet elucidated. For example, in fixed cells DPV15b is localized in the nucleus when conjugated to either peroxidase or antiperoxidase IgG. Consistent with the nuclear localization, observed in fixed cells, DPV15b is the most efficient DPV in driving LoxP nuclear recombination in living cells. In contrast, DPV3 is always observed to be cytoplasmic.

The internalization of large and small molecules was shown to differ in terms of their kinetics of internalization and temperature dependency. The data obtained are consistent with the notion that highly charged peptides (such as Tat together with DPV3 and DPV15b) have the capacity to directly translocate through the plasma membrane when conjugated to small conjugates, whereas larger conjugates can only enter cells via an energy-dependent endocytic process. These issues have to be considered when developing an effective strategy for the delivery of a given therapeutic molecule.

The properties of DPV1047 are distinct from the others. DPV1047 partially originates from a heparin binding protein (like the other Vectocell® peptides described in this work) but also from an anti-DNA auto-antibody. Unlike the other Vectocell® peptides it does not significantly internalize the reporter molecules assessed (PO, IgG, TMR or EGFP) in HeLa, HCT116 or CHO-K1 adherent cells, but it is the only peptide that is internalized in K562 suspension cells. The fact that K562 cells express only low levels of HSPGs and that poly-L-lysine is only able to partially block DPV1047 internalization, suggests that it is not just the cationic nature of this peptide that is responsible for its cell surface interaction and internalization. The K562 cell line might possess a mechanism of internalization that is caveolar endocytosis independent, which is consistent with the observation that the internalization of SynB1 and SynB3 cell penetrating peptides have been described to occur in these cells (Drin et al., 2003; Mazel et al., 2001).

In conclusion, novel human derived, Vectocell® peptides have been identified that show both enhanced and safe cell penetration characteristics. These new

peptidic sequences could deliver both small and large molecules for therapeutic purposes. Indeed, *in vivo* therapeutic validation of Vectocell® conjugates is currently being undertaken and confirms the therapeutic potential of these peptides.

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