CELLULAR Na^+,K^+-ATPase MEDIATES UNCONVENTIONAL EXPORT OF HIV-1 TAT PROTEIN

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Ph.D. Thesis

In

Molecular Biology

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Scuola Normale Superiore

2009

A Me,

Alla voglia di conoscere e

al piacere di capire

che mi accompagnano

da sempre

SYNOPSIS

The Tat (Trans activator) protein of the human immunodeficiency virus type 1 (HIV-1) is a 101 amino acid polypeptide acting as a powerful transcriptional activator of viral gene expression. The protein binds a highly structured region of nascent RNA transcripts and, from here, directs the assembly of processive transcription complexes and promotes chromatin modification at the HIV-1 LTR (Long Terminal Repeat) promoter. Besides this fundamental role in the control of HIV-1 gene expression, Tat also possesses the unusual property of trafficking in and out of the cells. In particular, the capacity of being internalized by the cells when present in the extracellular compartment, which depends on the integrity of the basic domain of the protein (aa 49-57), has been extensively characterized and constitutes the basis for its biotechnological utilization for the delivery of heterologous proteins, drugs, viral vectors and nanoparticles (Becker-Hapak *et al.* 2001; Tasciotti *et al.* 2003; Fittipaldi and Giacca 2005; Tasciotti and Giacca 2005). Some of the studies that elucidated the molecular mechanisms of extracellular Tat internalization also noticed that cells constitutively expressing Tat release this protein into the cell culture supernatant (Becker-Hapak *et al.* 2001; Tyagi *et al.* 2001; Tasciotti *et al.* 2003; Tasciotti and Giacca 2005). However, the mechanisms underlying extracellular Tat release have so far remained elusive.

The aim of this project was to explore the molecular mechanisms responsible for Tat export from the expressing cells, with the ultimate purpose to understand the significance of such mechanism in the biology of HIV-1 infection and exploit the ensuing information for the improvement of the trafficking properties of Tat-fused protein cargos.

This Thesis shows that:

1) When cells were transfected with expression vectors encoding fusion constructs corresponding to a reporter protein fused to full-length HIV-1 Tat or to an 11 amino acid peptide encompassing its basic domain, a significant fraction of these proteins was found in the cell culture supernatants. Secretion had a rapid kinetics, different from that of proteins secreted through the endoplasmic reticulum (ER)-Golgi route, and was dependent on the integrity of the Tat basic domain.

2) Neither drugs blocking canonical protein secretion nor drugs interfering with intracellular vesicle trafficking blocked extracellular Tat release, indicating that this process occurred through a Golgi-independent, unconventional secretion route.

3) Tat and Tat-fusion protein release was impaired by ouabain. This drug is a specific inhibitor of the cellular Na^+,K^+-ATPase, an enzyme built into the plasma membrane which catalyzes ATP hydrolysis coupled with Na+ and K+ transfer through the membrane against the electrochemical gradient. Ouabain is known to also impair the unconventional secretion of FGF-2 (Fibroblast Growth Factor 2) (Florkiewicz *et al.* 1998; Dahl *et al.* 2000).

4) The Tat basic domain was found to specifically bind the catalytic (alpha) subunit of the Na^+,K^+-ATPase. This interaction appeared essential for extracellular Tat release and was disrupted by ouabain treatment.

5) Interaction between Tat and the Na^+,K^+-ATPase required integrity of the Carboxy-terminal domain of the latter protein. In particular, this region contains three short intracytoplasmic peptide stretches (of 19, 12, and 16 amino acids) that are juxtaposed in the protein three-dimensional structure, which were all required to bind Tat.

6) Intracellular expression of a novel fusion protein corresponding to a linear combination of the three Tat-binding, Na^+,K^+-ATPase peptide regions blocked Tat transactivation.

7) A mixture of soluble synthetic peptides corresponding to the three Tat-binding, Na^+,K^+-ATPase peptide regions blocked Tat release.

8) The same mixture of peptides, when added to CD4+ T-cells infected with a wild type strain (HIV-1_{BRU}) impaired HIV-1 infection.

Taken together, these results shed light on the molecular mechanism responsible for extracellular Tat release, disclose the importance of inhibiting this process in the viral life cycle and identify a novel tool for pharmacological and/or gene-based therapy of HIV-1 infection.

Chapter 1

Introduction

INTRODUCTION

HIV life cycle

The Human Immunodeficiency Virus type 1 and 2 (HIV-1 and HIV-2) belongs to the Lentiviral genus of *Retroviridae*, and is the etiological agents of the Acquired Immunodeficiency Syndrome (AIDS). HIV establishes a persistent infection by means of provirus integration in the host genome and rapid mutation of viral genes encoding for proteins eliciting host immune response. Even in presence of effective antiviral treatment and strong immune response, however, the ability of the virus to establish a latent infection during the early stages of the disease grants the persistence of the virus in the host organism.

The HIV genome is 10 kilobases long and encodes a number of structural proteins as well as several regulatory and accessory proteins which are not found in gamma- and alpha-retroviruses (**Fig. 1.1**); consequently, HIV replication cycle is far more complex, showing many regulatory mechanisms and strategies aimed at viral persistence.

Viral replication requires the transcription of the proviral genome and the synthesis of several proteins necessary for the assembly and the budding of the viral progeny. HIV transcription depends upon the interaction between a number of cellular transcription factors and co-activators with regulatory sequences contained in the viral Long Terminal Repeat (LTR) promoter; such interactions lead to the assembly of a stable transcription complex stimulating several transcription rounds by RNA polymerase II.

The structural genes, *gag*, *pol* and *env*, common to all other retroviruses, encode the proteins necessary for virion assembly, as well as the enzymes required for genome replication, proviral integration and polyprotein cleavage. The *gag*, *pol* and *env* genes are transcribed into polyproteins that are subsequently processed by viral (Gag and Pol) or cellular (Env)

proteases. The *gag* gene encodes the core proteins (Capsid CA, Nucleocapsid NC and p6) and the Matrix protein (MA), while the *env* gene encodes the glycoproteins gp120 and gp41, and the *pol* gene encodes three essential enzymes for viral replication: Reverse Transcriptase (RT), Integrase (IN) and Protease (PR).

In addition to these structural genes, the HIV genome expresses several accessory and regulatory proteins: Vif (Viral infectivity Factor), Vpr (Viral Protein R), Vpu (Viral Protein U) (HIV-1), Vpx (Viral Protein X) (HIV-2), Tat, Rev and Nef (Negative Factor).

Fig. 1.1: HIV-1 genome

HIV has been reported to infect a broad range of cellular types *in vitro*; however, *in vivo*, infection is restricted to $CD4^+$ T-lymphocytes and cells of the monocyte-macrophage lineage.

Infection starts with the fusion between HIV envelope and the cell plasma membrane; the surface gp120 protein on the viral envelope binds the CD4 receptor on the host cell surface, inducing conformational changes and promoting the binding to chemokine receptors that serve as co-receptors for HIV-1 infection (Kwong *et al.* 1998). These co-receptors are both critical for virus entry, and crucial for determining the tropism among $CD4^+$ cell types. HIV-1 uses for its infection two chemokine receptors: CCR5, which binds macrophage-tropic (R5) viruses, and CXCR4, which binds T-cell tropic (X4) viruses. While R5 viruses are responsible of mucosal and intravenous transmission of HIV-1, X4 isolates appear only in the late stages of infection, with the appearance of the immunodeficiency (Scarlatti *et al.* 1997) .

Viral gp120 is bound to gp41, a transmembrane protein which assembles as a trimer on the virion envelope. The interaction between gp120, CD4 and the co-receptor induces a conformational change in gp41, leading to the exposition of three peptide fusion domains. These domains are able to spear the plasma membrane and promote the fusion of the viral envelope, leading to the release of the HIV core in the host cytoplasm (Chan and Kim 1998).

Upon entry, the virus undergoes the uncoating process, which generates the viral reverse transcription complex, composed by the viral genome, RT, IN, MA, NC, Vpr and other host proteins (Karageorgos *et al.* 1993); MA protein is then phosphorylated and interacts with actin microfilaments (Bukrinskaya *et al.* 1998), while Vpr serves to stabilize the reverse transcription complex (Ohagen and Gabuzda 2000).

The completion of the reverse transcription leads to the formation of the Pre-integration complex (PIC), which is composed of viral cDNA, IN, MA, Vpr and RT (Miller *et al.* 1997). As HIV, unlike classical retroviruses, is able to infect non-dividing cells, such as differentiated macrophages, the PIC needs to be translocated in the nucleus through an intact nuclear envelope. Viral proteins Vpr, IN and MA possess in their sequence a nuclear localization signal, and are all involved in mediating nuclear import of the PIC (Bukrinsky *et al.* 1993; Heinzinger *et al.* 1994; Gallay *et al.* 1997); however, whether these proteins act in a cooperative manner, or have individual roles in different target cells is still unclear.

Inside the nucleus, IN mediates the integration of the viral DNA in the cellular genome. IN, as well as other proteins involved in PIC formation, binds specific sequences at the end of the viral cDNA, named "att sites", and removes two nucleotides left by the terminal transferase activity of RT. No primary sequence of the cellular DNA has been shown as a preferential target for IN and integration seems to occur at random in the host genome.

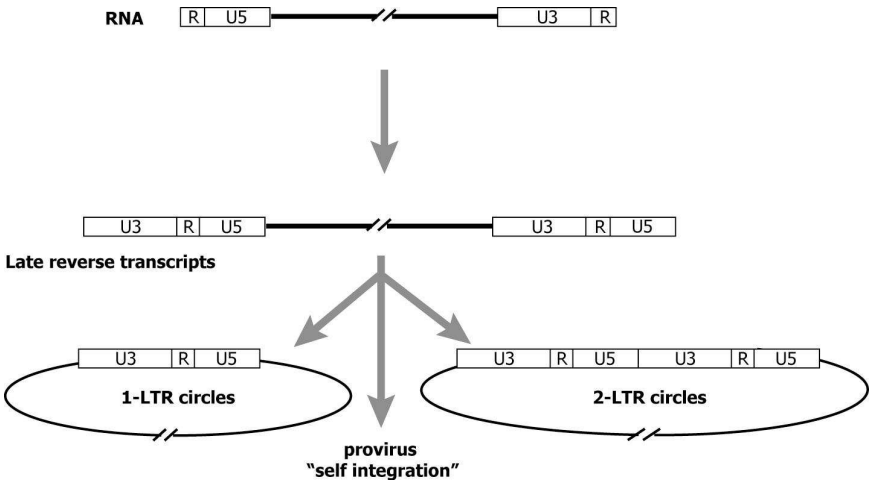

Fig. 1.2: scheme of the different fates of the viral DNA after retrotranscription (Butler *et al.* 2001)

Other than integration, the viral DNA may follow three different fates, neither leading to the formation of a functional provirus (**Fig. 1.2**). The viral ends may join to form a 2-LTR ring, or the viral genome may undergo homologous recombination, producing a single LTR ring, or the viral DNA may integrate into itself, forming a rearranged circular structure. All these circular structures are non infectious, even if some of them are transcribed and produce Tat and Nef proteins (Wu and Marsh 2001).

Eukaryotic DNA inside cells is assembled into nucleosomes to form chromatin, which can be found in at least two different functional forms: a condensed form, named heterochromatin, generally lacking transcriptional activity, and a decondensed form (euchromatin) that provides the necessary environment for DNA regulatory processes such as transcription. As HIV integrates randomly in the host cell genome, the provirus can be found in chromatin domains with different condensation states; integration in heterochromatic regions may lead to latent infections, while viral integration in euchromatin may lead to transcriptionally active form of infection (Adams *et al.* 1994).

The 5' LTR of the integrated provirus acts as the viral promoter, as it contains the binding sites for several positive transcription factors; in the absence of the viral Tat protein, however, the binding of such factors is not sufficient to promote the active transcription of viral genes. The binding of transcription factors to the promoter elements results in the correct positioning of RNA polymerase II complex at the initiation site and in the assembly of a pre-initiation complex. At this point transcription starts in a non-processive way, as the polymerase produces mostly short, non polyadenylated RNAs containing at the 5' end a hairpin structure, named trans-activation-responsive region (TAR). Tat is a powerful trans-activator of provirus transcription, acting by binding the TAR region and promoting the production of poly-adenylated, full-length RNA viral genome.

Tat-activated transcription gives birth to different transcripts derived from the splicing of the viral genome; the first transcripts to appear after infection are completely spliced and are exported into the cytoplasm following the fate of cellular mRNAs (Cullen 1998). These are the shortest viral mRNAs, and encode for Tat, Rev and Nef proteins. Incompletely spliced RNAs cannot be exported from the nucleus as they are blocked by the cellular machinery controlling the integrity of splicing processes; unspliced and single spliced transcripts persist in the nucleus due to the presence of defective splice sites, and to the inhibitory effected exerted by Rev on the splicing process (Luo *et al.* 1994; Powell *et al.* 1997). Longer transcripts encode for Gag, Pol and Env proteins, as well as they constitute the viral genome; thus, they are necessary for the production of a viral progeny. The export of these transcripts into the cytoplasm depends on the expression of the Rev protein (Pomerantz *et al.* 1992), which is able to shuttle between the nucleus and the cytoplasm. Rev binds the viral transcripts through the interaction with an RNA stem-loop structure named Rev responsive element (RRE), located in the env gene (Malim *et al.* 1990). Upon binding to the RRE, Env protein assembles in a multimer which associates with the cellular CRM-1 and Ran

proteins. Ran is a small GTP/GDP binding protein, whose GDP binding form is mainly located in the cytoplasm; Ran-GTP, instead, is mainly found in the nucleus. The Rev/CRM-1/Ran-GTP complex associated with the viral RNA interacts with the pore complex allowing nuclear export, coupled to the hydrolysis of Ran-bound GTP (Cullen 1998). By this mechanism, Rev promotes the cytoplasmic translocation of unspliced or single spliced viral RNAs, allowing the synthesis of all required viral proteins.

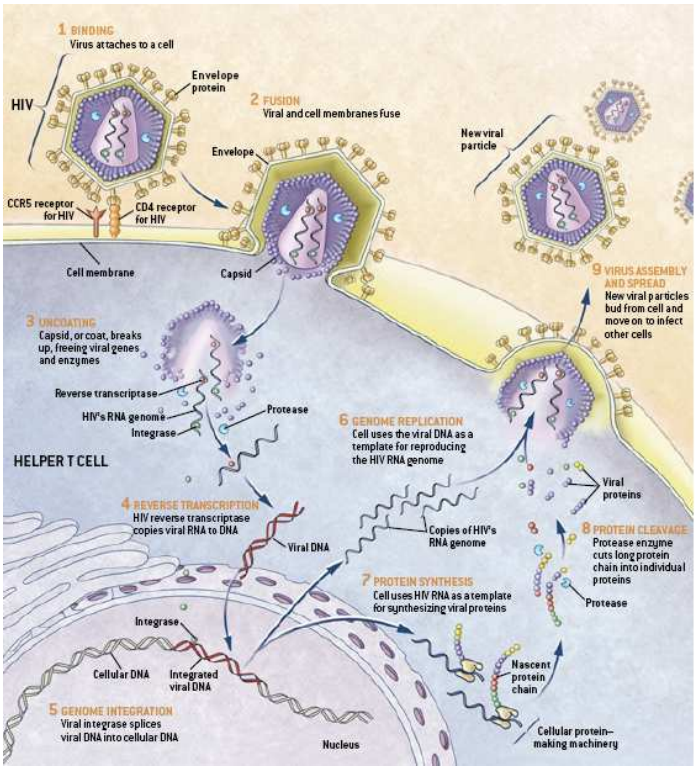

Fig. 1.3: overview of HIV-1 life cycle (reproduced from Quade Paul, Echo Medical Media)

Upon translation, all viral proteins necessary for virion assembly together with RNA genomes are transported to the plasma membrane, close to lipid rafts domain where the building of the viral particles takes place. The gp120/gp41 complex is translocated through the ER-Golgi pathway, while

the Gag-Pol polyproteins are targeted to the membrane by means of myristylation of Gag, resulting in the attachment of the secretory vesicles to the plasma membrane (Gottlinger *et al.* 1989). The resulting virions budding from the membrane are still incomplete, and their maturation is exploited by the viral Protease, which first cleaves Gag-Pol and then, from the Gag and Pol precursors, originates several products: the single core proteins, the MA protein and the viral enzymes. The proteolytic activity ends when the virion is detached from the host cell and results in the formation of mature infectious virions.

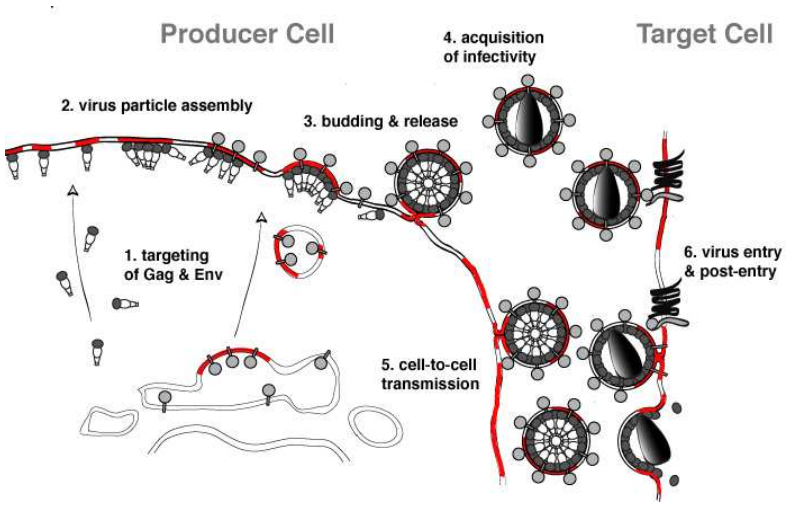

Fig. 1.4: Assembly of the new virions at the cell surface (Ono and Freed 2005)

During the replication cycle, the auxiliary proteins of HIV-1 play a crucial role in regulating the different steps of the intracellular viral pathway.

Nef is encoded by completely spliced transcripts and is targeted to the plasma membrane by myristylation of its N-terminus. The first demonstrated activity of Nef is the down-regulation of CD4 receptor. Even if the real advantage of removing the CD4 receptor is still unclear, this mechanism is

supposed to protect the infected cells from the immune system and to facilitate the release of the virions. In addition to CD4, Nef has been demonstrated to promote degradation of MHC-I that presents viral epitopes to cytotoxic T lymphocytes (CTLs), thus impairing the CTL-mediated immune response leading to the lysis of HIV infected cells (Collins *et al.* 1998). Vif protein is a small protein of 23 Kda affecting infectivity but not viral particle production. As the protein is encoded by single-spliced viral transcript, its expression is Rev-dependent. Upon expression, the protein accumulates in the cytoplasm and interacts with the plasma membrane through its C-terminal region. While mutant viruses lacking Vif show regular cell-to-cell transmission, they show diminished infectivity. It has been recently demonstrated that Vif promotes the degradation of APOBEC3G, a cellular factor involved in the RNA editing process (Sheehy *et al.* 2002); in the absence of Vif, this factor is packaged into virions and introduces point mutations in the viral RNA, thus impairing virion infectivity.

Vpr is expressed at later stages of the life cycle and is packaged in all HIV-1 virions by the p6 core protein. Vpr is incorporated in the PIC complex and contributes to its nuclear import, thus promoting HIV-1 replication in non-dividing cells (Popov *et al.* 1998). Moreover, Vpr induces cell cycle arrest in the G2 phase, by inhibiting the p34-cyclin B kinase activity (Re *et al.* 1995). As the LTR is more active in the G2 phase, viral transcription is enhanced in cells blocked by the action of Vpr.

Vpu is a transmembrane protein found only in HIV-1. like Nef, Vpu exerts CD4 downregulating activity, promoting its ubiquitination and subsequent proteasomal degradation (Margottin *et al.* 1998). In addition, Vpu enhances virus release inducing the budding and the detachment of virions from the plasma membrane.

For more detailed insights into other properties of the HIV-1 accessory proteins, see below.

Role of intercellular protein trafficking in the biology of complex retroviruses

The relatively simple life cycle delineated above is not a fully adequate description of the replication cycle of all the members of the Retroviridae family of viruses. Retroviruses belonging to the complex retroviruses category, which includes all lentiviruses and spumaviruses, as well as HTLV-I (Human T-cell Leukaemia Virus) and related viruses, are distinguished from MLV (Murine Leukaemia Virus) and other simple retroviruses by a number of characteristics. These include the peculiar genomic characterization with the presence of accessory genes, the complex pattern of splicing of the mRNA of genomic length and the special regulation of both transcription and splicing. In addition, the accessory proteins of these viruses exert a number of effects on the infected cells, ranging from modulation of the immune response to inhibition of apoptosis (Cullen 1991a; Cullen 1991c).

While the biological activity of regulatory proteins in complex retroviruses in terms of transcriptional regulation and/or modulation of viral functions has been widely dissected and understood, only in recent years the focus has shifted to the notion that several of these proteins (namely, HIV-1 Tat, Vpr and Nef, HTLV-1 Tax and HFV -Human Foamy Virus- Bet) can be detected in the extracellular environment, and could be responsible of a number of pleiotropic effects, often observed during the time course of the infection, and not directly related to the cytopathic effects of the viruses.

The notion that a protein could have distinct functions inside and outside the cell membrane is not new, and has been recently proposed that this duality of roles could be part of a general regulation of complex functions, such as organ development or tissue homeostasis (Radisky *et al.* 2009).

The biological significance of intercellular trafficking of these proteins is still unclear. However, this characteristic elicits interest, since it might be variably involved in the mechanisms of viral pathogenesis.

The main features of the retroviral proteins showing the capacity of intercellular trafficking are summarized in the following Sections.

HIV-1 Tat

Similar to most animal DNA viruses, upon infection of the host cell, HIV-1 needs to carry out transcription of viral genes using the cellular transcriptional apparatus as well as cellular transcription factors and co-activators.

The Tat proteins of HIV-1 and HIV-2 serve as powerful transcriptional activators of viral transcription; Tat is a small protein of 86-101 aminoacids (the widely used HXB2 strain contains a point mutations thereby stopping at aminoacid 86) (Neuveut and Jeang 1996; Jeang *et al.* 1999); the coding sequence is encoded by two exons. The expression of Tat is essential for the transcription of viral genes and thereby for viral replication.

So far no crystal structure of the protein has been produced; NMR (Nuclear Magnetic Resonance) spectroscopy (Bayer *et al.* 1995) and structural prediction point out a highly flexible structure and does not indicate any evident secondary structure. Five different domains have been identified, based on the amino acid distribution in the protein sequence, and on conservation studies comparing homologous proteins from other lentiviruses (**Fig. 1.5**). In the first exon (aa 1-72) there are an N-terminal acidic domain (aa 1-21), a cystein-rich domain (aa 22-37), a core region (aa 38-48) and a basic domain, highly conserved and rich in Arginine and Lysine residues (aa 49-57). The second exon starts at position 73 and has a variable sequence among different strains. The minimal LTR transactivating domain is fully contained in the first exon.

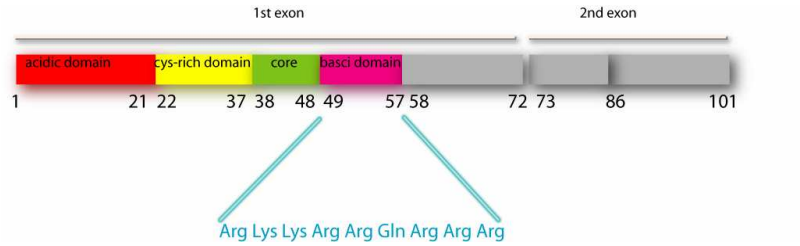

Fig. 1.5: HIV-1 tat protein domains (the sequence of the basic domain is highlighted)

Tat- mediated transcriptional activation

Following reverse-transcription and integration into the host genome, the HIV-1 proviral sequence is organized in a chromatin structure which is repressive of viral transcription (Marzio and Giacca 1999; He *et al.* 2002); such repression is relieved by a number of extracellular stimuli leading to cellular activation. Tat transactivation of viral gene expression is a critical step in the life cycle of the virus.

In spite of continuous HIV-1 replication in all phases of disease, the activity of the LTR promoter at the single cell level is strictly regulated and significantly correlated with the level of host cell activation. The expression of a sufficient amount of the Tat protein leads to a very strong activation of the LTR, making this sequence a highly efficient promoter of viral expression. At the 5' end of all viral mRNAs, Tat interacts with TAR, a region comprising nucleotides 1-60 (considering 1 the first nucleotide of the transcribed RNA) (Berkhout *et al.* 1989; Cullen 1990). The Tat-TAR interaction occurs between the basic domain of the protein and three nucleotides that form a bulge near the apex of the TAR stem (Rana and Jeang 1999). The control of transcription is obtained through the concerted action of several cellular transcription factors, bound to regulatory sequences in the LTR. The main function of Tat is to induce a chromatin modification at the LTR promoter, and to mediate the recruitment of an elongation-competent RNA polymerase II.

A number of cellular proteins have been reported to interact with Tat and to modulate its transcriptional activity. These proteins include general transcription factors (TATA binding protein -TBP-, TAFII250, TFIIB, TFIIH) (Kashanchi *et al.* 1994; Veschambre *et al.* 1995; Parada and Roeder 1996; Veschambre *et al.* 1997; Weissman *et al.* 1998), RNA polymerase II (Wu-Baer *et al.* 1995), Sp1 (Jeang *et al.* 1993), the cyclin subunit of positive transcription elongation factor (PTEFB) cyclinT1 (Wei *et al.* 1998), and several different co-activators possessing histone acetyl-transferase (HAT) activity.

In particular, Tat activates HIV transcription from the LTR through at least two different mechanisms (Marcello *et al.* 2001).

The first one involves cellular HATs and their recruitment at the level of the LTR promoter; here they can relieve the repression induced by chromatin conformation on the HIV promoter (Verdin 1991; Verdin *et al.* 1993). Such repressive activity and the consequent viral latency have been ascribed to the positioning of nucleosomes in the promoter region. Whichever the site of integration is, nucleosomes in the 5' LTR are positioned with respect of the *cis*-acting regulatory elements, and define two nucleosome-free regions, encompassing nucleotides -256 to -3 and +141 to +256 (Van Lint *et al.* 1996); these regions are separated by a nucleosome named nuc-1 blocking efficient transcription in the silent provirus. Nuc-1 is rapidly and efficiently disrupted during transcriptional activation, leaving a large open chromatin region (**Fig. 1.6**).

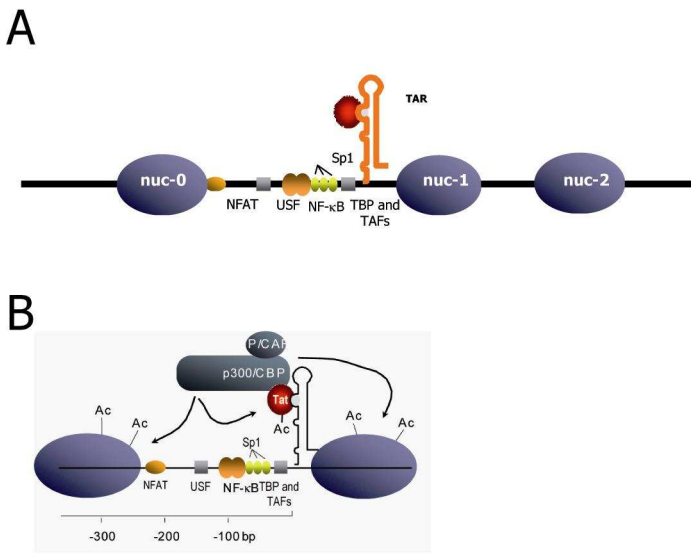

Fig. 1.6: Tat recruits HATs thus determining the acetylation of the N-terminal tails of histone 3 and 4
(**A**) Nuc-1 blocking efficient transcription in the silent provirus
(**B**) Nuc-1 is rapidly and efficiently disrupted during transcriptional activation

The positioning of nuc-1 on the transcription start site, and its disruption during transcriptional activation show the critical role played by chromatin modification in viral latency and the subsequent switch to active viral replication.

Complexes containing HATs mitigate nucleosomal repression at the level of specific promoters by means of acetylation of the N-terminal tails of histones, thus inducing destabilization of histone-DNA interactions. The HAT proteins involved in the TAR-dependent Tat transactivation include p300 and the 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-derepresssible-5 (GCN-5) (Col *et al.* 2001), the TIP60 protein (Kamine *et al.* 1996), and the general transcription factor TAFII250 (Weissman *et al.* 1998). As a consequence of Tat-mediated recruitment of HATs at the level of the viral promoter, LTR-proximal

nucleosomes are acetylated and viral expression is activated (Lusic *et al.* 2003).

The second mechanism mediating Tat-induced activation is the interaction with human cyclin T1 (Wei *et al.* 1998), the cyclin subunit of CDK9 (Cyclin Dependent Kinase 9) in PTEFB. A homologous of this complex was originally identified in D. melanogaster as a kinase necessary for the transcription of several genes (Marshall and Price 1992).

HIV-1 LTR contains several binding sites for a number of transcription factors (such as AP-1 (activator protein 1), COUP-TF (Chicken ovalbumin upstream promoter transcription factor), Ets, LEF-1 (lymphoid enhancer binding factor 1) NFAT (Nuclear factor of activated T-cell 1), Rel/NFkB, Sp1, USF (upstream stimulatory factor) and TFIID). However, in the absence of Tat expression, there is no viral expression and the LTR promoter produces only short, non polyadenylated RNA comprising the TAR structure (Cullen 1991b). DNA footprinting experiment have demonstrated that such DNA sites are bound to cellular transcription factors in both silent and activated HIV-1 infected cells (Demarchi *et al.* 1993), suggesting a mechanism of transcriptional regulation going beyond chromatin accessibility to transcription factors. The inability to produce full length mRNA is due to the lack of processivity of the hypo-phosphorilated RNA polymerase II. When Tat is expressed, it interacts with both the TAR element and the cyclin T1, thus promoting CDK9-mediated phosphorylation of the RNA polymerase II CTD (Carboxy Terminal Domain) and overcoming the low processivity of the enzyme. The interaction between Tat and cyclin T1 increases the specificity and the affinity of binding of Tat to the TAR element, thus forming a ternary complex which guarantees the presence on the HIV promoter of the kinase activity required for efficient viral transcription (**Fig. 1.7**). Moreover, Tat shifts the equilibrium towards CTD phosphorylation, by inhibiting a CTD phosphatase (Marshall *et al.* 1998).

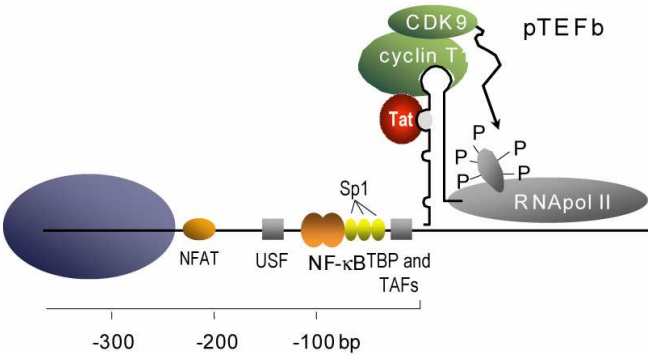

Fig. 1.7: Tat binding to cyclinT1/CDK9 complex promotes the hyperphosphorylation of the RNA polymerase II CTD

PTEFB kinase activity also results in the dissociation of NTEF, negative transcription elongation factor which associates with the CTD impairing the processivity of RNA polymerase II (Fujinaga *et al.* 2004). This complex is found associated with the viral LTR in the early phases of viral transcription (Ping and Rana 2001): one of its components, named DSIF (DRB sensitivity inducing factor) specifically binds the hypophosphorylated CTD, while NELF (negative elongation factor) associates with the TAR region through an RNA recognition domain. When Tat recruits PTEFB at the site of viral transcription, CDK9 phosphorylates DSIF, NELF and the CTD thus relieving NTEF mediated inhibition of RNA polymerase II processivity.

Tat also possesses a direct role in the activation of viral gene expression by modifying the site of CDK9-induced phosphorylation on the CTD of RNA polymerase II (Zhou *et al.* 2000), and stimulating the activity of the mRNA capping enzyme Mce1 (Chiu *et al.* 2002). In the absence of Tat, CDK9 only phosphorylates the CTD on Ser2; when Tat is present, however, CDK9 changes its substrate specificity and phosphorylates the CTD at Serine2 and Serine5, thus promoting polymerase hyperphosphorylation. As the mammalian capping enzyme (Mce1) only binds phosphorylated CTD, this

hyperphosphorylation results in an added stability of Mce1 to the transcription machinery leading to a higher guanylyl transferase activity.
Moreover, Tat binds directly to Mce1 with its C-terminal domain, stimulating its enzymatic activities and promoting its recruitment to the transcription initiation site. Finally, capping promotes efficient splicing and polyadenylation of viral RNAs protecting them from exonucleolytic decay.
The activity of Tat on cellular HATs in not limited to the promotion of their association with the LTR promoter: Tat is also a substrate for acetylation by p300/CBP, PCAF and GCN5 (Kiernan *et al.* 1999; Ott *et al.* 1999; Col *et al.* 2001; Bres *et al.* 2002; Dorr *et al.* 2002; Mujtaba *et al.* 2002). p300/CBP and GCN5 acetylate lysines 50 and 51 within the basic, RNA binding domain, regulating the binding with the TAR structure; pCAF instead acetylates lysine 28 in the activation domain, thus modulating the interaction of Tat with the CDK9/cyclin T1 complex. Mutation in these lysines impairs virus replication.
The effects of acetylation at different lysine residues are various: acetylation of lysine 28 impairs Tat interaction with PCAF, while strengthening the binding with PTEFB; while acetylation of Lys 50 promotes the dissociation from the TAR (Kiernan *et al.* 1999) and creates a new binding site for PCAF (Mujtaba *et al.* 2002).
The proposed model (Bres *et al.* 2002) (Figure 1.8) for the events triggered by Tat at the 5' LTR is the following: non acetylated Tat binds PCAF and is acetylated at lysine 28; following acetylation, Tat dissociates from PCAF and binds PTEFB, and the latter complex interacts with the TAR RNA, leading to the hyperphosphorylation of the CTD of RNA polymerase II, which is associated with the site of initiation. Subsequently, p300 acetylates lysine 50, inducing the dissociation from the TAR and the formation of a TAT/PTEFB/PCAF complex which remains associated with the elongation machinery. GCN5 also acetylates Tat on Lysine 50 and 51, leading to increased activation of the LTR (Col *et al.* 2001).

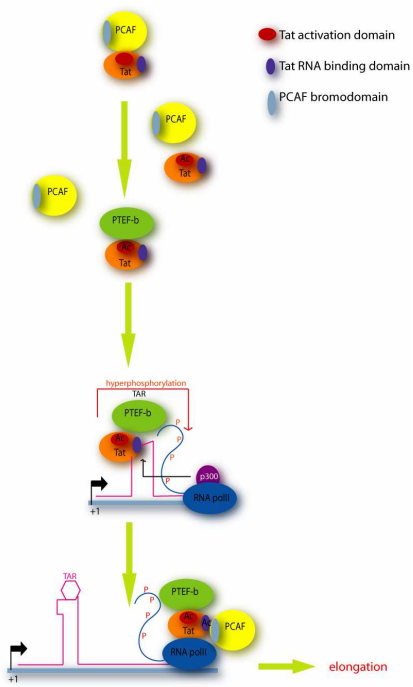

Fig. 1.8: proposed model for the modulation of Tat activity by cellular HATs PCAF and p300 (Bres *et al.* 2002)

Pleiotropic activities of extracellular Tat

Besides being a powerful regulator of viral transcription, Tat also possesses the peculiar property of entering cells when present in the extracellular medium (Frankel and Pabo 1988; Green and Loewenstein 1988). The early experiments were performed by assessing the ability of exogenous Tat to activate an LTR-driven reporter gene, thus implying that not only the protein is able to cross the cell membrane, but also that it is transported in the nucleus in a transcriptionally active form (Frankel and Pabo 1988).

In addition to entering cells when present in the extracellular compartment, that has been demonstrated to be secreted from expressing cells, following an ER-Golgi independent pathway as its sequence lacks an N-terminal signal

peptide necessary for "classical" secretion (Chang *et al.* 1997; Tyagi *et al.* 2001).

Recent findings have suggested that Tat is displayed on the envelope of HIV-1 virions (Marchio *et al.* 2005). Thus, once it is released from HIV-1 infected cells, Tat interacts with heparan sulfate proteoglycans (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 2005) and virion budding occurs through these membrane microdomains, producing viruses with cholesterol-rich membranes (Nguyen and Hildreth 2000; Campbell *et al.* 2001). The presence of Tat on the viral surface might have an effect on HIV infectivity by facilitating virus adsorption with its HSPG binding activity (Nappi *et al.* 2009). Moreover, recent evidence suggest that the presence of Tat bound to extracellular HSPGs of endothelial cells, may promote adhesion and subsequent extravasation of lymphoid cells (Urbinati *et al.* 2009).

The fact that a viral peptide possesses the ability to easily cross cell membranes thus allowing its intercellular trafficking raises several questions about whether this ability could have been evolved to enhance viral infectivity, or to somehow interfere with the host immune response. A reasonable possibility is that HIV-1 evolved this property in order to prime non infected cells for their primary infection, thus creating a tissue environment suitable for viral replication. Several evidences have been reported on the ability of Tat to induce the transcription of cellular genes. In this manner, secreted Tat present in the extracellular environment might exert a number of pleiotropic effects. Indeed, Tat induces the secretion of cytokines (Zauli *et al.* 1992; Lotz *et al.* 1994; Nabell *et al.* 1994; Scala *et al.* 1994; Westendorp *et al.* 1994; Opalenik *et al.* 1995) and their receptors (Pocsik *et al.* 1992; Puri and Aggarwal 1992; Purvis *et al.* 1992), modulates the survival, proliferation and migration of several cell types (Ensoli *et al.*

1990; Ensoli *et al.* 1993; Zauli *et al.* 1993; Lafrenie *et al.* 1997), possesses angiogenic activity both *in vitro* and *in vivo* (Albini *et al.* 1995; Albini *et al.* 1996; Corallini *et al.* 1996), and inhibits antigen-specific lymphocyte proliferation (Viscidi *et al.* 1989; Subramanyam *et al.* 1993).

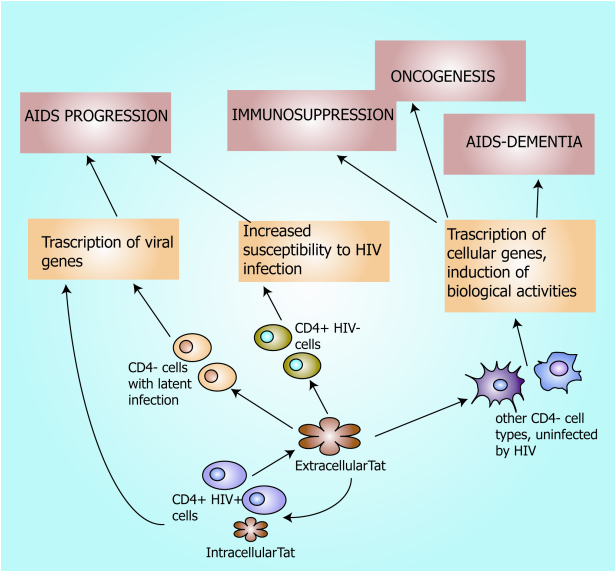

Fig. 1.9: Role of intra- and extracellular Tat in AIDS progression and in AIDS-associated pathologies

Probably some of the above mentioned effects have important implications for the pathogenesis of HIV disease in an autocrine or paracrine fashion, in particular in the neuropathogenic role of HIV in the infection of the central nervous system (Sabatier *et al.* 1991; Taylor *et al.* 1992; Weeks *et al.* 1995). Whether these effects are mediated by the interaction of Tat with cell surface receptors (Vascular endothelial growth factor receptor -VEGFR- 1 and 2, integrin and chemokine receptors above others) and the consequent activation of intracellular signal transduction, or are a consequence of the

transcriptional activation following Tat internalization remains poorly understood.

In addition to the above effects, Tat release might be involved in the induction of immunosuppression during the course of HIV disease.

Neurodegenerative disorders

AIDS-related neurodegenerative disorders are found in one third of adults and half of the children affected with the disease. Productive infection of the central nervous system (CNS) occurs in macrophages and microglia, but is also found in astrocytes. The deterioration of brain tissue (leading do dysfunction of cognition, dementia and eventually paralysis) however is found even in absence of a productive infection in the neuronal tissue, or of malignancies and opportunistic infections (Price *et al.* 1988). The discrepancy between the lack of detectable HIV-1 and the severity of brain damage in infected individuals has led to the hypothesis that damage might due to the release of viral products from HIV-infected cells as macrophages or microglia; such molecules could trigger a pathological alteration of neuronal cells by disrupting the normal pattern of gene expression.

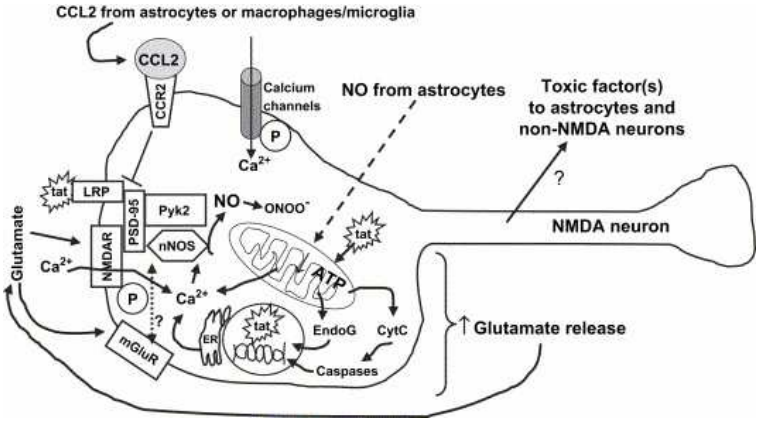

Fig. 1.10: Tat toxic activities on neuronal cells (King *et al.* 2006)

Transforming growth factor-β1 (TGF-β1) gene expression is stimulated in HIV-1 positive individuals (Kekow *et al.* 1990); it has been suggested that this growth factor could act as a chemoactractant, recruiting infected monocytes in the brain, thus enhancing CNS dysfunction, and altering the expression of neurotoxic cytokines. Moreover, monocyte infiltration in the CNS, even in the absence of monocyte infection, is associated with AIDS-induced dementia, probably due to the toxic effect exerted by monocyte-derived molecules.

As the Tat protein, upon secretion from infected cells, can penetrate uninfected cells, it could alter the expression of several genes. For example, its upregulating effect on TGF-β1 (Rasty *et al.* 1996) and MCP-1 (monocyte chemoactractant protein-1) (Conant *et al.* 1998) were demonstrated both in vitro and in the mouse brain, thus suggesting a key role for secreted Tat in monocytic infiltration and in subsequent neurotoxin release.

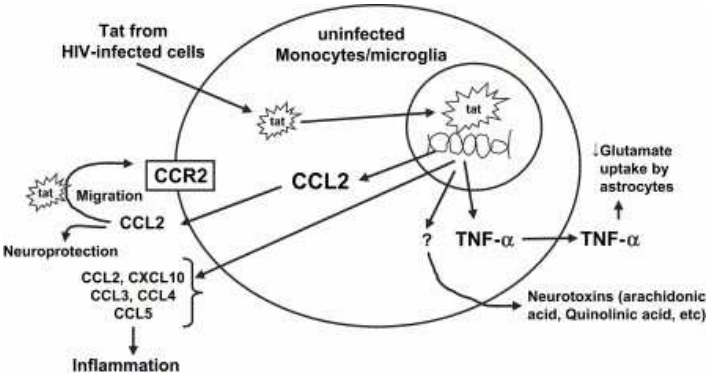

Fig. 1.11: Tat interaction with uninfected monocytes (King *et al.* 2006)

Extracellular Tat also up-regulates CCR5 chemokine receptor on human peripheral blood monocytes, an event capable of enhancing the migratory response and the transmigration of these cells across a tissue model of blood-brain barrier in vitro (Weiss *et al.* 1999). Moreover, Tat is able to

induce the expression and the extracellular release from activated macrophages and astrocytes of Tumor Necrosis Factor-α (TNF-α), the levels of which closely correlate with the severity of dementia (Chen *et al.* 1997; New *et al.* 1998).

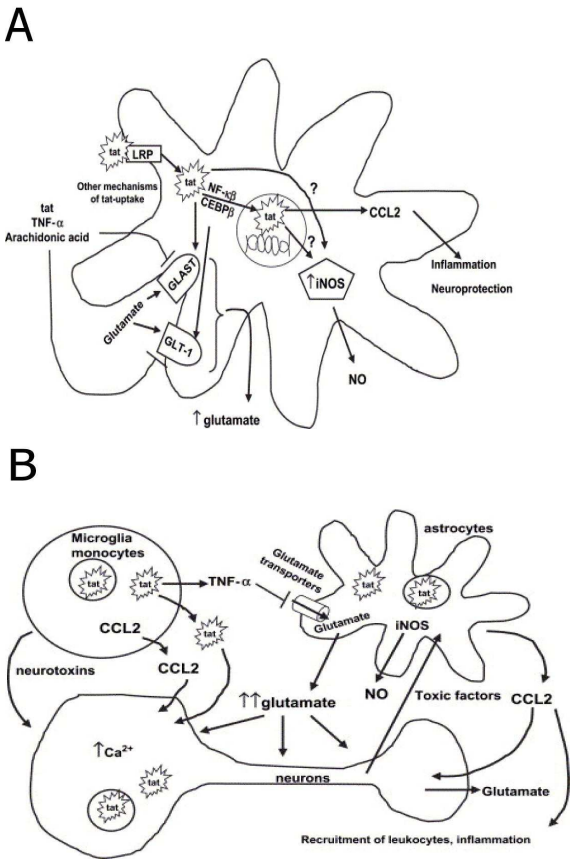

Fig. 1.12: Tat neurotoxic activities
(**A**) Tat interaction with astrocytes
(**B**) Tat neurotoxic effect is mediated by an interplay between different cell types triggered by Tat pleiotropic activities (King *et al.* 2006)

In order to better define the functional damage induced by Tat in the CNS, several groups have examined both neuronal injury and spatial learning. They found that the intracerebroventricular injection of Tat leads to the

attenuation of spatial learning and correlates with the suppression of Long Term Potentiation (LTP) (Li *et al.* 2004). Unlike what had been previously described by other authors (Sabatier *et al.* 1991), in this study the intra-hippocampal injection of Tat did not lead to neuronal cell loss, thus suggesting that Tat induces neuronal dysfunction before causing neuronal cell death.

Kaposi's sarcoma

Kaposi's sarcoma (KS) is an angioproliferative disease very frequent and aggressive when found in HIV infected individuals. All forms of KS possess similar histological features, showing the presence of spindle-shaped cells, vascular smooth cell, endothelial cells, fibroblasts, inflammatory cells and high vascularization. KS lesions are associated with infection by HHV8 (human herpes virus 8) and are characterized by a complex interplay of cytokines (Interleukin (IL)-1, IL-6, Interferon (IFN) - α, TNF-α), angiogenic growth factors (among others, FGF-2, vascular endothelial growth factor (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 in HIV infected individuals originally led to the hypothesis that somehow HIV infection itself might play an important role in favoring KS onset and progression.

Tat mimics the activity of fibronectin and vitronectin by binding their cell surface receptors through its RGD (Arg-Gly-Asp) motif; however, the heparin binding activity of its basic domain is also involved in the process of migration (Barillari *et al.* 1993).

Moreover, Tat is able to mimic extracellular proteins involved in adhesion, migration and growth of both KS and endothelial cells, thus potentially enhancing angiogenesis and KS progression. However Tat is not able to induce a response in primary endothelial cells unless they are activated by

inflammatory cytokines (IFN-γ, TNF-α and IL-1β) (Fiorelli *et al.* 1999), which in turn activate the release of FGF-2.

In addition to cellular adhesion, integrins play a key role in angiogenesis, by modulating migration, apoptosis and response to growth factors of endothelial cells. Tat RGD domain is able to induce endothelial cells migration, mimicking the effect of vitronectin and fibronectin, stimulating the expression of matrix metalloproteinase (MMP)-2 (Toschi *et al.* 2001), and of focal adhesion kinases necessary for the invasion of the extracellular matrix. In addition to these activities, the binding of Tat to integrins enhance the cellular response to FGF-2 (Barillari *et al.* 1999).

The basic domain of Tat binds extracellular matrix heparan-sulfate proteoglycans (HSPGs) thus facilitating the interaction between the RGD domain and the integrins; on the other hand, binding of Tat to the HSPG retrieves soluble FGF-2 from the extracellular matrix, thus promoting cell growth (Folkman *et al.* 1988) (similar to Tat, FGF-2 binds HSPG upon secretion). Finally, the basic domain can bind the VEGFR-2, thus stimulating its phosphorylation and promoting endothelial cells proliferation (Mitola *et al.* 2000).

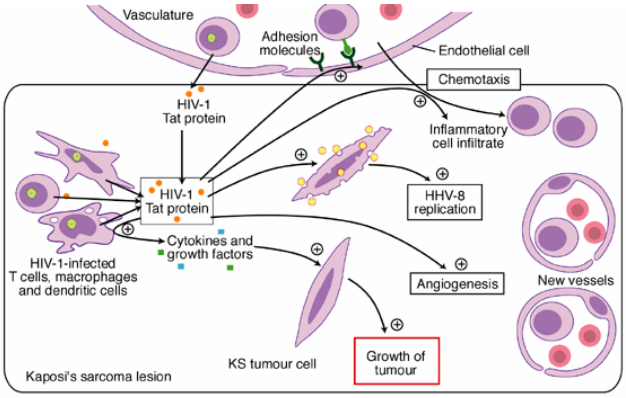

Fig. 1.13: Model for the role of Tat in the pathogenesis of acquired AIDS-associated Kaposi's sarcoma (Foreman 2001)

The immune response

Several studies on HIV-infected patients showed a marked increase of the inflammatory response in the brain tissue, due to the effect of Tat on the expression of several pro-inflammatory cytokines and growth factors, such as the aforementioned IFN-γ, TNF-α, IL-1β, as well as TGF-β, IL-2, IL-6, IL-8, IL-10, CXC-chemokine receptor 4, IL-2 and IL-4 and TNF-α receptors.

Moreover, HIV infected individuals display a significant immune hyperactivation, leading to lymphocyte proliferation, expression of T cell activation antigens and increased cytokine expression.

During the early stages of HIV infection, Tat is selectively taken up by mature dendritic cells (Fanales-Belasio *et al.* 2002), where it induces a upregulation of the major MHC genes and of Th1 cytokines and β chemokines, therefore promoting the antigen presenting capacity of these cells, and enhances the recruitment of T cells by means of reprogramming gene expression in dendritic cells (Izmailova *et al.* 2003). Since the chemokines induced by Tat recruit activated T cells and macrophages, HIV can use the dendritic cells as vehicles to infect its ultimate targets of infections.

As HIV cannot infect resting T- cells, Tat could also facilitate the spread of HIV infection by upregulating the expression of IL-2, thus promoting the priming of naive T-cells (Ott *et al.* 1997).

A number of different and sometimes controversial studies suggest several immunosuppressive activities for extracellular Tat, which may account for the immunosuppression observed in AIDS disease.

These activities range from apoptosis of peripheral blood cells (Li *et al.* 1995), to impairment of natural killers (NK) cells (Poggi *et al.* 1998; Zocchi *et al.* 1998), to the inhibition of antigen-specific T-cell activation response (Viscidi *et al.* 1989), to the upregulation of the production in peripheral blood monocytes of IL-10 (Bennasser and Bahraoui 2002), a highly

immunosuppressive cytokine the levels of which correlate with disease progression in HIV infected individuals.

Despite this intense research on the properties of extracellular Tat, however, the issue whether Tat might be directly involved in the induction of immunosuppression in HIV-1 infected patients still remains unsolved.

HIV-1 Vpr

Vpr is a small protein of 96 aminoacids (14 kDa) rich in basic residues, which is well conserved in HIV-1, HIV-2 and SIV (Simian Immunodeficiency Virus) (Tristem *et al.* 1992). Its real functions during the natural course of infection are still under debate; nonetheless, the role of Vpr in AIDS pathogenesis appears crucial, as *vpr* null mutants show decreased viral replication and delayed disease progression in rhesus monkeys experimentally infected with SIV (Tristem *et al.* 1992). As a result of extensive study in a number of *in vitro*, *in vivo* and *ex-vivo* systems, Vpr has been shown to play, despite its small size, a wide number of functions during viral replication, ranging from nuclear import of the pre-integration complex, to cell cycle progression, to transactivation of the viral LTR as well as of host genes.

Intracellular activities of Vpr

Along the viral life cycle, a first activity of Vpr is to influence the fidelity of reverse transcription: in addition to a potential role in the initiation step of the reverse transcription (Stark and Hay 1998), Vpr was shown to modulate the in vivo mutation rate of HIV-1 by interacting with the nuclear form of uracil DNA glycosylase (UNG2) (Mansky *et al.* 2000), an enzyme involved in the base excision repair pathway that specifically removes the RNA base uracil from DNA. The association of Vpr with UNG2 in virus-producing cells allows the incorporation of a catalytically active enzyme into HIV-1 particles, where UNG2 may directly influence the reverse transcription accuracy (Mansky *et al.* 2000), contributing to the ability of HIV-1 to replicate in

primary macrophages and nondividing cells (which express low levels of UNG and contain relatively high levels of dUTP) (Sandgren *et al.* 2002).

Despite the lack of any identifiable canonical nuclear localization signal (NLS), Vpr displays evident karyophilic properties and is rapidly targeted to the host cell nucleus after infection (Lochelt *et al.* 1993). One possibility is that Vpr primarily serves to dock the PIC at the nuclear envelope, while IN and MA act in cooperation with the central DNA flap to target the viral DNA to the nucleus.

In addition to its non conventional NLS for targeting into the nucleus, Vpr is a dynamic mobile protein able to shuttle between the nucleus and cytoplasmic compartments (Jenkins *et al.* 2001; Sherman *et al.* 2001; Sherman *et al.* 2003). The exact role of the nuclear export signal (NES) in the function of Vpr is not known but since Vpr is rapidly imported into the nucleus after biosynthesis, the NES could redirect it into the cytoplasm for subsequent incorporation into virions during the late budding step of the virus life cycle (Jenkins *et al.* 2001; Sherman *et al.* 2003).

A further important biological activity of SIV and HIV Vpr proteins is related to their ability to induce an arrest in the G2 phase of the cell cycle of infected proliferating human and simian T cells (Di Marzio *et al.* 1995; He *et al.* 1995; Jowett *et al.* 1995; Re *et al.* 1995; Bartz *et al.* 1996; Planelles *et al.* 1996). The biological significance of this arrest during the natural infection is not well understood, but as the HIV-1 LTR seems to be more active in the G2 phase, the G2 arrest may confer a favorable cellular environment for efficient transcription of HIV-1. Such increase transcriptional activity from the viral LTR in arrested cells expressing Vpr (Subbramanian *et al.* 1998; Gummuluru and Emerman 1999; Hrimech *et al.* 1999), could be mediated through *cis*-acting elements, found in the LTR promoter which are bound by Vpr.

Though no specific DNA sequence targeted by Vpr has been yet identified (Zhang *et al.* 1998; Kichler *et al.* 2000), Vpr displays high affinity for nucleic

acids; Vpr may function as an adaptor molecule for an efficient recruitment of transcriptional co-activators (such as GRE, p300/CBP) to the HIV-1 LTR promoter and thus enhances viral replication. Additionally, it may be involved in the activation of host cell genes inducing cellular pathways in relation with the AIDS pathogenesis.

HIV infection causes a depletion of $CD4^+$ T cells in AIDS patients, which results in a weakened immune system, impairing its ability to fight infections. The major mechanism for $CD4^+$ T cell depletion is programmed cell death, or apoptosis; even though the exact contribution of Vpr as a pro-apoptotic factor responsible for the T cell depletion observed in the natural course of HIV infection is still unknown, it was repeatedly evidenced that Vpr has cytotoxic potential and is able to induce apoptosis in many in vitro systems. However, controversial results indicating that Vpr can also act as negative regulator of T cell apoptosis have been reported (Ayyavoo *et al.* 1997; Conti *et al.* 1998).

Other laboratories have then shown that synthetic Vpr, as well as truncated Vpr polypeptides, are able to induce apoptosis by directly acting on the mitochondria, leading to the permeabilization of the mitochondrial membrane and subsequent dissipation of the mitochondrial transmembrane potential ($\Delta\Psi m$) (Jacotot *et al.* 2000). This direct effect of Vpr is related to its ability to interact physically with the adenine nucleotide translocator (ANT), a component of the permeability transition pore of mitochondria localized in the inner mitochondrial membrane. The interaction between Vpr and ANT triggers permeabilization of the inner membrane followed by permeabilization of the outer mitochondrial membrane with consequent release of soluble intermembrane proteins, such as cytochrome c and apoptosis inducing factors, in the cytosol.

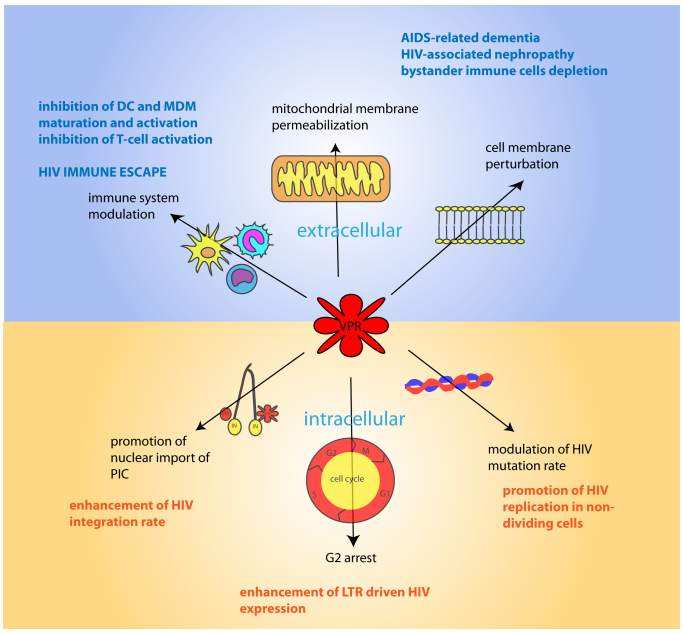

Fig. 1.14: Overview of Vpr biological activities

Cytotoxic effect of extracellular Vpr

Extracellular or free Vpr that exists in cell-free and virus-free state was detected in great amounts in sera and cerebrospinal fluid (CSF) of HIV-1 infected patients (Levy *et al.* 1994). However, it has not yet been clearly established whether extracellular Vpr results from breakdown of infected cells and virus particles or release from infected cells. Interestingly, some studies found that purified intracellular Vpr could enter the cells when added to cultured cells (Levy *et al.* 1995; Huang *et al.* 2000).

The carboxyl-terminal end of Vpr was shown to cause structural defects in cell membranes, indicated by osmotic sensitivity and gross cell enlargement (Macreadie *et al.* 1995); these effects were dependent on the sequence HFRIGCRHSRIG, containing two H(S/F)RIG motifs (Macreadie *et al.* 1995). When extracellular peptides containing the above sequence were added, they rapidly entered yeast cells and caused cell membrane permeabilization

and death in a variety of yeast cells (Macreadie *et al.* 1996). Extracellular addition of synthetic Vpr peptides containing the H (F/S) RIG repeat motif showed similar consequence in mammalian cells. When this peptide was added externally to human $CD4^+$ cells, it induced mitochondrial membrane permeabilization, dissipation of mitochondrial transmembrane potential, morphological changes, the formation of apoptotic bodies and breaks of DNA chain (Arunagiri *et al.* 1997).

According to these last findings, the existence of circulating Vpr in infected individuals, might also explain the toxic effects exerted by Vpr on bystander, uninfected cells.

Vpr and AIDS dementia complex

Relatively high levels of Vpr were detected in the CSF of HIV-infected patients with neurological defects (Levy *et al.* 1995); as macrophages and microglia of human brain are infiltrated during HIV-1 infection (Nottet and Gendelman 1995; Nottet 1999), these cells are thought to release extracellular Vpr to the CSF. Additionally, infected astrocytes may also release Vpr (for a review on the effect of HIV on the neural tissue, see (Jones and Power 2006)).

Based on several studies demonstrating the neurotoxicity of extracellular Vpr, the protein was then shown to exhibit channel-forming capacity in the membrane of intact cells when added extracellularly. Moreover, recombinant extracellular Vpr associated directly with the plasmalemma of hippocampal neurons caused a large inward cation current and depolarization of the plasmalemma, finally resulting in cell death (Piller *et al.* 1998). The first 40 amino acid residues of N-terminal domain of Vpr (N40) affect the ion channel activity thus showing cytotoxic effects, while the C-terminal domain is involved in rectification of Vpr currents. Moreover, the N40 has the cytotoxic ability similar to intact Vpr, including depolarization of the

plasmalemma and cell death in cultured hippocampal neurons (Piller *et al.* 1999).

Extracellular Vpr also showed toxic effects on brain activity; when added to mixed embryonic rat brain cultures, Vpr-induced cell death was observed. Similarly, Vpr-induced cell death was also observed in enriched primary cortical rat astrocytes, and it was considered that death was mainly caused by necrotic mechanism (Huang *et al.* 2000). On the other hand, extracellular Vpr was shown to induce the death of human neuronal cells through apoptosis (Patel *et al.* 2000).

HIV-1–associated nephropathy

HIV-associated nephropathy (HIVAN) is unique in that the etiology of podocyte injury is genetically well defined, namely caused by HIV-1 itself. Patients with HIVAN present with heavy proteinuria and rapid progression to end-stage renal failure with collapsing focal segmental glomerulosclerosis and microcystic tubular dilation (Bourgoignie 1990; Humphreys 1995; Klotman 1999; Ross and Klotman 2002; Kimmel *et al.* 2003; Weiner *et al.* 2003; Ross and Klotman 2004).

Transgenic mice carrying the HIV-1 long terminal repeat, *tat* and *vpr*, but not mice that carrying an HIV-1 genome defective in *vpr*, develop glomerulosclerosis. Selective podocyte expression of either Vpr or Nef alone can induce podocyte injury that leads to glomerulosclerosis; in addition, a prominent synergistic effect of these two proteins on podocyte injury was reported (Zuo *et al.* 2006).

Role of Vpr in AIDS-related insulin resistance/lipodystrophy syndrome

Recent advances in the development of the nucleotide and non-nucleotide analogues acting as reverse transcriptase inhibitors (NRTIs) and the non-peptidic viral protease inhibitors (PI), and their introduction in the management of patients with AIDS, either alone or in combination, have

dramatically improved the clinical course of the disease and prolonged life expectancy in patients with AIDS. The increase in life expectancy associated with the long-term use of the above antiviral agents, however, has generated novel morbidities and complications. Central among them is the quite common AIDS-related insulin resistance and lipodystrophy syndrome, which is characterized by a striking phenotype and marked metabolic disturbances.

A role for the Vpr protein in these disorders has been suggested by a recent study which investigated the capacity of Vpr to co-activate the glucocorticoid receptor, which potentiates the action of glucocorticoid hormones, thereby inducing tissue glucocorticoid hypersensitivity (Kino and Chrousos 2004). Vpr interacts with novel 14-3-3 proteins, promoting their interaction with Cdc (Cell division cycle) 25 and subsequently suppressing their transcriptional activity by segregating Cdc25 into the cytoplasm. The same study also showed that Vpr suppresses the association of 14-3-3 with other partner molecules, in particular the FoxO (Forkhead box O) transcription factors. Since the FoxO proteins function as negative transcription factors for insulin, Vpr may cause resistance of tissues to insulin. Through these two newly identified functions of Vpr, namely, coactivation of glucocorticoid receptor activity and inhibition of insulin effects on FoxO proteins, Vpr may participate in the development of AIDS-related insulin resistance/lipodystrophy syndrome.

Effects on immune system

Cellular immunity, specifically MHC-restricted CTL responses, is thought to play an intrinsic role in protection and clearance of many viral infections. Though HIV-1 infection is controlled by the immune response initially, the immune system fails to clear the virus and ultimately loses control of viremia through unclear mechanisms (Koup *et al.* 1994; Pantaleo *et al.* 1994; Fauci 1996; Barouch *et al.* 2000; Piguet and Trono 2001; Letvin *et al.* 2002; Letvin

and Walker 2003; Nickle *et al.* 2003). Furthermore, there is evidence that the host immune response is compromised early in HIV infection.

Vpr could be a possible important player in the compromised immune control of HIV as it exerts significant effects on cellular proliferation, differentiation, regulation of apoptosis, modulation of cytokine production and transcription in vitro. Many of these Vpr-mediated cellular events have been observed in a wide variety of cell lineages, suggesting that Vpr targets basic eukaryotic cellular pathways.

As an extracellular delivered protein, Vpr down-modulates the expression of several immunologically important molecules including CD40, CD80, CD83 and CD86 costimulatory molecules on MDM and MDDC (monocyte-derived dendritic cells) (Muthumani *et al.* 2005), suggesting that Vpr could interfere with DC (dendritic cells) functions or maturation, or both.

The potential consequences of failed APC maturation during HIV infection could be significant: for instance, Vpr-mediated CD40 repression could contribute to the inability of the host immune system to continue to mount an effective response against HIV, as the engagement of CD40L on APC by CD40 on $CD4^+$ or $CD8^+$ cells were shown to 'condition' the APCs for antigen-specific CTL activation, and facilitate induction of a memory T cell response.

The CD80 and CD86 molecules are surface glycoproteins and members of the immunoglobulin superfamily, which are only expressed on professional APCs (Sharpe and Freeman 2002). Blocking these costimulatory signals leads to T cell unresponsiveness; therefore, despite efficient migration of APCs into regional lymph nodes, the effects of Vpr may influence both presentation and activation of T cells, thus influencing viral clearance. This unique property of Vpr could ensure infection in a T cell rich environment without immune clearance and favor viral propagation.

Similarly, it has been observed that Vpr affects the expression of the CD33 antigen, a transmembrane surface protein specific for myeloid lineage cells, which can strongly influence the antigen presentation properties of DCs.

Thus, Vpr can affect dendritic cell/macrophage maturation and activation *in vitro*, and this effect could *in vivo* lead to diminished T-cell activation and consequently T cell non-responses, providing an interesting mechanism for HIV evasion from the host's immune system.

In conclusion, the release from infected cells, the capacity to enter other cells or form ion channels in the membrane of living cells, and the cytotoxic effects of extracellular Vpr strongly suggest that Vpr has cytotoxic effects on bystander cells during HIV infection, thus potentially contributing to the severe multiorgan complications often observed during the course of AIDS.

HIV-1 Nef

The accessory protein Nef was originally discovered as a "negative factor" that inhibited viral replication (hence the name). Later, it became clear that Nef positively affects viral replication and infectivity (Miller *et al.* 1994a; Miller *et al.* 1994b). HIV-1 and SIV Nef proteins are small (25- to 34-kDa), myristoylated proteins that reside both in the cytoplasm and in association with the cytosolic face of cell membranes.

A structural model of the conformation of the full-length Nef polypeptide predicts that the surface of the protein consists of a linear array of potential protein-protein interaction domains and that this surface is quite flexible. Interestingly, it has been speculated that the overall flexibility of Nef enables the protein to switch between multiple conformations and that its structural organization may be dictated by its binding partner(s).

Even if no enzymatic activity has been directly attributed to the Nef protein, extensive studies of Nef biology have revealed several conserved motifs that mediate physical association with cellular factors. Consequently, Nef could function as a molecular adaptor, altering cellular pathways (such as protein trafficking events, signal transduction cascades, and apoptotic pathways) via multiple protein-protein interactions. As a matter of fact, it has been widely

demonstrated that Nef contributes to HIV pathogenesis by several mechanisms.

Intracellular activities of Nef

Nef was shown to promote viral infection by activating $CD4^+$ T lymphocytes, making them more susceptible to infection, by means of altering signal transduction pathways downstream of the T-cell receptor (Skowronski *et al.* 1993; Baur *et al.* 1994). Nef has been reported to interact with the T-cell signaling pathway leading to the upregulation of Fas ligand (FasL) (Xu *et al.* 1999) and therefore promoting CTL apoptosis. In addition, Nef may play a role in the spread of HIV-1 through its specific effects on DCs, which can capture HIV-1 particles through a DC-specific receptor (DC-SIGN) and later transmit the virus to target cells without becoming productively infected (transcytosis).

HIV-1 Nef reduces the surface expression of MHC-I (Schwartz *et al.* 1996), preventing the exposure of viral antigens on the surface of HIV-infected cells, thus allowing HIV-infected cells to escape recognition and lysis by anti-HIV CTLs in vitro (Collins *et al.* 1998). There is evidence that the ability of Nef to disrupt MHC-I antigen presentation is very important for viral disease pathogenesis in vivo (Carl *et al.* 2001; Munch *et al.* 2001; Swigut *et al.* 2004).

By affecting the MHC-I surface expression, Nef may protect the HIV-infected cell from both adaptive and innate cell-mediated immunity.

The CD4 protein is a coreceptor required for HIV infection; however, its continued presence on the surface of an HIV-infected cell after viral entry is problematic because coexpression of CD4 and the viral envelope disrupts the trafficking of both proteins; moreover, the presence of CD4 on the cell membrane reduces the ability of the newly formed particle to properly bud and escape the infected cell and therefore reduces viral infectivity. Nef

dramatically decreases the half-life of CD4 that has reached the cell surface, by targeting CD4 for degradation in an acidic compartment.

Several models for Nef-mediated CD4 transport have been proposed, each based on a familiar premise: Nef binds the cytoplasmic tail of CD4 and recruits a cellular factor(s) to transport CD4 from the cell surface to lysosomes for degradation.

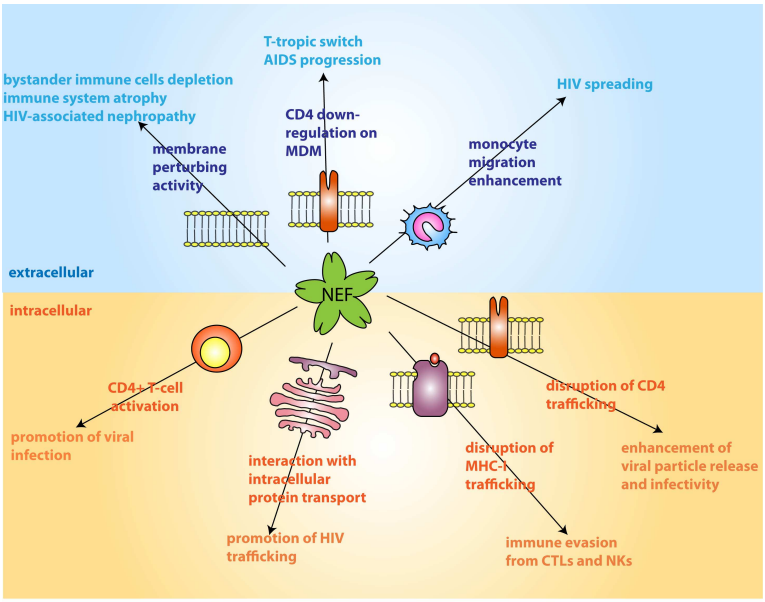

Fig 1.15: Overview of Nef biological activities

Pathological effects of extracellular Nef

Cell depletion, often observed in the immune system of HIV infected individuals, is likely to be predominantly a bystander effect because the number of cells dying far outnumbers that of the HIV-infected cells, and is not confined to CD4 cells. There is increasing evidence of indirect killing of bystander cells by extracellular or cell surface-associated components of HIV infected cells.

Perturbation of membrane structure

The myristylated N-terminal region of Nef has severe membrane disordering properties (Curtain *et al.* 1994), and, when present in the extracellular medium, causes rapid lysis of a wide range of $CD4^+$ and $CD4^-$ cells *in vitro*, suggesting a role for extracellular Nef in the depletion of bystander cells, tissue atrophy, and in delaying the death of HIV-infected cells.

Thymic atrophy and loss of T cells in peripheral lymphoid organs, with resultant immunodeficiency, has been seen in transgenic mice expressing the *nef* gene alone (Skowronski *et al.* 1993; Lindemann *et al.* 1994; Hanna *et al.* 1998) suggesting that Nef could be directly responsible for the depletion of lymphocytes and thymocytes during HIV infection. A number of studies suggest that the cytopathic effects of Nef could be related to its being targeted to the plasma membranes and other cellular membranes by the sequence at the N-terminus which has an N-myristoyl group attached at Gly-2. The N-terminal region of Nef shows striking homology to the bee venom mellitin, and when myristylated at the N-terminus has a dramatic disordering effect on lipid bilayers (Curtain *et al.* 1994). Full length myristylated Nef is released into the extracellular medium when expressed in mammalian cells (Guy *et al.* 1990), where it could exert cytolytic effects. As a matter of fact, addition of myristylated N-terminal peptides of Nef to the extracellular medium of human erythrocytes, $CD4^+$ CEM (Human acute lymphoblastic T-cell Leukaemia) cells or PBMC (peripheral blood monocyte cells), caused very rapid hemolysis or cell lysis in a dose dependent manner (Curtain *et al.* 1997).

The myristylated N-terminal Nef peptide-mediated death of a variety of cells could, at least in part, explain the death of various cell types within lymphoid organs during HIV-infection, while soluble or cell surface associated Nef could possibly be responsible for bystander cell killing. The early appearance of Nef-specific antibodies and cytotoxic T lymphocytes in the sera of two-thirds of HIV infected people (Ameisen *et al.* 1989; Reiss *et al.* 1989;

Cheingsong-Popov *et al.* 1990) suggests that Nef must be available in the extracellular medium. In agreement, Nef has been shown to be present in significant quantities in the sera of HIV-infected individuals (Fujii *et al.* 1996), and on the surface of infected $CD4^+$ cells (Fujii *et al.* 1993). Its presence in the extracellular medium could be due to the release of myristylated Nef in membrane vesicles, or to its incorporation as a component of viral particles. As viral particles mostly consist of the p20 fragment of Nef (Pandori *et al.* 1996), the myristylated N-terminal fragments (p7) is probably cleaved off into the extracellular medium, so that significant quantities of p7 fragment could be available in the extracellular medium in view of the large amounts of virus produced in lymphoid organs during HIV infection (Pantaleo *et al.* 1993; Veazey *et al.* 1998).

The severe depletion of $CD4^+$ cells in SCID mice engrafted with HIV-infected human fetal liver and thymus (Liv/Thy) and PBMCs, but the lack of such cell depletion in tissues infected with *nef*-deleted HIV (Jamieson *et al.* 1994; Gulizia *et al.* 1997), points to a direct *in vivo* role for Nef in cytopathicity. A cytopathic role for Nef is also supported by the observation of multiple AIDS-like pathologies such as thymic atrophy, loss of T cells and immunodeficiency in transgenic mice expressing the *nef* gene alone (Skowronski *et al.* 1993; Hanna *et al.* 1998). These studies clearly suggest that HIV Nef and the N-terminal region of Nef are directly involved in development of AIDS pathologies including cell depletion and tissue atrophy.

M-tropic to T-tropic switch

Increasing interest has been devoted to the effect of Nef on monocyte–macrophages in AIDS pathogenesis. Treatment of human MDM with recombinant HIV-1 Nef protein (rNef) induces a strong inhibition of the replication of either macrophage (M-) or dual-tropic HIV-1 strains (Alessandrini *et al.* 2000). The resistance to HIV replication induced by rNef treatment in MDM favors the spread of T-tropic over M-tropic HIV strains in

doubly infected $CD4^+$ lymphocyte–MDM co-cultures (Alessandrini *et al.* 2000).

These data support the intriguing possibility that extracellular Nef might contribute to AIDS pathogenesis by inducing resistance to M-tropic HIV replication in MDM, thereby facilitating the switch from M- to T-tropic HIV that correlates commonly with AIDS progression.

How could the switch be achieved? MDM exposed to rNef showed downregulation of CD4 display, reproducing a typical effect widely observed in $CD4^+$ cells endogenously expressing Nef; it is conceivable that factors released in vivo by MDM as a result of an inflammatory stimulus (either dependent or not on HIV infection) attract and activate both infected and uninfected $CD4^+$ lymphocytes that can be induced to productive HIV replication; released Nef may be internalized by uninfected macrophages that, as a consequence, are induced to produce additional amounts of HIV stimulating factors (included chemokines) becoming, at the same time, resistant to HIV, by means of CD4 downregulation.

This mechanism may be at least in part the basis of the switch from M- to T-tropic HIV strains frequently observed in seropositive patients progressing to AIDS.

Extracellular Nef increases the migration of monocytes

Infiltration of HIV-1-infected and uninfected monocytes/macrophages in organs and tissues is a general phenomenon observed during the progression of the disease. In a recent study (Lehmann *et al.* 2006) it was shown that extracellular Nef increases migration of monocytes with an effect which is (i) concentration-dependent, (ii) reaches the order of magnitude of that induced by formyl-methyonyl-leucyl-proline (fMLP) or CC chemokine ligand 2 (CCL2)/monocyte chemotactic protein (MCP)-1, (iii) is inhibited by anti-Nef monoclonal antibodies as well as by heat, and (iv) depends on a concentration gradient of Nef.

These data suggest that extracellular Nef may contribute to disease progression as well as HIV-1 spread by affecting migration of monocytes.

HIV-associated nephropathy: role of Nef

As mentioned above, HIVAN is the leading cause of end-stage renal failure in HIV-1 seropositive patients (Bourgoignie 1990; Humphreys 1995; Klotman 1999; Ross and Klotman 2002; Kimmel *et al.* 2003; Weiner *et al.* 2003; Ross and Klotman 2004). Podocyte dysfunction appears to be a direct result of HIV-1 protein expression, specifically Nef and Vpr as well as specific host factors that have yet to be elucidated (Zuo *et al.* 2006). The mechanism by which Nef induces podocyte proliferation and dedifferentiation has been traced to its ability to activate several signaling pathways including Src-Stat3 and ras-raf-MAPK-1, and -2 (He *et al.* 2004). Activation of the cAMP/PKA (Protein Kinase A) pathway with all-trans-retinoic acid appears to modulate these changes and returns podocytes to a differentiated, nonproliferating phenotype.

In a study in which transgenic mice that expressed individual HIV-1 genes in podocytes were generated, animals that expressed Vpr or Nef developed podocyte injury. In contrast, none of the transgenic mice that expressed Vif, Tat, Rev, or Vpu developed podocyte injury (Zuo *et al.* 2006).

As all *nef:vpr* double-transgenic mice showed more severe podocyte injury and glomerulosclerosis than all *vpr* or *nef* single-transgenic mice in the same litter, Vpr and Nef each can induce podocyte injury with a prominent synergistic interaction.

HTLV-1 Tax

HTLV-I is the etiologic agent of adult T cell Leukaemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP).

Mitotic division of infected cells appears to be the major route of expansion of HTLV-1 since transmission of the virus is inefficient. The major

mechanism involved in the leukaemogenic process is transactivation (see below) and not insertional activation or oncogene transduction as seen in animal leukaemias caused by gamma- and alpha-retroviruses. In addition to the canonical retroviral structural genes, there is a region at the 3′ end of the genome which encodes a number of accessory proteins that play important roles in the transformation process; these include Tax, Rex, p12, p13, p30, and HBZ.

HTLV-1 Tax is a 40-kD phosphoprotein encoded by a multiply spliced RNA. At its amino-terminal end, the protein contains zinc-finger-like cysteine-rich and histidine-rich motifs that are important for trans-activation (Smith and Greene 1991; Grassmann *et al.* 2005; Giam and Jeang 2007).

Tax is present predominantly in the nucleus due to its NLS residing at its amino terminus. However, a substantial portion of the protein is also present in the cytoplasm due to its NES (Alefantis *et al.* 2003). Tax, which acts as a dimer, was originally discovered as a transactivator of viral RNA transcription from a promoter located at the 5'LTR (Felber *et al.* 1985), but later proved to modulate the synthesis or function of a wide range of cellular regulatory proteins (Bex and Gaynor 1998; Iha *et al.* 2000; Jeang 2001; Yasunaga and Matsuoka 2007; Boxus *et al.* 2008).

Tax intracellular activities: oncogenesis

While the pathogenesis of ATL remains incompletely understood, the viral regulatory protein Tax appears to play a central role as it has been shown to be able to transform primary lymphocytes and rat fibroblasts; in addition, transgenic mice models have confirmed the oncogenic properties of the protein *in vivo*. Tax functions primarily through protein-protein interactions, and its transformation properties are a consequence of the ability of the protein to deregulate the transcription of genes and signaling pathways involved in cellular proliferation, cell cycle control and apoptosis, including transcription factors such as CREB/ATF (cAMP response element binding

protein/activating transcription factor), NF-kB, AP-1, SRF (Serum response factor) and NFAT; and distinct cellular-signaling pathways, involving PDZ domain-containing proteins, Rho-GTPases and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), and TGF-β pathways.

NF-κB activation by Tax results in the upregulation of expression of a large number of cellular genes involved in cell proliferation, including several cytokines and their corresponding receptor genes, including IL2 and the IL2-Rα, which leads to polyclonal proliferation of HTLV-1-infected cells by autocrine and paracrine mechanisms (Ballard *et al.* 1988; Good *et al.* 1996). In addition, NF-κB stimulation causes increased expression of proteins with anti-apoptotic function including Bcl-xL and survivin (Kamihira *et al.* 2001; Mori *et al.* 2001a; Mori *et al.* 2001b). Many of the cellular genes deregulated by Tax are involved in cell cycle control, apoptosis, and DNA repair.

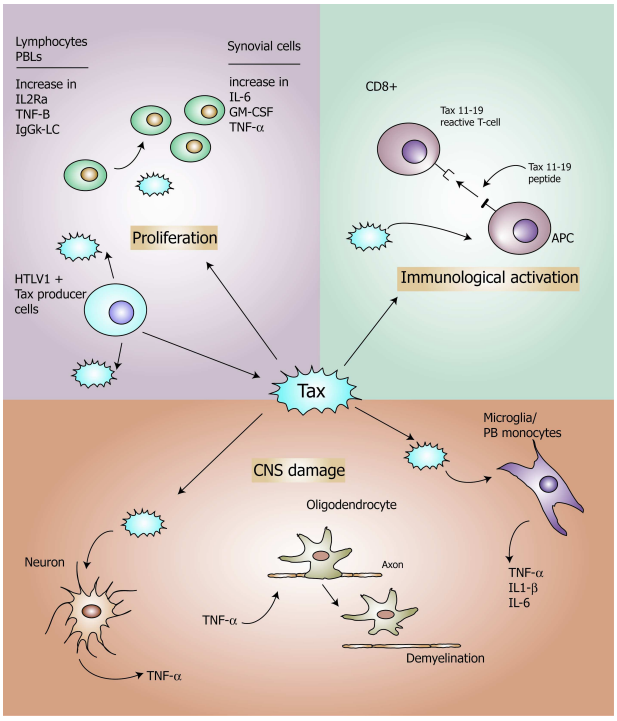

Fig 1.16: Tax effects

Biological effects of extracellular Tax

HTLV-1 Tax, similar to other retroviral proteins, is likely able to perform a variety of functions while regulating viral gene expression and promoting the survival of the virus within the human population.

All Tax-mediated processes are dependent on Tax localization to the nucleus where it interacts with a number of cellular transcription factors during its course of nuclear functions. However, there is mounting evidence suggesting that Tax may shuttle between the nucleus and cytoplasm, localize to several cytoplasmic organelles with subsequent secretion from both Tax-transfected cells as well as HTLV-1-infected cells.

Consistent with the concept of cellular secretion, it has been recently confirmed that Tax is detected in the cell culture media of Tax-transfected cells where Tax secretion was, at least in part, the result of a canonical cellular secretory pathway. This observation was supported by additional experimental evidence demonstrating that the presence of extracellular Tax was significantly decreased following treatment with the secretory pathway inhibitor brefeldin A (BFA) (Alefantis *et al.* 2005b).

The process of Tax secretion has not been studied in great detail. While the presence of Tax in the extracellular space has been demonstrated, there have been few reports pertaining to the mechanism of Tax secretion (Alefantis *et al.* 2005a; Alefantis *et al.* 2005b). In this respect, the cytoplasmic organelles to which Tax has been shown to localize, such as the ER and Golgi complex, as well as the amino acid signals responsible for Tax targeting to these organelles, clearly warrants further examination. Full-length Tax is secreted from both baby hamster kidney cells and a human kidney tumor cell line, suggesting that Tax enters the classic ER-Golgi secretory pathway in a leaderless manner, distinct from the pathways exploited by HIV-1 Tat (described below) and HFV Bet (see below) which seem not to rely on the ER-Golgi machinery (Alefantis *et al.* 2005b).

Regardless of the process Tax utilizes to gain entry into the secretory pathway, Tax likely begins its egress through the secretory pathway in the ER. In this regard, Tax contains several amino acid sequences similar to those utilized by other proteins to initiate the secretory process through the ER. This signal may enable Tax to be sorted within the ER and transported to the Golgi in COPII vesicles.

Neurotoxic effects.

The activity of extracellular Tax has been central to many theories regarding the progression of HAM/TSP. Hypothetically, the presence of extracellular Tax in the CNS may be the result of either infiltrating HTLV-1- infected cells ($CD4^+$ or $CD8^+$ T cells or, possibly, other immune cell populations), including cells of the monocyte/macrophage lineage, or resident CNS cell populations that have been infected with HTLV-1 after entry of the virus into the CNS compartment.

While neurons are not believed to harbor HTLV-1, both astrocytes and microglia have been shown to be infected by HTLV-1 in vitro and in vivo, so it is plausible that Tax could be released from infected astrocytes shortly after infection, as Tax expression in astrocytes has been demonstrated to have consequences for the astrocytes themselves as well as for surrounding cells. Whatever the source of extracellular Tax, its effects on resident CNS cell populations, particularly neurons, may contribute to the etiology of HAM/TSP: extracellular Tax has been demonstrated to induce both the production and secretion of TNF-α from NT2-N cells, a cell line model of mature primary human neurons (Cowan *et al.* 1997), an effect observed with an extracellular concentration of Tax that has been shown to approximate that produced by HTLV-1-infected cells (Lindholm *et al.* 1992; Cowan *et al.* 1997).

Release of TNF-α may result in both an autocrine and paracrine cytokine-mediated destruction of neuronal tissue and of cells supporting the neuronal

system. Secretion of cytokines in response to extracellular Tax is not limited to neurons, as other types of resident CNS cell populations have been shown to respond to extracellular Tax, such as primary adult human microglial cells which were shown to produce and secrete TNF-α, IL-1β, and IL-6 in response to extracellular Tax (Dhib-Jalbut *et al.* 1994). The effects of exposure to extracellular Tax have not been limited to the CNS, since primary human peripheral blood macrophages have also been shown to produce and secrete TNF-α, IL-1β, and IL-6 in response to exposure to extracellular Tax (Dhib-Jalbut *et al.* 1994).

The effects of extracellular Tax on a particular cell may also depend on the stage of cellular development. For example, extracellular Tax induces NT2-N cells, terminally differentiated cells from the NT2 teratocarcinoma cell line, to produce TNF-α. However, NT2 precursor cells do not produce TNF-α in the presence of extracellular Tax, suggesting that factors specific to more developmentally mature neurons are necessary for either the detection of Tax and subsequent activation of the TNF-α pathway, or factors that act in conjunction with Tax (Dhib-Jalbut *et al.* 1994). It is not known whether the effects of Tax on NT2-N cells occur as the result of Tax acting at the cell surface or after internalization.

An equally plausible mechanism for the destruction of CNS cells observed in HAM/TSP may involve infiltration of HTLV-1-infected $CD4^+$ T lymphocytes into the CNS. It is possible that circulating HTLV-1-infected $CD4^+$ T cells migrate to the CNS, where they release Tax into the extracellular environment resulting in a deregulation of CNS function. Thus, infected astrocytes and infected circulating T lymphocytes may act as Tax-producer cells resulting in the up-regulation and secretion of other toxic molecules by resident CNS populations. Finally, extracellular Tax from Tax-producing cells may be engulfed by professional APCs. Tax peptide presented in the context of class I MHC by APCs such as DCs would result in lysis of Tax-expressing cells by Tax-specific $CD8^+$ T cells. Either mechanism, production of toxic

molecules or specific cell lysis or both could result in significant CNS damage similar to that observed in HAM/TSP.

Finally, evidence has also been presented suggesting that extracellular Tax is able to gain entry into the cell where it may potentially resume its normal intracellular role as a transcriptional transactivator (Lindholm *et al.* 1992).

Pro-inflammatory activity

Secretion of cytokines in response to extracellular Tax is not limited to neurons, as other types of cell populations have been shown to respond to extracellular Tax. Specifically, extracellular Tax has been demonstrated to induce the production and release of several inflammatory cytokines, including TNF, IL-6, and granulocyte macrophage colony stimulating factor (GM-CSF) (Dhib-Jalbut *et al.* 1994); primary human peripheral blood macrophages have also been shown to produce and secrete, in response to exposure to extracellular Tax, these cytokines each having deleterious effects on neighboring cells. Several reports have demonstrated that soluble extracellular Tax induces several biological effects on target cells, including the production and secretion of pro-inflammatory cytokines (Dhib-Jalbut *et al.* 1994; Cowan *et al.* 1997). The major obstacles in treatment of patients with ATL include drug resistance and development of opportunistic infections indicating that cell-mediated immunity is severely impaired since HTLV-1-transformed cells are resistant to most apoptosis inducing agents (Shimoyama 1994; Matsuoka 2002).

Additionally, extracellular Tax has been shown to induce the production of Tax-specific antibodies and Tax-specific CD8+ T cells (Kubota *et al.* 2000), both of which may promote the inflammatory effects observed in HAM/TSP.

HFV Bet

The genome of Foamy viruses (FVs) contains several open reading frames (ORFs) at the 3' end of the genome, in addition to canonical *gag*, *pol*, and

env genes (Flugel *et al.* 1987). Only two of these ORFs are known to encode proteins (discussed further below). The genome of FVs is similar to that of other complex retroviruses, such as HIV, and contains many of the *cis*-acting sequences utilized by other retroviruses (Erlwein *et al.* 1998). Interestingly however, FVs present some peculiarities that distinguish them from the rest of the retroviral family (Lecellier and Saib 2000). For example, despite the presence of the classical early and late phases in FV infections, thus far no structural or functional Rev/Rex homologues have been described for these complex retroviruses, and the function of these proteins seems to be replaced by a *cis*-acting sequence in the FV *pol* domain in the viral mRNA.

FVs encode auxiliary proteins from the 3' end of their genome in addition to the structural and enzymatic *gag*, *pol*, and *env* genes; in the case of human foamy virus (HFV), the prototype of FVs, two additional open reading frames (ORFs) have been described, ORF1 and ORF2. Tas (transactivator of spumaviruses, originally called Bel1), a 36- kDa nuclear phosphoprotein, which is encoded by ORF1, transactivates viral gene expression by directly binding the viral DNA on specific sequences in the long terminal repeat (LTR) and in the internal promoter (Venkatesh *et al.* 1993; Blair *et al.* 1994; He *et al.* 1996). Although the product of ORF2, named Bel2, has been reported (Bieniasz *et al.* 1995), its existence has never been confirmed.

The auxiliary Bet protein of the prototypic HFV is a 482- amino-acid (aa) protein generated by alternative splicing that fuses the first 88 residues of Tas to 394 aa of ORF2 (Muranyi and Flugel 1991). Highly expressed in acute-infection and chronically infected cells, Bet was also shown to be secreted by producing cells and internalized by naive ones (Giron *et al.* 1998). Although the functions of Bet in the replication cycle remain unknown, its integrity is absolutely required for efficient replication of the feline foamy virus (Alke *et al.* 2001) and for that of HFV to a much lesser extent (Yu and Linial 1993; Alke *et al.* 2001). Besides its involvement in

acute infection, Bet was shown to be implicated in the establishment and/or maintenance of viral persistence.

As HFV Bet-expressing cell lines were shown to be resistant to productive HFV infection (Bock *et al.* 1998), the effect of Bet seems to take place during the early steps of infection, after virus entry but before provirus establishment. This protein is distributed in both the cytoplasm and the nucleus of HFV-infected or Bet-transfected cells, and the nuclear targeting results from the presence of a bipartite nuclear localization signal at the C-terminal region, sufficient to direct heterologous reporter proteins to the nucleus (Lecellier *et al.* 2002).

Properties of extracellular Bet

HFV Bet follows an unconventional route to exit the cell since its secretion is not affected by BFA, a drug which disrupts the trafficking between the ER and the Golgi complex (Lippincott-Schwartz *et al.* 1989); once secreted, the protein can be internalized and has been shown (Lecellier *et al.* 2002) to target the nuclei of recipient cells. Finally, these inter- and intracellular movements were also observed for the equine foamy virus Bet protein, strongly suggesting that these remarkable features are conserved among FVs (Lecellier *et al.* 2002).

That Bet spreads to surrounding cells and moves into the nucleus suggests that these properties are relevant to some aspects of the virus cycle. It has been reported that Bet expression, prior to FV infection, blocks the replication cycle at early stages (Bock *et al.* 1998). The nuclear localization of Bet suggests that this protein affects viral replication at nuclear stages, in particular at the import of the preintegration complex or provirus integration steps. Finally, through its secretion property, Bet could act from a distance to limit FV propagation, one possible mechanism to establish viral persistence. Indeed, while a Tas-defective provirus (ΔHFV) is detected in chronically infected hematopoietic cell lines, viral persistence is, in that case,

associated with a constant production of infectious viruses (Yu *et al.* 1996), suggesting that the molecular basis of viral persistence seems to depend on the cell type (Alke *et al.* 2001). Moreover, deleterious mutations in the Bet gene were detected in a simian FV isolated from a case of persistent zoonotic infection (Callahan *et al.* 1999). Therefore these observations suggest that several mechanisms have been developed by FVs to persist in their hosts.

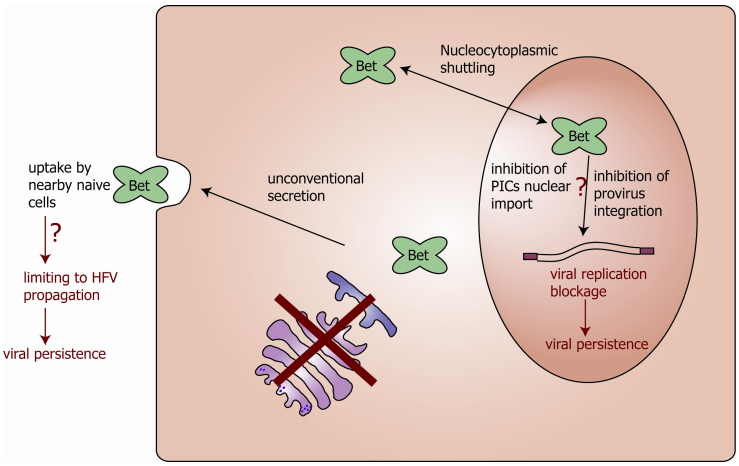

Fig. 1.17: Bet biological activities

Internalization of extracellular Tat and other proteins and peptides

Several studies over the last few years have shown how Tat and other proteins, including the *D. melanogaster* homeoprotein Antennapedia, and the VP22 of Herpes simplex, are rapidly internalized by cells.

This property can be ascribed to the presence of short peptides, which have then be used for the transduction of proteins inside cells, and have been collectively named "Protein Transduction Domains" (PTDs) or "cell penetrating peptides" (CPPs). PTDs (or CPPs) typically have a composition rich either in positvely charged amino acids such as Lysine or Arginine

(polycationic PTDs), or an alternating pattern of polar and hydrophobic residues (amphipatic PTDs).

Historically, the first CPPs were found in the primary structure of HIV Tat and Antennapedia, which properties will be further described below.

Antennapedia belongs to the Homeoproteins class of transcription factors, which binds DNA through a specific sequence of 60 amino acids called the homeodomain. The third helix of the antennapedia homeodomain is involved in the translocation process and, in particular, the minimal protein transduction domain (PTD) of Antp, called penetratin, is the 16-mer peptide (Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys) corresponding to residues 43–58 of the third helix (Derossi *et al.* 1994).

The internalization of Antp peptide is thought to follow non-saturable, dose-dependent kinetics and is believed to occur efficiently both at 37°C and at 4°C, suggesting and internalization mechanism independent of endocytosis (Derossi *et al.* 1994; Vives *et al.* 1997; Pooga *et al.* 1998).

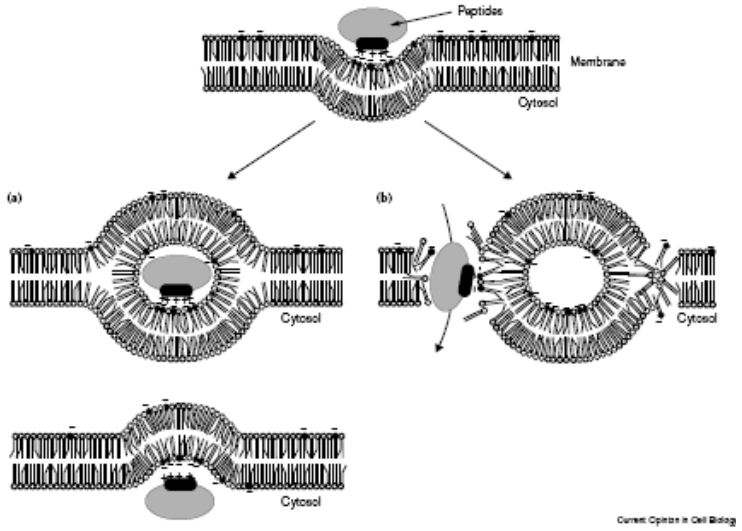

Fig. 1.18: proposed mechanism for membrane translocation of Antp peptide (Prochiantz 2000)

The translocation of Antp does not involve receptors since both the reverse helix and the helix with D-enantiomers for the Antp peptide 43–58 were translocated across biological membranes.

Due to the peculiar ability of Antp peptide to cross cell membrane in an energy-independent way, the formation of inverted micelles (**Fig. 1.18**) was suggested for their translocation through lipid bilayers (Derossi *et al.* 1996; Vives *et al.* 1997).

VP22 is a major structural component of HSV-1 (Herpes Simplex Virus) possessing a remarkable property of transport between cells (Elliott and O'Hare 1997). VP22 is involved with different functions such as intercellular transport, binding to and bundling of microfilaments, inducing cytoskeleton collapse, nuclear translocation during mitosis, and binding to chromatin and nuclear membrane. VP22 has been demonstrated to mediate the trafficking of protein cargos to the nuclei of recipient cells (Elliott and O'Hare 1997); later investigations, however, have questioned such ability (Fang *et al.* 1998) observing that the protein transduction activity could be an artifact of cell fixation (Aints *et al.* 1999; Elliott and O'Hare 1999); even if other works in the following years seem to have proven that VP22 ability to cross cell membranes does in facts exist (Lemken *et al.* 2007), the matter of whether VP22 could be able to transduce protein cargos remains under debate.

The capacity of Tat to enter the cells depends upon the integrity of the so called basic domain, an arginine rich stretch of 9 aminoacids that also corresponds to the nuclear localization signal and to the TAR-binding domain of the protein; as a matter of fact, the works on Tat and Tat11 PTD demonstrated the specific role of cell surface proteoglycans, such as heparan sulfate (HS), as cellular receptors allowing the uptake of Tat and Tat11 in time-, concentration-, and temperature-dependent manner (Tyagi *et al.* 2001; Console *et al.* 2003).

As for the internalization of Tat, while some works showed the involvement of processes like macropinocytosis and clathrin mediated endocytosis

(Lundberg *et al.* 2003; Caron *et al.* 2004), Tat-mediated intracellular delivery of protein cargos seems to proceed mainly via the energy-dependent, caveolar-dependent endocytosis, with subsequent enhanced escape from endosome into the cell cytoplasm (Ferrari *et al.* 2003; Fittipaldi *et al.* 2003).

Cell surface receptors for extracellular Tat internalization

It was first 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. 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 $>1\times10^7$ 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 (Mann and Frankel 1991; Rusnati *et al.* 1997; Rusnati *et al.* 1998; Rusnati *et al.* 1999; Hakansson and Caffrey 2003). Heparin is a close structural homologue of the heparan sulfate glycosaminoglycan (GAG), a major constituent of cell surface and extracellular matrix proteoglycans (Yanagishita and Hascall 1992), thus suggesting that membrane-bound HS proteoglycans (HSPG) might be involved in the Tat uptake and internalization process.

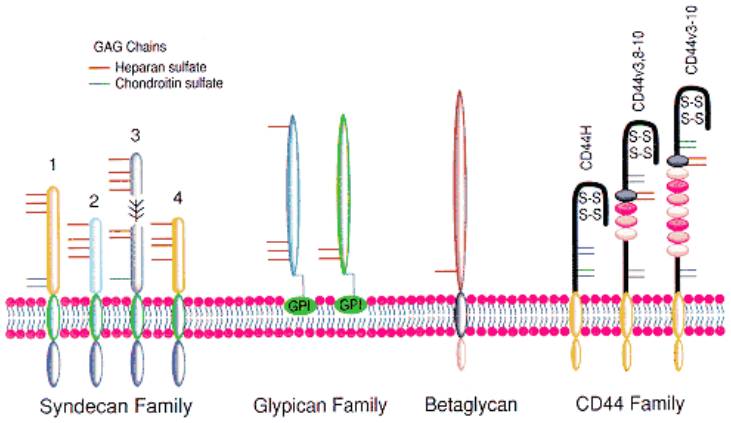

Fig. 1.19: structure of the most common HSPGs of extracellular matrix

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. 1.20**) (Tyagi *et al.* 2001).

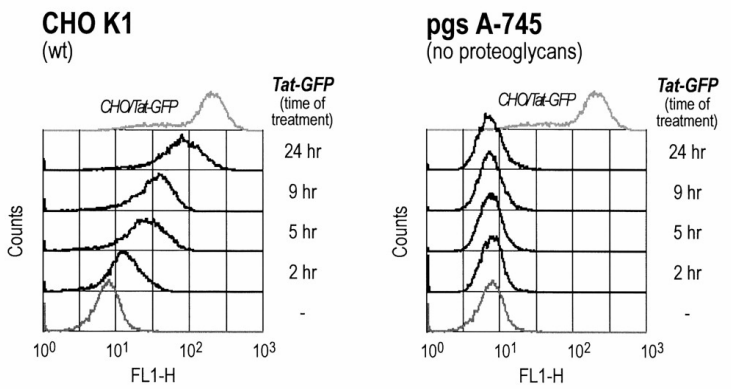

Fig. 1.20: **Hamster cell lines, genetically defective in different biochemical steps of HSPG production, fail to internalize exogenous Tat** (Tyagi *et al.* 2001).

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 binds 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 peptide motif for heparin/HS binding consists of a region rich in basic amino acids flanked by hydrophobic residues (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).

Tat binding to heparin/HS is not only determined by ionic interactions (Maccarana *et al.* 1993; Spillmann *et al.* 1998) but also requires specific structure recognition. 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 and 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.

Molecular mechanism of Tat internalization

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. The best-characterized endocytosis 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). Clathrin is involved in several receptor-mediated endocytosis 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. 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 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.

Recent studies (Ferrari *et al.* 2003; Fittipaldi *et al.* 2003) 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 (**Fig. 1.21**).

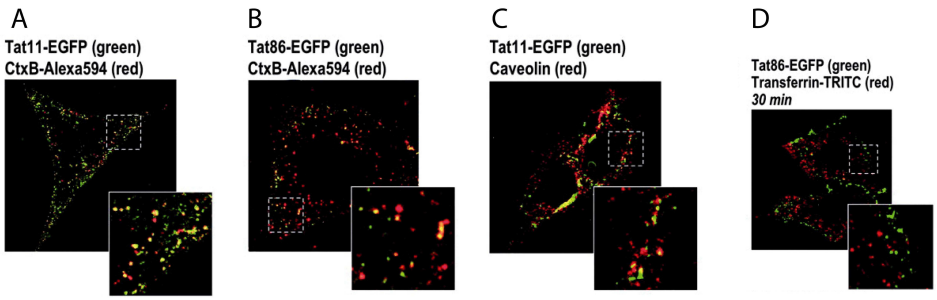

Fig. 1.21: **Internalization of Tat fusion proteins occurs through a process of caveolar endocytosis**
Internalized Tat co-localizes with Ctx-B (**A** and **B**) and caveolin (**C**), thus indicating that Tat endocytosis proceeds through caveolar lipid rafts; no localization occurs with transferrin (**D**), a protein known to be internalized by clathrin-mediated endocytosis (Fittipaldi *et al.* 2003)

The question of what might be the connection between HSPGs, acting as membrane receptors for extracellular Tat and caveolar endocytosis remains open; one of the two families of cell surface HSPGs is the glypicans, which lack a membrane-spanning domain and are anchored to the external surface of the plasma membrane via glycosylphosphatidylinositol (GPI). As GPI-anchored proteins are highly enriched in lipid rafts on the cell surface, a prediction could that the internalization of Tat might occur following its interaction with the sugar moiety of this HSPG.

A crucial question that still remains to be answered is how the internalized Tat molecules escape the endosomal compartment and translocate to the nucleus. Whichever the molecular mechanism of nuclear translocation might be, it is clear that the efficiency of the process is limiting and that only a tiny fraction of the internalized protein escapes the endosomal compartment.

Very recently, a model for Tat membrane insertion in response to acid pH has been proposed based on in vitro studies using model membranes (Yezid *et al.* 2009); upon acidification below pH 6.0, Tat inserts into membranes with a process relying on its Trp11. The conformational change leading to the exposition of Trp11 on the protein surface, allowing its interaction with membranes, is induced by low pH and involves a pH sensor made by Glu/Asp2 (the first acidic reside) and residues 55-57 (end of the basic domain)

Tat as a vehicle for transcellular transduction of proteins, nucleic acids and other cargos *in vitro* and *in vivo*

Given the potency of the Tat basic domain in mediating the cellular uptake of small and large macromolecular cargos, it is not surprising that, over 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 (Kabouridis *et al.* 2002; Wang *et al.* 2002; Krosl *et al.* 2003), but also of larger molecules or particles, including magnetic nanoparticles (Lewin *et al.* 2000), phages and retroviral vectors (Eguchi *et al.* 2001; Gratton *et al.* 2003), liposomes (Torchilin *et al.* 2001) and plasmid DNA (Ignatovich *et al.* 2003), with variable efficiency.

Besides these applications in cultured cells, Tat-mediated transcellular protein transduction has been shown to occur also in living animals *in vivo*, 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). As this observation was not confirmed in subsequent studies, however, the question about the possible therapeutic applications of Tat-mediated transcellular protein transduction *in vivo* remains open.

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. An appealing possibility in this field is to extend the bystander effect of the enzyme also to non-transduced cells by fusing Herpes Simpex Thymidine Kinase (HSV-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; Tasciotti and Giacca 2005).

Protein secretion

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 (Ensoli *et al.* 1993; Chang *et al.* 1997). Thus, the delivery of genes encoding for Tat fusion proteins *in vivo* permits the release of these proteins outside of the expressed cells, thus allowing an extension of the desired effect.

The following Chapters describe the mechanisms of canonical and non-canonical protein secretion by the cells.

"Classical" protein secretion: an overview

The secretory and endocytic pathways of eukaryotic organelles consist of multiple compartments, each with a unique set of proteins and lipids. Specific transport mechanisms are required to direct molecules to defined locations and to ensure that the identity, and hence function, of individual

compartments are maintained. Thus the localization of proteins to specific membranes is complex and involves multiple interactions.

Proteins contain structural information that targets them to their correct destination and many targeting signals have now been defined; the organelles of the secretory pathway are involved in reading this signals and sorting of proteins to a variety of intracellular membrane compartments and the cell surface. For example, proteins that are transported within the secretory pathway are alternatively secreted from the cell, bound to the plasma membrane, sorted to lysosomes, or are retained as "residents" in any of the organelles. Within the ER, newly synthesized proteins are scrutinized to ensure they are correctly folded, undergo initial glycosylation before being packaged into transport intermediates or vesicles, and then moved forward to the entry site of the Golgi apparatus. Secretory proteins are then transported through the Golgi cisternae to the *trans*-Golgi network (TGN), or Golgi exit site; at the TGN proteins are sorted according to their final destinations. The TGN is also the site where the biosynthetic and endocytic pathways converge: molecules that are internalized from the cell surface in endocytic vesicles and transported to the early endosome where they can be recycled to the plasma membrane (such as recycling receptors), or transported to the TGN or to the lysosome via the late endosomes for degradation. Thus, the TGN and the early endosome represent the two major sorting stations of the cell.

Protein transport in the secretory and endocytic pathways is a multi-step process involving the generation of transport carriers loaded with defined sets of cargo, the shipment of the cargo loaded transport carriers between compartments, and the specific fusion of these transport carriers with a target membrane. These processes involve a complex interplay of protein and lipid interactions (for an extensive review of the components of the secretory pathway, and of the molecular mechanisms underlying the formation, the transport and the sorting of vesicles to the different

destinations, as well as of the molecules involved in the regulation of such processes, see (van Vliet *et al.* 2003)).

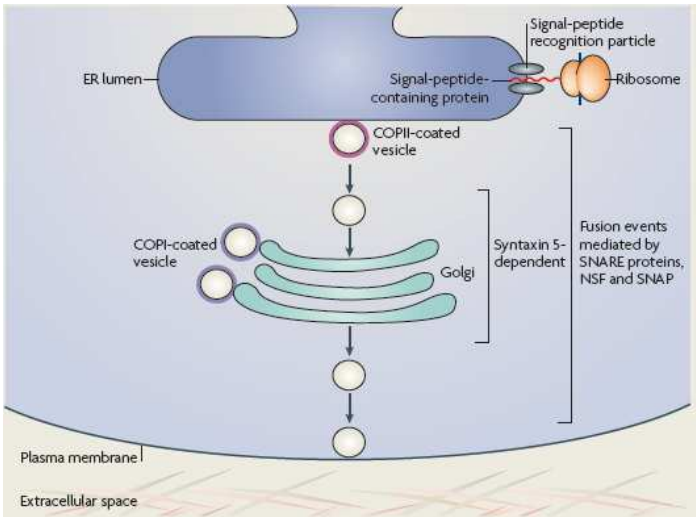

Fig. 1.22: Overview of the overall process of classical secretion (Nickel and Rabouille 2009)

Protein translation begins on free ribosomes in the cytosol. Proteins destined for secretion or for residence along the secretory pathway are targeted to the ER lumen by an N-terminal signal sequence (Walter and Johnson 1994). As the nascent polypeptide chain emerges from free ribosomes it is bound by the signal recognition particle (SRP) that directs the ribosome and nascent chain to the ER membrane. The ribosome-nascent chain-SRP complex then binds to the cytosolic side of the ER membrane via interactions with the Sec61p complex and SRP receptor (Rapoport *et al.* 1996; Matlack *et al.* 1998). The polypeptide chain is cotranslationally translocated into the lumen of the ER through the Sec61p membrane channel, referred to as the translocon, with the assistance of the translocating-chain associating membrane (TRAM) protein (Gorlich and Rapoport 1993). As the nascent chain is translocated into the lumen of the

ER the N-terminal signal sequence of soluble proteins is cleaved by a signal-specific peptidase (Martoglio and Dobberstein 1998). Transmembrane proteins are cotranslationally inserted into the membrane of the ER (Do *et al.* 1996). Within the lumen of the ER, chaperon molecules assist the folding as well as the quality control of the nascent polypeptide chains.

The majority of plasma membrane and secretory proteins are glycosylated: N-linked oligosaccharides are added to the growing polypeptide chain as it enters the ER (Kornfeld and Kornfeld 1985); O-linked glycosylation begins in the ER or *cis*-Golgi where a GalNAc residue is added to a serine or threonine residue by polypeptide N-acetylgalactosaminyl transferase (Marth 1996).

ER-Golgi trafficking

Proteins are exported from the ER at specialized exit sites called transitional elements or transitional ER (tER) (Palade 1975), smooth ribosome-free sections of the ER adjacent to the rough ER. tER-derived transport vesicles subsequently fuse to form a network of vesicular tubular clusters (VTCs) (Bannykh *et al.* 1996), also known as pre-Golgi intermediates (Saraste and Kuismanen 1992) or the ER-Golgi intermediate compartment (ERGIC). The ERGIC compartment is a major sorting station, recycling ER proteins in retrograde vesicles as well as delivering secretory cargo to the *cis*-Golgi (Warren and Mellman 1999). Peripheral ERGICs move along microtubules to the Golgi region where they fuse to form the *cis*-Golgi network, (Saraste and Kuismanen 1992; Presley *et al.* 1997).

Secretory cargos leaving the ER are packaged into vesicles possessing a specialized protein coat known as COP (Coat Protein) II; much debate has occurred over the years as to whether this transport process is selective or whether cargo is transported as part of a bulk flow process. It appears more likely that cargo leaves the ER both by selective mechanisms and bulk flow: highly abundant proteins could be transported by bulk flow mechanisms, while rare proteins would be packaged into COPII vesicles by selective

uptake (Warren and Mellman 1999). Following budding from the ER, COPII vesicles lose their coats and fuse to form ERGICs (Aridor *et al.* 1995). Another COP coat, called COPI, assembles on the membranes of the ERGICs generating vesicles for retrieval of ER residents.

The Golgi apparatus

The Golgi apparatus consists of a series of flattened membrane cisternae, called the Golgi stack, bordered by two tubulo-vesicular networks, the *cis*-Golgi network (CGN) and the *trans*-Golgi network (TGN). Long membrane tubules interconnect multiple Golgi stacks, which are arranged around the nucleus, close to the centrosome (Thyberg and Moskalewski 1999). The *cis* face of the Golgi, which receives cargo from the ER, is likely to arise from the fusion of several ERGIC elements and, as such, assists in sorting secretory cargo from ER residents as described in the previous section. The TGN sorts and packages proteins into membrane carriers destined for the plasma membrane, endosomes and regulated secretory granules. The TGN is functionally and morphologically distinct from the Golgi stack (Griffiths and Simons 1986): the TGN is a tubular reticular network and has coated buds whose coats differ from those found in the Golgi stack (Boman 2001); moreover, there are a number of distinct processing activities that are restricted to the TGN. These include: sialylation (Chege and Pfeffer 1990), proteolytic processing (Molloy *et al.* 1994), tyrosine sulphation (Baeuerle and Huttner 1987).

The TGN is a highly dynamic compartment involved in sorting of cargo for delivery to multiple destinations (**Fig. 1.23**): proteins are packaged into membrane carriers for transport to regulated secretory granules and the endosome/lysosome system (Keller and Simons 1997; Traub and Kornfeld 1997), or the apical and basolateral membranes in polarized cells. Proteins can also be recycled from the TGN to earlier compartments in the secretory pathway (Cole *et al.* 1998).

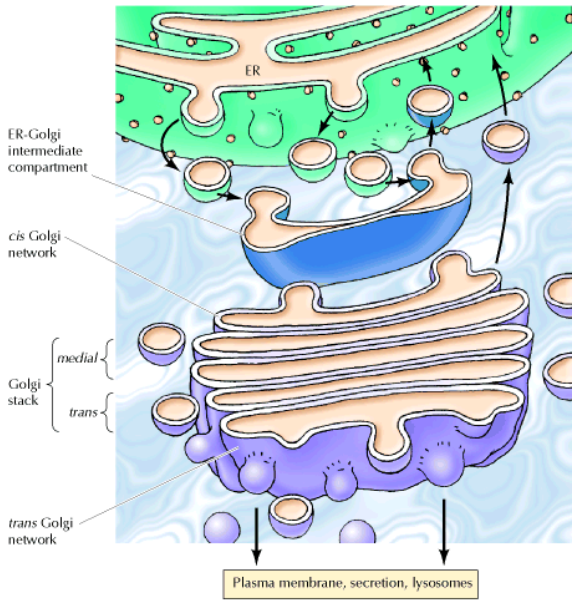

Fig. 1.23: vesicular trafficking between the ER and the Golgi apparatus (*The Cell - A Molecular Approach* Cooper, G. Sinauer Associates, Inc.; 2000)

A large number of glycosidases and glycosyltransferases reside within the Golgi. These glycosylation enzymes modify the asparagine linked structure that arrives from the ER; the presence of a particular glycosyltransferase is often used as a marker for a particular compartment.

Cisternae progression, proposed in the 1950s, and later refined by Morre (Morre and Ovtracht 1977), suggests that the individual Golgi cisternae move progressively through the stack carrying anterograde cargo.

Recent studies showed that vesicular transport and cisternae maturation need not be mutually exclusive and may in fact be occurring simultaneously (Pelham and Rothman 2000). Large molecules and protein aggregates too large to fit into vesicles could be transported inside maturing cisternae, while bidirectional vesicles could provide a "fast track" for cargo transport through the Golgi (Orci *et al.* 2000). Molecular tethers observed linking COPI vesicles

to Golgi membranes would constrain the vesicles, limiting the transfer of cargo to adjacent cisternae in the stack: budding from one cisterna and randomly fusing with an adjacent cisterna, these vesicles would result in net flow of cargo through the Golgi as newly synthesized cargo is continually added at the *cis* face and removed at the *trans* face.

Proteins destined for regulated secretion aggregate in the TGN where they are packaged into immature secretory granules (ISGs) (Thiele *et al.* 1997). Clathrin coated vesicles form on these ISGs and recycle TGN proteins, resulting in further concentration of the secretory proteins in a manner analogous to anterograde cargo concentration by removal of ER proteins from ERGICs in COPI vesicles. Mature secretory granules are stored in the cytosol until the cell receives a signal to release their contents. Signaling triggers the transport of secretory granules and their fusion with the plasma membrane thereby releasing their contents (Burgess and Kelly 1987).

Lysosomal enzymes are transported from the TGN in specialized vesicles.

Unconventional pathways of protein secretion

The vast majority of soluble proteins are characterized by an N-terminal signal peptide that promotes their trafficking through the ER and Golgi apparatus, and their subsequent delivery to the cell surface by vesicular transport. Nonetheless, over the last 15 years two types of non-conventional protein transport to the cell surface of eukaryotic cells have been revealed. The first type regards proteins that, despite having a signal peptide-driven targeting to the ER, also reach the cell surface in a COPII or Golgi independent pathway; crucial membrane bound proteins, such as CD45 (Baldwin and Ostergaard 2002) and CFTR (cystic fibrosis transmembrane conductance regulator) (Yoo *et al.* 2002), belong to this group. On the other hand, a number of secretory proteins, with defined extracellular functions, have been shown not to contain functional signal peptides and do not represent substrates for the ER membrane translocation

machinery (Cleves 1997; Hughes 1999; Nickel 2003; Prudovsky *et al.* 2003; Nickel 2005), thus pointing out the existence of an alternative pathway for protein secretion, independent from the ER-Golgi system (for a review of the mechanism of unconventional secretion, see (Nickel and Rabouille 2009)).

Signal peptide containing proteins: bypassing COPII vesicles or Golgi apparatus

Despite ER-Golgi system being very efficient and highly regulated machinery for the export of protein towards the cell membrane and the extracellular space, some signal-peptide containing proteins, directed to the ER, are able to exit this compartment bypassing either the Golgi compartment, or the COPII vesicles.

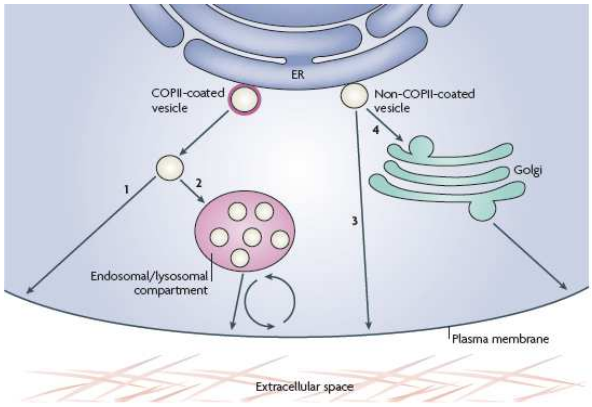

Fig. 1.24: Unconventional export of signal-peptide-containing proteins (Nickel and Rabouille 2009)

In the first case, proteins, despite containing a signal peptide, do not seem to be sensible to BFA, known to be an inhibitor of the ER-Golgi trafficking (Misumi *et al.* 1986). Most likely, after translocation into the ER, proteins are directly incorporated in COPII coated vesicles and targeted to the plasma membrane (**Fig. 1.24,** mechanism 1): such mechanism has been hypothesized for CFTR (Yoo *et al.* 2002); alternatively, COPII-coated vesicles

could be directed to an endosomal/lysosomal compartment that, in turn, fuses with the plasma membrane (**Fig. 1.24**, mechanism 2) (Nickel and Rabouille 2009).

The second case refers to proteins which are targeted to the plasma membrane through non-COPII-coated vesicles: this is the case of yeast Hsp (Heat shock protein) 150 (Fatal *et al.* 2002; Karhinen *et al.* 2005), voltage-sensitive potassium channel (Kv4 K^+) (Hasdemir *et al.* 2005) and ER degradation-enhancing α-mannosidase like protein 1 (EDEM1) (Cali *et al.* 2008). Such vesicles could be directly targeted to the plasma membrane (**Fig. 1.24**, mechanism 3) or be exported through the Golgi apparatus (**Fig. 1.24**, mechanism 4).

Secretion of proteins lacking the signal peptide for ER targeting: the "unconventional pathway"

A number of cytoplasmic, nuclear or pathogen proteins, lacking an ER-signal peptide, have been shown to be exported from cells through an ER-Golgi independent pathway. Accordingly, the extracellular appearance of such molecules is not compromised in the presence of BFA. These observations led to the postulation of the existence, in eukaryotic cells, of secretory mechanisms that are fully functional in the absence of an intact ER/Golgi system and therefore have been collectively termed "unconventional" secretory processes (Cleves 1997; Florkiewicz *et al.* 1998; Andrei *et al.* 1999; Hughes 1999; Landriscina *et al.* 2001b; Gardella *et al.* 2002; Nickel 2003; Backhaus *et al.* 2004; Hunter-Lavin *et al.* 2004; Schafer *et al.* 2004; Nickel 2005). The basic observations (summarized in (Cleves 1997; Hughes 1999)) that led to the proposal of alternative pathways of eukaryotic protein secretion are (a) the lack of conventional signal peptides in the secretory proteins examined, (b) the exclusion of these proteins from classical secretory organelles such as the ER and the Golgi combined with the lack of ER/Golgi-dependent post-translational modifications such as N-glycosylation

and (c) resistance of these export processes to BFA, a classical inhibitor of ER/Golgi-dependent protein secretion (Misumi *et al.* 1986; Lippincott-Schwartz *et al.* 1989; Orci *et al.* 1991). Intriguingly, unconventional secretory proteins comprise a group of molecules of significant biomedical relevance such as the proangiogenic growth factor FGF-2, a direct-acting stimulator of angiogenesis, produced in many tumors and metastasis (Nugent and Iozzo 2000). Other examples include lectins of the extracellular matrix (galectin family) involved in tumor-mediated immune suppression (Perillo *et al.* 1995), inflammatory cytokines such as IL-1β (Braddock and Quinn 2004) and macrophage migration inhibitory factor (MIF) (Lue *et al.* 2002) as well as a family of stage-regulated surface molecules from Leishmania parasites termed hydrophilic acylated surface proteins (HASPs) implicated in host cell infection (McKean *et al.* 2001).

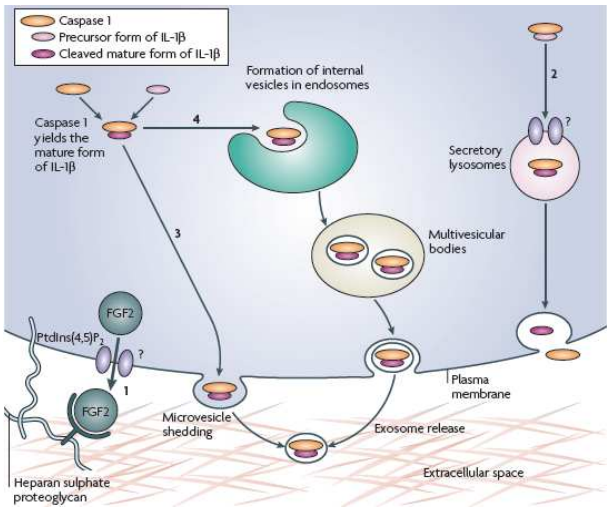

Fig. 1.25: The process of non classical protein secretion (Nickel 2003; Nickel and Rabouille 2009)

It is nowadays clear that the possibility of exporting proteins that lack the canonical N-terminal secretion signal is widespread (e.g. in mammalian cells,

bacteria, lower eukaryotes and protozoa) and seems to be a general phenomenon that could operate by several routes and affect many proteins. The most relevant proteins known to be secreted by unconventional means will be discussed in detail below.

protein name	*function*	*organism type*
FGF-1	Mitogenic and angiogenic growth factor	mammals
FGF-2	angiogenic growth factor	mammals
galectin-1	extracellular matrix	mammals
HASPB	Leishmania outer wall component	leishmania
IL-1B	cytokine	mammals
MIF	cytokine	mammals
Bet	viral accessory protein	Human Foamy Virus
Tat	viral regulatory protein	Human Immunodeficiency Virus
VP22	viral accessory protein	Herpes Simplex Virus
visfatin	insulin-mimetic,proinflammatory secreted factor	mammals
Ovine Mx1	antiviral protein	mammals
thioredoxin	cytokine	mammals
Syntaxin-2	epithelial morphogen, mediates membrane fusion	mammals
En2	homeoprotein	mammals
HMG	homeoprotein	mammals
GFP	bioluminescence	soft coral Renilla reniformis; jellyfish Aequorea victoria

Table 1.1: state of the art on the main proteins known to be secreted by unconventional routes in various organisms

Cytokines: interleukin-1β, thioredoxin and macrophage migration inhibitory factor

In 1987, Dinarello and colleagues first demonstrated that IL-1, a cytokine (Dinarello 1991; Dinarello 1997) lacking a classical signal peptide for ER/Golgi-mediated protein secretion (Auron *et al.* 1984), is exported from activated human monocytes (Auron *et al.* 1987). Two isoforms of IL-1 termed IL-1α and IL-1β have been described which represent proteolytically processed forms derived from two related but distinct precursors (Dinarello 1997).

Most studies targeted toward the molecular mechanism of interleukin 1 export have been focused on the β-isoform. A detailed molecular analysis of the export process revealed that IL-1β does not make use of an unconventional pathway of translocation into the lumen of the ER but rather appears to utilize a secretory mechanism independent of ER/Golgi-related vesicular transport (Rubartelli *et al.* 1990). This process was shown to be distinct from unspecific release as for example only the processed form of IL-1β (17 kDa) can be detected in cellular supernatants whereas the precursor (33 kDa) is retained by IL-1β-expressing cells. Moreover, under the experimental conditions applied, only the β-isoform was found to be secreted, whereas the α-isoform could not be detected in cellular supernatants (Rubartelli *et al.* 1990). However, despite apparently utilizing a distinct secretory mechanism, it was later found that IL-1α is also exported (Watanabe and Kobayashi 1994).

Though IL-1β is found in certain intracellular vesicles, as judged by protease protection experiments, these structures appear to be unrelated to the ER/Golgi system as IL-1β secretion was not inhibited but rather stimulated by BFA (Misumi *et al.* 1986; Orci *et al.* 1991). Consistently, IL-1β was found not to be glycosylated, despite bearing consensus sequences for glycosylation. Intracellular vesicles proposed to play a role in IL-1β secretion have been shown to be related to an endolysosomal compartment that

releases its content upon fusion with the plasma membrane (Andrei *et al.* 1999). These observations are consistent with the fact that IL-1β secretion is sensitive to methylamine, a drug known to interfere with endosomal recycling (Rubartelli *et al.* 1990). Based on pharmacological studies an ABC transporter, ABC1, has been implicated in the overall process of IL-1β secretion (Hamon *et al.* 1997; Zhou *et al.* 2002) and therefore might mediate IL-1β translocation from the cytoplasm to the lumen of the endolysosomal compartment. Interestingly, ABC inhibitors also appears to block non classical secretion of macrophage migration inhibitory factor (Flieger *et al.* 2003), an inflammatory cytokine mediating a number of immune and inflammatory diseases, e.g. bacterial septic shock (Bernhagen *et al.* 1998; Swope and Lolis 1999). The potential function of ABC transporters in these processes might be related to that of bacterial ABC transporters that mediate protein secretion of, for example, hemolysin (Hughes *et al.* 1992; Kuchler and Thorner 1992).

Thioredoxins are ubiquitous intracellular enzymes that catalyze thiol-disulfide exchange reactions (Holmgren 1989). Additionally, extracellular populations of thioredoxin have been detected that, similar to IL-1β and migration inhibiting factor, follow an ER/Golgi-independent route of secretion (Rubartelli *et al.* 1992; Sahaf and Rosen 2000). This observation is consistent with additional physiological roles of thioredoxin such as its function as a mitogenic cytokine that requires extracellular localization (Pekkari *et al.* 2000). Secretion of thioredoxin appears to be mediated by a pathway distinct from IL-1β as it could neither be detected in intracellular vesicles, nor was the secretion process reported to be inhibited by reagents that interfere with the function of ABC transporters. However, as with IL-1β, secretion of thioredoxin is inhibited by methylamine and stimulated by BFA (Rubartelli *et al.* 1992). Interestingly, the redox state of thioredoxin does not influence its unconventional export (Tanudji *et al.* 2003).

Pro-angiogenic growth factors: FGF-1 and FGF-2

FGF-1 and FGF-2 belong to a large family of heparin-binding growth factors (Burgess and Maciag 1989) that, apart from their mitogenic activity, are key activators of tumor-induced angiogenesis (Nugent and Iozzo 2000). The majority of the members of the FGF family are exported by ER/Golgi-dependent secretory transport. However, FGF-1 and the 18 kDa isoform of FGF-2 have been shown to be secreted by an alternative pathway (Jackson *et al.* 1992; Mignatti *et al.* 1992; Trudel *et al.* 2000). While it was first assumed that angiogenic growth factors might be released from mechanically injured tissue to promote wound healing, a process that requires angiogenesis, various lines of evidence demonstrate that FGF-1 and FGF-2 are exported from cultured cells in the absence of appreciable amounts of cell death (Jackson *et al.* 1992; Mignatti *et al.* 1992; Florkiewicz *et al.* 1995; Trudel *et al.* 2000; Engling *et al.* 2002). Like IL-1β, FGF-1 is increasingly secreted under stress conditions such as heat shock treatment (Jackson *et al.* 1992; Shin *et al.* 1996). In contrast, FGF-2 export is not affected under these experimental conditions (Mignatti and Rifkin 1991). While serum starvation has been reported to inhibit export of both FGF-2 and IL-1β, it was found to actually induce secretion of FGF-1 (Shin *et al.* 1996). Similarly, methylamine has been found to block export only of FGF-2 and IL-1β with no apparent effect on FGF-1 export (Rubartelli *et al.* 1990; Mignatti and Rifkin 1991; Shin *et al.* 1996). It was reported that expression of the IL-1α precursor inhibits FGF-1 release in response to temperature stress (Tarantini *et al.* 2001); in contrast, expression of the mature form of IL-1α did not affect FGF-1 export, suggesting that IL-1α processing is somehow related to FGF-1 biogenesis. However, whether FGF-1 and IL-1α utilize similar export mechanisms remains unclear.

These observations point to some common characteristics in the export of the cargo proteins discussed, but it seems unlikely that the same machinery mediates secretion of all these factors: while IL-1β has been reported to be

secreted by a vesicular non classical export pathway, FGF-1 and FGF-2 are likely to be directly translocated from the cytoplasm into the extracellular space. While there is also one report pointing to a role of large granules involved in FGF-2 export based on immune-EM (Electron Microscopy) analysis of mast cells (Qu *et al.* 1998), and another research group (Taverna *et al.* 2003; Taverna *et al.* 2008) demonstrated that in Sk-Hep1 cells FGF-2 release is mediated by the shedding of membrane vesicles, this issue remains controversial, as intracellular FGF-2 has been localized to the cytoplasm in many FGF-2-secreting cell types with no apparent localization in vesicular structures. Moreover, very recently a report on the characterization of the unconventional pathway of FGF-2 and Galectin-1 secretion (Seelenmeyer *et al.* 2008) puts into question the effective role of the so called membrane blebbing of vesicles in the release of the factors cited above.

With regard to the protein components involved in the overall processes of non classical export pathways, most is known about the secretion of FGF-1. As noted above, FGF-1 export is significantly increased in response to stress conditions such as heat shock treatment and serum starvation. It was shown that secreted FGF-1 isolated from cell culture supernatants in the form of homodimer represents a latent, inactive form that can be reactivated (Jackson *et al.* 1995). These observations led to the discovery of a specific cysteine residue (Cys30) in FGF-1 that is required for both dimer formation and non classical export of FGF-1 (Jackson *et al.* 1995; Tarantini *et al.* 1995). Upon heat shock treatment, two intracellular proteins have been shown to associate with the latent FGF-1 homodimer in the cytoplasm. These are a cleavage product of the transmembrane protein synaptotagmin consisting of its cytoplasmic domain (p40-Syt1) and the Ca^{2+}-binding protein S100A13. Apparently, they are exported together with FGF-1 (Landriscina *et al.* 2001b). A direct role of p40-Syt1 and S100A13 in FGF-1 export has been proposed as both repression of p40-Syt1 expression by antisense techniques

and the expression of a dominant-negative S100A13 mutant attenuate FGF-1 export. As with FGF-1 dimer formation, oxidation by Cu^{2+} cations has been demonstrated to trigger the formation of a complex consisting of FGF-1, p40-Syt1 and S100A13 (Landriscina *et al.* 2001a), while more recently, stress-induced formation of the intracellular FGF-1–p40-Syt1–S100A13 complex has been demonstrated to cause a redistribution of cytoplasmic FGF-1 to the inner surface of the plasma membrane (Prudovsky *et al.* 2002). These results suggest that FGF-1–p40-Syt1–S100A13 complex formation is the first step in the FGF-1 export pathway, followed by direct translocation of this protein complex across the plasma membrane. However, the machinery mediating this last step remains unknown.

Compared to FGF-1 export, much less is known about the mechanism and the role of specific proteins with regard to the overall process of FGF-2 export from mammalian cells. To date the only protein that has been proposed to play a role in FGF-2 export is the Na^+,K^+-ATPase (Florkiewicz *et al.* 1998). This conclusion was based initially on the observation that cardiac glycosides such as ouabain partially inhibit FGF-2 export (Smith *et al.* 2001). Further experiments demonstrated that the expression of an ouabain-resistant α 1 subunit mutant of the Na^+,K^+-ATPase rescues FGF-2 export in the presence of ouabain (Dahl *et al.* 2000). Moreover, a direct or indirect physical interaction between the α subunit and FGF-2 was detected based on co-immunoprecipitation, though this association could only be observed upon co-overexpression of both proteins (Florkiewicz *et al.* 1998). Together with the result that overexpression of the α subunit interferes with FGF-2 export (Florkiewicz *et al.* 1998), these observations are reasonably supportive of a role for the Na^+,K^+-ATPase in the overall process of FGF-2 export. On the other hand, ouabain treatment (typically used at 10–100 μm) causes only partial inhibition of FGF-2 export, whereas concentrations of ouabain of less than 5 μm (IC50 ≈ 1 μm) completely inhibit the ATP-dependent translocation of cations catalyzed by the Na^+,K^+-ATPase. Thus,

the membrane potential generated by the Na^+,K^+-ATPase is not required for FGF-2 export (Florkiewicz *et al.* 1998). Based on these observations, it has been proposed that the α/β heterodimers that constitute a functional Na^+,K^+-ATPase in terms of ion transport might be able to form higher ordered complexes that catalyze FGF-2 export in a membrane potential-independent manner. Alternatively, the α subunit alone might associate with other so far uncharacterized factors as part of a novel complex that mediates FGF-2 export. Unfortunately, no progress has yet been made in identifying such molecular structures.

Recently, it has been pointed out that FGF-2 export could be driven by direct translocation from the cytoplasm across the plasma membrane into the extracellular space (Schafer *et al.* 2004). These findings imply that quality control measures operate at this level of the export pathway, and indeed, evidence has been reported that FGF-2 secretion occurs in a folded state (Backhaus *et al.* 2004). These findings offer the fascinating possibility that the so-far uncharacterized targeting signal of FGF-2 is somehow integrated in its three-dimensional structure; the export apparatus might only accept cargo molecules that are properly folded and, in turn, would ensure quality control during protein translocation across the plasma membrane.

If no vesicular intermediate is involved in the process of secretion, how would FGF-2 be recruited at the plasma membrane before being released? This question has partially been addressed very recently, as a direct role of Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) has been demonstrated in unconventional secretion of FGF-2 (Temmerman *et al.* 2008). Briefly, FGF-2 was shown to bind this phosphoinositide (which is the principal species associated with plasma membrane) in a lipid background that resembles the plasma membrane; FGF-2 secretion was impaired by reducing cellular levels of $PI(4,5)P_2$, and likewise, variant forms of FGF-2, deficient for the binding to the phosphoinositide, were severely impaired with regards to export efficiency. The authors propose that a transient interaction between FGF-2

and PI(4, 5)P2, associated to the inner leaflet of the plasma membrane, might represent the initial step which leads to the membrane translocation and subsequent release of FGF-2 in the extracellular space.

A further addition to this model regards the possible role of the extracellular receptor of HSPGs as a "molecular trap" which may drive the export of FGF-2 outside the cells, as FGF-2 mutants that cannot bind to heparan sulfates are not secreted, and cells that do not express functional HSPGs cannot secrete wild-type FGF-2 (Nickel 2007).

Galectins: components of the extracellular matrix

The members of the galectin protein family are abundant β-galactoside-specific lectins of the extracellular matrix implicated in many cellular processes such as regulation of cell proliferation, differentiation and apoptosis (Perillo *et al.* 1998; Rabinovich *et al.* 2002). The best characterized members of this family are galectin-1 and galectin-3 which are present as soluble proteins in the cytoplasm in a wide range of vertebrate cell lines and tissues. Secreted galectins are found either bound to the extracellular surface of the plasma membrane or as abundant components of the ECM (Sato *et al.* 1993; Mehul and Hughes 1997; Seelenmeyer *et al.* 2003). Cell surface association of galectins is mediated by both N- and O-glycosylated β-galactose-terminated oligosaccharide side chains of glycoproteins as well as by galactose-containing glycolipids such as GM1. Secreted galectins are thought to affect processes such as cell differentiation by cell surface counter receptor-mediated signaling (Perillo *et al.* 1998). Classical counter receptors of galectin-1 include laminin, fibronectin and cell-type specific receptors such as T cell CD43 and CD45, and it has recently been shown that the tumor-specific cell surface antigen CA125 also represents a galectin counter receptor that preferentially binds galectin-1 (Seelenmeyer *et al.* 2003). This way, tumor cells could differentially interact with the extracellular matrix, a process crucial for tumor progression.

Similar to interleukin 1β, FGF-1 and FGF-2, galectins apparently do not contain signal peptides in their primary structure suitable for ER/Golgi-mediated secretion. Consistently, galectins are synthesized on free ribosomes in the cytoplasm and galectin secretion has been shown not to be blocked by inhibitors of the ER/Golgi-dependent pathway such as BFA and monensin (Lindstedt *et al.* 1993; Sato *et al.* 1993; Hughes 1999). Unlike IL-1β, galectin-1 and galectin-3 do not appear to be packaged into intracellular vesicles prior to export (Sato *et al.* 1993; Mehul and Hughes 1997; Hughes 1999); rather, galectin-1 and galectin-3 have been shown to accumulate directly below the plasma membrane, followed by an export mechanism that appears to involve the formation of membrane-bound vesicles that pinch off before being released into the extracellular space (Sato *et al.* 1993; Mehul and Hughes 1997; Hughes 1999). This mechanism also distinguishes galectin export from FGF-1 and FGF-2 export, as there is no evidence that these proteins are packaged into exosomes (see above). However, there is no information describing exactly what causes galectin-1 and galectin-3 to accumulate at specific spots underneath the plasma membrane, and what actually causes the formation of exosomes into which these proteins appear to be packaged in an active fashion. Very recently, moreover, it has been shown that membrane blebbing is neither induced nor it is necessary for the release of Galectin-1 from expressing cells (Seelenmeyer *et al.* 2008); thus, the molecular mechanism which leads to the release of Galectins from cells is still under debate.

It has been proposed that the interactions between Galectin-1 and its β-galactoside counter receptors are an integral part of the export mechanism itself, as it has been reported that both Galectin-1 mutants deficient in β-galactoside binding, and mutant cell lines deficient in the biogenesis of Galectin counter receptors are defective with regards to Galectin-1 secretion (Seelenmeyer *et al.* 2005). These data estabilish that the targeting motif of Galectin-1 is primarly defined by its β-galactoside-specific carbohydrate

recognition domain. Galectin counter receptors could act either at an intracellular level in order to recruit cytoplasmic galectins to the non classical export pathway, or at an extracellular level by exerting a pulling force to promote directional transport of galectins across the plasma membrane.

Other secretory proteins exported by non conventional means: HIV-Tat, Herpes simplex VP22 and foamy virus Bet

Besides the classical examples of ER/Golgi-independent protein secretion described above, other proteins have been reported to be secreted by non conventional means. Such proteins include virus-encoded factors (HSV VP22, HIV Tat and HFV Bet) that are critical for the respective viral replication cycle. The molecular mechanism of the translocation process of these proteins does not seem to involve a proteinaceous machinery as the Antp PTD has been shown to cross artificial protein-free membranes (see above). Another unusual feature of protein transduction domains is their apparent ability to translocate across membranes even at 4°C, an observation consistent with a membrane translocation mechanism independent of proteinaceous machinery. In all cases, however, protein transduction domains appear to function in unconventional modes of protein uptake by mammalian cells. Similar to herpes simplex VP22, a secreted auxiliary protein termed Bet encoded by FVs has been shown to spread between cultured cells (Lecellier *et al.* 2002) (see above). Interestingly, both VP22 and Bet are found in the cytoplasm of expressing cells whereas they are targeted to the nucleus of cells that received the protein by intercellular spreading (Elliott and O'Hare 1997; Lecellier *et al.* 2002). In both cases, this process is not affected by BFA, suggesting that export of VP22 and Bet from expressing cells does not involve the ER/Golgi system.

In the case of HIV-Tat, a region in its primary structure, termed the basic transduction domain, appears to enable the protein to traverse membranes (see above). In addition to entering cells when present in the extracellular

compartment, in facts, Tat has been demonstrated to be secreted from expressing cells, following an ER-Golgi independent pathway as its sequence lacks an N-terminal signal peptide necessary for "classical" secretion (Chang *et al.* 1997; Tyagi *et al.* 2001).

The use of an alternative pathway of secretion has been suggested to be a way to avoid the oxidizing milieu of the ER lumen (Rubartelli *et al.* 1990). Since cysteine residues present in Tat, IL-1β and FGF-2 are essential for their bioactivity, the requirement of a reducing milieu for secretion is one of the reasons for alternative secretion pathways. Native extracellular Tat is biologically active, as found for IL-1β and FGF-2, and can bind heparin, through its Tat basic peptide. *In vivo* HSPG may also exert a protective activity and be able to store biologically active Tat. Therefore, the understanding of the mechanisms of its release and extracellular fate may help in the investigation of new strategies to inhibit its activity.

Leishmania HASPB

Another quite remarkable example of non classical protein export from eukaryotic cells is the mechanism of cell surface expression of *Leishmania* HASPB (hydrophilic acylated surface protein B) which is found associated with the outer leaflet of the plasma membrane only in the infectious stages of the parasite lifecycle (Flinn *et al.* 1994; Pimenta *et al.* 1994). The protein is synthesized on free ribosomes in the cytoplasm and becomes both myristoylated and palmitoylated at its N-terminus, which is the molecular basis of how HASPB is anchored in the membrane. In the proposed model, HASPB is transferred from the cytoplasm to the outer leaflet of the Golgi membrane, from where it is transported to the plasma membrane via conventional vesicular transport (Denny *et al.* 2000). This process would insert HASPB into the inner leaflet of the plasma membrane. At present it is completely unclear how HASPB is then translocated across the membrane, resulting in the insertion of the two acyl chains in the outer leaflet of the

plasma membrane. Intriguingly, heterologous expression of various HASPB fusion proteins in mammalian cells revealed the existence of a machinery that is capable of translocating the protein across the plasma membrane (Stegmayer *et al.* 2005), demonstrating a conserved pathway among lower and higher eukaryotes. No endogenous mammalian cargo proteins that make use of this type of export system have been yet identified.

Homeodomain-containing transcription factors and HMG (high mobility group) chromatin-binding proteins

As an additional example of non classical protein export, two classes of proteins involved in the overall process of regulated gene transcription have been proposed to operate as extracellular factors even though they are normally localized to the nucleus of mammalian cells (Joliot *et al.* 1998; Muller *et al.* 2001). For the transcription factor Engrailed homeoprotein isoform 2 (En2), a potential paracrine signaling activity was postulated as a subpopulation of En2 has been localized to the cell periphery in caveolae-like structures (Joliot *et al.* 1997) In addition, a small but significant portion of total cellular En2 was found to reside in membrane-bound vesicles as judged by protease protection experiments (Joliot *et al.* 1997). Therefore, it was reasoned that En2, despite lacking a conventional ER signal peptide, might be secreted at a certain rate. This hypothesis was tested experimentally by coculturing COS cells expressing the chicken orthologue of En2 (cEn2) with rat primary neurons demonstrating intercellular transfer of cEn2 (Joliot *et al.* 1998). The internalization of homeodomain-containing proteins apparently differs from classical endocytosis, as it seems to occur by direct translocation across the plasma membrane.

About 5% of total cellular En2 becomes externalized by COS cells which is about the portion that is also found to be protected against protease treatment. An 11-amino acid sequence within the homeodomain of En2 has been identified that, when removed, causes a block in export of the

corresponding mutant protein (Joliot *et al.* 1998). This phenotype correlates with the disappearance of the mutant protein from the protease-protecting organelle, which probably represents a kind of a secretory compartment. While the homeodomain-derived peptide was originally thought to represent a signal for non classical export, this view has to be re-evaluated as it might only trigger cytoplasmic localization of En2 and may not be required afterwards for externalization of En2 (Maizel *et al.* 1999).

HMG (High Mobility Group) proteins are intranuclear factors that mediate the assembly of site-specific DNA-binding proteins within chromatin (Bianchi and Beltrame 2000). As a surprising finding, but similar to the homeodomain-containing transcription factors described above, HMGB1 is secreted during certain physiological processes such as inflammation: specifically, monocytes have been shown to export HMGB1 upon stimulation with bacterial lipopolysaccharides (Wang *et al.* 1999). As antibodies against HMGB1 suppress LPS-induced endotoxemia, and injection of HMGB1 protein into mice causes toxic shock, HMGB1 apparently acts as a mediator of endotoxin lethality in mice (Wang *et al.* 1999). Interestingly, HMGB1 export competence appears to be a special property of a limited number of cell types (such as monocytes and macrophages) as many cell types including lymphocytes are not capable of secreting HMGB1 (Wang *et al.* 1999). Again, similar to homeodomain-containing transcription factors, extracellular HMGB1 has also been shown to act as both an autocrine and paracrine signaling molecule promoting differentiation processes of the HMGB1-secreting cell or other cells nearby (Passalacqua *et al.* 1997).

HMGB1 does not contain a signal peptide for translocation into the ER (Muller *et al.* 2001); similar to IL-1β, FGF-2 and galectin-3, a rise in intracellular Ca^{2+} triggers HMGB1 export (Muller *et al.* 2001). HMGB1 has been observed to redistribute from the nucleus to the cytoplasm upon activation of monocytes; a detailed ultrastructural analysis revealed that redistributed HMGB1 localizes to an endolysosomal compartment from which

secretion can be triggered by stimuli known to promote lysosomal exocytosis (Gardella *et al.* 2002). However, unlike IL-1β secretion from monocytes, HMGB1 release is induced by lysophosphatidylcholine. Moreover, the kinetics of IL-1β and HMGB1 release from monocytes differ significantly, with IL-1β being secreted early after monocyte activation and HMGB1 at a later stage. These results have been taken to indicate that lysosomal exocytosis might involve distinct populations of endolysosomal vesicles, thereby allowing different kinetics of cargo release (Gardella *et al.* 2002).

Cytoplasmic clearance of unfolded proteins by non classical secretion

The mitochondrial matrix protein Rhodanese, a monomeric sulfotransferase that, following synthesis on free ribosomes in the cytoplasm, is normally imported into mitochondria represents another unusual example of non classical protein export from mammalian cells. When overexpressed in HEK-293 cells from a strong viral promoter, about 40% of total Rhodanese was found to be secreted into the culture medium (Sloan *et al.* 1994). Export was shown to occur in the absence of appreciable amounts of cell death and to depend on neither the mitochondrial targeting sequence of Rhodanese nor a functional ER/Golgi system (Sloan *et al.* 1994). Based on the observation that Rhodanese acquires its enzymatic activity only after import into the mitochondrial matrix (and that the signal peptide cannot be an inhibitor of enzymatic activity as it is not cleaved off in the matrix), it was concluded that the population present in the cytoplasm remains unfolded before import into mitochondria. Therefore, it has been postulated that the export pathway detected for Rhodanese represents a mechanism for clearing the cytoplasm of unfolded proteins that apparently accumulate upon overexpression. More recently, a potentially similar example of cytoplasmic clearance of an unfolded protein population possibly generated by overexpression has been observed (Tanudji *et al.* 2002). In this case, an unfolded subpopulation of transiently overexpressed GFP (Green Fluorescent

Protein) was found to be secreted in a BFA-insensitive manner. This effect has not been observed in stable cell lines that express moderate levels of GFP in a doxicycline-dependent manner (Engling *et al.* 2002). However, these different observations are not necessarily inconsistent as in the latter case an unfolded population of GFP is unlikely to exist. Interestingly, methylamine and other drugs known to inhibit non classical export of substrates such as IL-1β, FGF-2, thioredoxin, and the galectins (see above) do not block externalization of Rhodanese or unfolded GFP, again suggesting the existence of distinct molecular mechanisms of unconventional protein secretion.

Non classical secretion in yeast.

Yeast cells have a thick wall that maintains cell shape, protects against osmotic stress and requires modification for cell growth. Covalently linked cell-wall proteins carry the typical N-terminal signal peptide that drives their export; moreover, several proteins carrying an N-terminal secretion signal peptide, upon release from the plasma membrane, are presumably trapped in the cell wall at some stage.

However, the evidence accumulated during the past decade using the current yeast models, clearly shows that many proteins that lack an N-terminal signal peptide also reach the cell surface.

In particular, *C. albicans* and *S. cerevisiae* share 11 signal-less proteins in common; a further 26 have been described only in the former, compared to five identified in the latter yeast species only. Many of the surface proteins lacking the N-terminal signal peptide are also found in the cytoplasm, where they perform well-known functions (**Table 1.2**).

Protein (gene)	Cytoplasmic function	Function at cell wall	Ca[a]	Sc[a]	Refs
Enolase (*ENO*)	Glycolysis	Changes with drug exposure in *Ca*	+[b]	+	[12,14,16,41]
Phosphoglycerate kinase (*PGK1*)	Glycolysis	Unknown	+	+	[12,17,42,43]
Phosphoglycerate mutase (*GPM1/2*)	Glycolysis	Changes with drug exposure in *Ca*; binds to plasminogen	+	+	[12,23,41,43]
Glyceraldehyde-3-phosphate dehydrogenase (*TDH1–3*)	Glycolysis	Binds to laminin and fibronectin; enzymatic activity; binds to plasminogen	+	+	[12,23,44]
Fructose 1,6-bisphosphate aldolase (*FBA1*)	Glycolysis	Antigen	+	+	[12,13,43]
Alcohol dehydrogenase (*ADH1*)	Glycolysis	Binds to plasminogen; binds to fibronectin and vitronectin	+	+	[12,17,24,41, 43,45,46]
Pyruvate decarboxylase (*PDC11*)	Glycolysis	Unknown	+	+	[12,17,43]
Triose phosphate isomerase (*TPI1*)	Glycolysis	Unknown	−	+	[12,43]
Hsp70 (*SSA1/2*)	Chaperone	Histatin 5 and defensin 1 receptor	+	+	[12,17,19,47–49]
Hsp70 (*SSB1/2*)	Chaperone	Unknown	+	+	[12,13,17,43]
HSP70 (*SSZ1*)	Chaperone	Unknown	+	−	[12]
Heat-shock protein (*SSC1*)	Stress, protein folding and protein import	Unknown	+	−	[16]
HSP70 (*SSC1*)	Stress	Unknown	+	−	[17]
Protein phosphatase (*SSD1*)	Cell-wall integrity	Unknown	+	−	[12]
Catalase (*CTA1*)	Reactive O_2 metabolism	Binds to plasminogen	+	−	[23]
Thioredoxin peroxidase (*TSA1*)	Redox homeostasis	Binds to plasminogen	+	−	[17,23]
Cytochrome *c* haem lyase (*CYC3*)	Cytochrome *c* synthesis	Preferential hyphal cell wall	+	−	[50]
Cell-surface hydrophobicity protein (*CSH1*)	Aryl-alcohol dehydrogenase	Cell-surface hydrophobicity	+	−	[17,51]
Pyruvate carboxylase (*PYC2*)	Gluconeogenesis	Unknown	+	−	[17]
Translational elongation factors (*TEF1*)	Protein synthesis	Unknown	+	−	[12,17]
Translational elongation factors (*TEF2*)	Protein synthesis	Unknown	+	−	[12,17]
Translational elongation factors (*EFT3*)	Protein synthesis	Unknown	+	−	[12,17]
Transketolase (*TKL1*)	Glycolysis	Unknown	+	−	[17]
14-3-3 protein (*BMH1*)	Regulation	Unknown	+	+	[12,17,43]
S10 ribosomal protein (*RPS6*)	Translation	Unknown	+	−	[17]
Succinate dehydrogenase (*SDH2*)	Tricarboxylic acid cycle	Unknown	+	−	[17]
HSP90 and 47-kDa fragment (*HSP90*)	Stress	Unknown	+	−	[17]
Pyruvate kinase (*PYK1*)	Glycolysis	Unknown	+	+	[17,43]
GDP-mannose phosphorylase (*SRB1/PSA1*)	Glycosylation	Unknown	+	−	[17]
S-adenosylmethionine synthase (*SAM2*)	Methionine metabolism	Unknown	+	−	[17]
Microsomal ATPase (*CDC48*)	Secretion, peroxisome formation and retrotranslocation of ubiquitinated proteins	Unknown	+	−	[12]
Myo-inositol-1-phosphate synthase (*INO1*)	Lipid biosynthesis	Unknown	+	−	[12]
General amino acid permease (*GAP3*/Orf6.713/19.3195)	Metabolism	Unknown	+	−	[12]
Kel1p (*KEL1*)	Cell fusion and morphogenesis	Unknown	−	+	[43]
Glutathione reductase (*GLR1*)	Oxidative stress	Unknown	−	+	[43]
Thiol-specific peroxiredoxin (*AHP1*)	Redox homeostasis	Unknown	−	+	[43]
Family of flavodoxin-like proteins (*YDR032C/PST2*)	Unknown. Responds to oxidative stress	Unknown	−	+	[43]
Part of spliceosome (*PRP46*)	mRNA metabolism	Unknown	−	+	[43]
UTP–glucose-1-1-phosphate uridylyl transferase (*UGP1*)	Protein glycosylation	Unknown	+	−	[17]
Coproporphyrinogen haem oxidase (*HEM13*)	Haem biosynthesis	Unknown	+	−	[17]
Glycogen phosphorylase (*GPH1*)	Glycogen metabolism	Unknown	+	−	[17]
Ubiquitin carboxyl-1-terminal hydrolase (*UBP1*)	Protease	Unknown	+	−	[17]
F1F0 ATPase F1 α subunit (*ATP1*)	Proton transport	Unknown	+	−	[17]
Probable NADPH dehydrogenase (EBP1)	'Old yellow' enzyme; binds to echinocandin	Unknown	+	−	[17]

[a]Abbreviations: *Ca*, *C. albicans*; *Sc*, *S. cerevisiae*.
[b]Plus (+) symbol refers to proteins detected at the yeast cell surface of *C. albicans* or *S. cerevisiae*; minus (−) symbol refers to proteins that are not detected.

Table 1.2 : N-terminal secretion-signal-less proteins detected at the cell surface of *Candida albicans* and *Saccharomyces cerevisiae* (Nombela *et al.* 2006)

The available evidence indicates that signal-less proteins are targeted outside the membrane as a result of alternative secretion processes that are not based on the canonical secretion-signal peptide.

The extracellular presence of several members of at least three groups of proteins with different functions has been characterized:

- a first group consists of enzymes involved in glycolysis;
- a second group consists of heat-shock proteins with the capacity to function as chaperones;
- a third group consists of translation elongation factors, i.e. proteins that interact with complexes of other proteins and RNA.

The presence of so many non-conventional proteins at the yeast cell surface demands experimental proof that these proteins have the capacity to target the external medium; indeed, this has been confirmed in some cases.

As proteins belonging to all the three groups have been described as important components of microbial virulence of several distinct bacteria and microorganisms, the connection between non-conventional surface proteins with microbial virulence reinforces the interest in the phenomenon of alternative secretion.

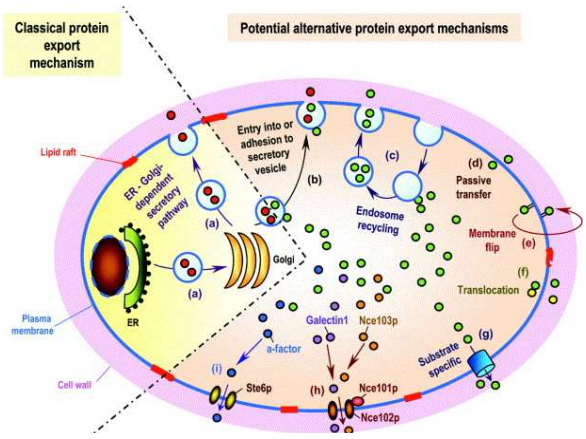

Fig. 1.26 : potential routes of non conventional secretion in yeast
(Nombela *et al.* 2006)

Given the diversity of non-conventional cell-surface proteins in yeast, several possibilities for the release of these proteins have been proposed (**Fig. 1.26**).

Targeting motifs and regulation of non classical protein export

In many cases of intracellular protein sorting, short, linear amino acid sequences have been identified that serve as sorting motifs, including N-terminal signal peptides for ER translocation and the N-terminal targeting signals of mitochondrial proteins (Schatz and Dobberstein 1996). Currently, very limited information is available about motifs directing proteins to the various pathways of unconventional protein secretion described above. The most defined one is that of Leishmania HASPB, which consists of a linear sequence of 18 amino acids at the extreme N-terminus referred to as HASPB-N18 (Denny *et al.* 2000). This sequence is both necessary and sufficient to direct a corresponding fusion protein to the HASPB export pathway in both parasites and mammalian cells. The most recent studies suggest that acylation might only be required to initially insert the protein into the membrane, and that the translocation that follows requires an interaction of the proteinaceous part of HASPB-N18 with the putative export machinery. Based on these characteristics, the HASPB export pathway appears to be unrelated to all other previously described examples of non classical protein export. As the pathway is functional in mammalian cells, endogenous substrates are likely to exist. However, the 18-amino acid sequence found at the N-terminus of HASPB is not only absent from other secretory proteins exported by unconventional means but is also not found in any mammalian protein.

Similar to Leishmania HASPB, the N-terminus of galectin-3 has been proposed to contain targeting information for non classical export (Menon and Hughes 1999). When the first 120 amino acids of galectin-3 are deleted, the residual portion of the protein is no longer secreted. Conversely, addition

of this N-terminal segment to a cytosolic protein directs the corresponding fusion protein to the galectin-3 export pathway. A short sequence comprising residues 89–96 (based on the hamster amino acid sequence) was identified that, upon deletion, impairs galectin-3 export. However, the addition of this small peptide to a cytosolic protein is not sufficient to direct the resulting fusion protein to the galectin-3 export pathway suggesting that, besides the critical role of this short segment, other determinants for non classical export exist in the N-terminal part of galectin-3 (Menon and Hughes 1999). When compared to the galectin-1 amino acid sequence, no significant homologies can be found within the N-terminal 120 amino acids between the two proteins.

In contrast to HASPB and galectin-3, the C-terminal half of FGF-1 has been implicated in its temperature- and stress-induced release (Shi *et al.* 1997). A domain comprising a stretch of amino acids from position 83–154 (based on the human FGF-1 orthologue) appears to prevent the protein from entering the nucleus, a step which has been suggested to be a prerequisite for unconventional export. When such domain was substituted with the corresponding C-terminal domain of FGF-2, however, secretion of the resulting hybrid protein was no longer observed. These data have been taken to mean that FGF-1 and FGF-2 are exported by distinct pathways (Shi *et al.* 1997), which is consistent with the observation that only FGF-1 release can be triggered by temperature stress. The actual targeting motifs for non classical export have not been revealed for either FGF-1 or FGF-2.

For the homeodomain-containing transcription factor En2, it has been suggested that an 11-amino acid motif within the homeodomain may function as a signal for non classical export (Joliot *et al.* 1998). As discussed above, this sequence was later found to be part of a nuclear export signal suggesting that nuclear export of En2 is a prerequisite for its unconventional secretion. At this point, it is not clear whether this segment of En2 (residues 146–169) is part of a signal sequence for non classical export or whether

this domain regulates access of En2 to its export pathway. In either case, the information for En2 export must lie within the En2 homeodomain, as this part of the protein alone is an efficient substrate for intercellular transfer.

Similar to En2 export, many of the proteins described here are exported in a regulated fashion. For example, IL-1β and HMGB1 can be released from monocytes upon stimulation with reagents that induce an inflammatory response (Rubartelli *et al.* 1990; Muller *et al.* 2001). At the same time, En2, IL-1β and HMGB1 are those factors among unconventionally secreted proteins that appear to be exported from an endosomal subcompartment (Joliot *et al.* 1998; Andrei *et al.* 1999; Gardella *et al.* 2002), which might be interpreted as some kind of storage mechanism from which regulated secretion of these factors can be triggered. On the other hand, as discussed above, non classical export of FGF-1 and galectin-1 are also regulated inducible processes, yet there is no evidence that these factors are packaged into intracellular vesicles prior to secretion. The export process of galectin-1 has been shown to be regulated based on cell differentiation (Lutomski *et al.* 1997). Similarly, FGF-2 export has recently been shown to be triggered by the expression of the Epstein–Barr virus (EBV) protein LMP-1 (Wakisaka *et al.* 2002). This mechanism requires a functional NF-κB signaling pathway, possibly indicating that LMP-1-mediated stimulation of FGF-2 export involves the induction of a protein machinery based on *de novo* synthesis.

As illustrated in **Fig. 1.27**, at least four distinct pathways of unconventional protein secretion may exist in mammalian cells that are fully functional in the absence of an intact ER/Golgi system. These pathways are in general attractive targets, because it may be possible to identify inhibitors that do not interfere with the essential function of the classical secretory pathway.

However, a biochemical analysis of the molecular machineries of non classical protein export has proven difficult because in many cases the export process is relatively inefficient.

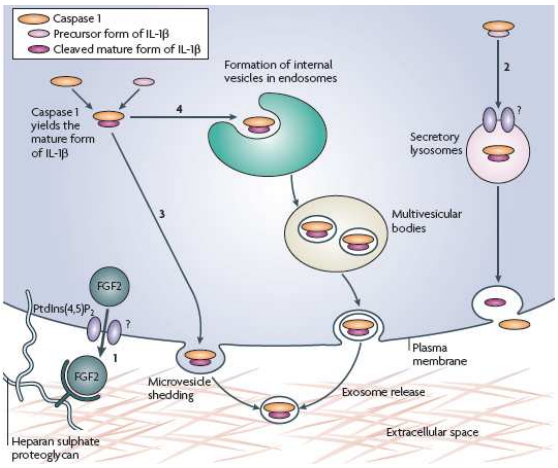

Fig. 1.27: proposed mechanism for non classical protein secretion (Nickel and Rabouille 2009)

In particular, it is not yet clear (with the noticeable exception of FGF-2 (Backhaus *et al.* 2004)) whether unfolding of the various cargo proteins is required for unconventional secretion and whether the non classical pathways of secretion could have a quality control machinery for secreted proteins. In the case of the classical secretory pathway, quality control occurs at the level of the ER in that secretory proteins not being folded properly do not have access to transport vesicle-mediated exit form this compartment (see above) and are targeted for degradation. For FGF-2 and HASPB, whose export mechanisms seem to rely on plasma membrane resident transporters (see above), it is likely that protein translocation is in some way coupled to a mechanism that ensures the secretion of properly folded protein. Evidence comes from experiments using FGF-2 fusion proteins with dihydrofolate-reductase (DHFR); aminopterine-mediated conformation stabilization of FGF2-DHFR did not have any impact on export efficiency suggesting that FGF-2 membrane translocation does not occur in an unfolded state (Backhaus *et al.* 2004). Moreover, very recently, Torrado and colleagues (Torrado *et al.* 2009) demonstrated that the folded

conformation is necessary for FGF-2 to interact with $PI(4,5)P_2$ which constitutes the first step leading to membrane translocation (see above); thus, at least for FGF-2, the existence of an intrinsic quality control mechanism which ensures secretion of the protein in a properly folded conformation can be hypothesized.

Caspase-1: a common regulator of unconventional protein secretion?

Until very recently, a common regulator involved in the secretion process of multiple proteins was lacking; however, in 2008, the work of Keller et al. (Keller *et al.* 2008) reported that active caspase-1 might be involved as a regulator in the unconventional secretion of several proteins, including IL-1β, IL-1α , thioredoxin, and FGF-2.

Caspase-1 has a well-established function in inflammation via the activation of proinflammatory cytokines (Dinarello 1998). In addition, it has been reported to mediate either cell death (Cookson and Brennan 2001) or survival (Gurcel *et al.* 2006) upon infection by certain pathogens. The authors found that activation of caspase-1 by inflammasome complexes is directly linked to unconventional secretion of a variety of leaderless proteins, most likely via a direct or indirect physical interaction.

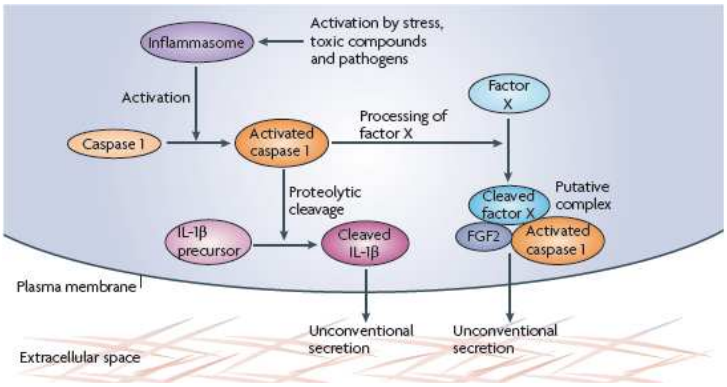

Fig. 1.28: proposed role for caspase-1 in the secretion of inflammatory cytokines (Nickel and Rabouille 2009)

Interestingly, many of the proteins that are secreted in a caspase-1-dependent manner are involved in inflammation, cytoprotection, and/or tissue repair. Therefore, activation of caspase-1 by various environmental challenges, e.g., UV radiation, infection, or wounding, seems to be coupled to the release of proteins, which might help to restore the cell and tissue homeostasis. Following this intriguing hypothesis, caspase activation would not only induce inflammation via secretion of proinflammatory cytokines, but also regulate the extracellular levels of proteins involved in tissue repair and cytoprotection. Therefore, it may provide a direct link between inflammation and regenerative processes.

Emerging roles of GRASP proteins

GRASP proteins associate with the Golgi apparatus and have been implicated in the stacking of Golgi cisternae, vesicle tethering, and mitotic progression, however their specific functions are unclear.

Recently, GRASP function has been studied in *Dictyostelium discoideum* - which has a single GRASP homolog GrpA – by targeted gene disruption, revealing that a GrpA-deficient strain grows at wild-type rates and exhibits normal trafficking of several secretory and lysosomal proteins (Kinseth *et al.* 2007). However, despite the Golgi gross structure, as seen by fluorescent microscopy, is unperturbed in GrpA-deficient cells, these cells are defective in producing viable spores. This defect was traced back to a strong block in secretion of the acyl-CoA binding protein AcbA, which is cleaved outside the cell to produce a spore differentiation factor. AcbA lacks a conventional secretory signal sequence and, in GrpA-deficient cells, accumulates in the cytoplasm. Therefore, at least in *Dictyostelium*, GRASP is not generally required for the activity of the Golgi in conventional secretion but, instead, is required for an unconventional secretory pathway. This new twist on GRASP function may open new views and investigations on the role of GrpA homologues in higher eukaryotes.

In a possible scenario, AcbA is transported from the cytosol into the internal vesicles of endosomal multivesicular bodies (MVBs), which subsequently fuse with the plasma membrane to release the internal vesicles as exosomes. GrpA would be needed at some stage of this process: (Figure 1A) GrpA may shuttle between the Golgi and the plasma membrane and operate as part of the MVB fusion machinery; in this model, GRASPs act as a go-between to coordinate the activities of the conventional and unconventional secretory pathways. In the second model (Figure 1B), GrpA operates exclusively at the Golgi and is selectively required for the biosynthetic transport of a plasma membrane component of the MVB fusion machinery. Hence, further insight into *Dictyostelium* sporulation may illuminate the role of GRASPs in protein transport.

Of course, variations on these models can be envisioned. For example, GrpA function might be required at the step of AcbA internalization into MVBs or for an unconventional secretory pathway that is independent of MVBs. Regardless, the elegant work of Kinseth et al. (2007) has revealed a surprising link between conventional and unconventional secretion.

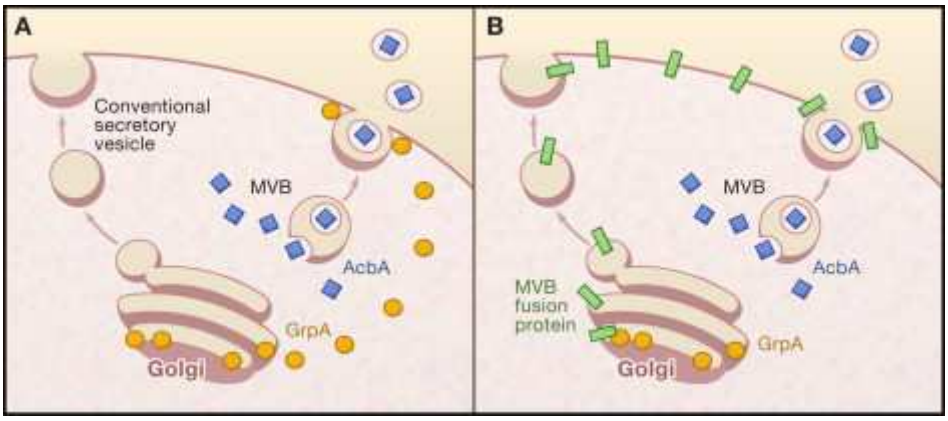

Fig. 1.29 : GrpA in Unconventional Secretion (Levi and Glick 2007)

Interestingly, the *D. melanogaster* GRASP protein has been recently shown to be required for the unconventional secretion of integral plasma membrane α integrins (Schotman *et al.* 2008), a signal-peptide-containing protein, requiring specific spatially restricted deposition for membranes to undergo remodeling. At the membrane, α integrins induce the formation of new focal adhesion sites that are required for epithelium integrity.

In a *D. melanogaster* GRASP mutant, however, the α integrin (but not the β integrin) subunits are not properly deposited at the plasma membrane. Instead, they are retained intracellularly and, as a result, the epithelium is disrupted, suggesting the existence of a GRASP-dependent secretory route that is specific for α integrin. Furthermore, this route bypasses the Golgi complex as it is not sensitive to BFA and is fully functional in the absence of syntaxin 5. Of note, the Golgi-independent transport of α integrins only occurs during epithelium remodeling, while under all other conditions, α and β integrins are transported as heterodimers through the conventional secretory pathway (Schotman *et al.* 2008). Why α integrin transport switches from conventional to non-conventional mechanisms during epithelium remodeling is currently not understood.

Although this transport process is clearly different from AcbA secretion, GRASP might have a similar role to GrpA, for example, acting as a plasma membrane-resident tethering factor for ER-derived carriers that are enriched in α integrins or bind to α integrin tails in the ER, or could be involved in the sorting or chaperoning of a fusion factor to the plasma membrane, thereby mediating the fusion of late endosomes or the deposition of integrins at the plasma membrane. GRASP could also have a common role in the unconventional delivery of integrins to the plasma membrane and in the release of AcbA into the extra cellular space, similarly to the proposed role for GrpA in tethering AcbA enriched endosomes (**Fig. 1.30**).

The underlying mechanisms driving GRASP anchoring at the plasma membrane, GRASP mediated tethering of the incoming membrane

compartment, eventual homologous functions of mammalian GRASPs, remain to be elucidated.

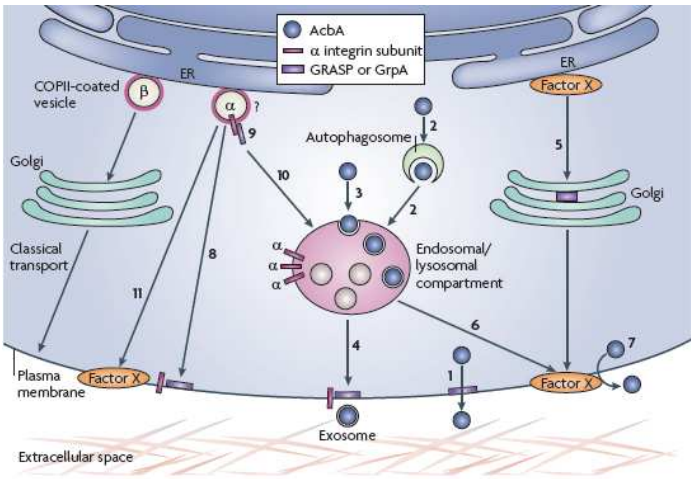

Fig. 1.30: proposed role for GRASP in unconventional secretion (Nickel and Rabouille 2009)

Unconventional secretion and evolution: integrating complex functions

The maintenance of organ homeostasis and the control of an appropriate response to environmental alterations require the intimate coordination of cellular functions and tissue organization. Recently, it has become clear that an important component of this coordination is provided by proteins that can have distinct but linked functions on both sides of the plasma membrane. These proteins often transit the plasma membrane by mechanisms that are distinct from the classical ER-Golgi secretory process, and their multifunctionality is puzzling - why should a protein with a well-known function in one context function in such a distinct way in another? One proposed reason for this is so that the protein can coordinate the organization and maintenance of a global tissue function (Radisky *et al.* 2009). Although the locations of their distinct molecular activities are

separated by the physical barrier of the membrane, their dual roles might coordinate a single tissue function and thus be equally important in an integrated overall purpose.

Although not all of the proteins that are released by unconventional secretion have distinct but linked functions, unconventional mechanisms of secretion might be important in enabling dual but integrated functionality for single proteins: in some cases, an alternative secretion pathway might be required when transport through the ER-Golgi results in protein modifications that interfere with the extracellular function of a protein, as it has been shown for FGF-2 (Wegehingel *et al.* 2008).

Moreover, the lack of an exocytosis signal sequence and the use of unconventional secretory pathways by proteins that have paired functions allow for greater flexibility in compartmental distribution: whereas the presence of a strong exocytosis signal in the protein would require extraordinary mechanisms by the cell to retain the protein, unconventional pathways might allow for flexible and dynamic regulation of protein localization in response to various internal and external stimuli, and thus might facilitate more intimate linkage of intracellular and extracellular functions.

Speculatively, it has been hypothesized (Radisky *et al.* 2009) that unconventional secretion mechanisms might have developed early in evolution, before the formation of the ER–Golgi pathway. For these early cells, it would have been crucial to coordinate cellular function with intercellular communication. These mechanisms might have been retained because they allow for rapid connection between diverse processes.

Na^+,K^+-ATPase structure, functions and regulation

Na^+,K^+-ATPase (Na-pump) is an enzyme embedded in the plasma membrane which catalyzes ATP hydrolysis coupled with it Na^+ and K^+

transfer through the membrane against the electrochemical gradient, in accord with the following equation:

$$\mathbf{ATP + 3Na_{in}^{+} + 2K_{out}^{+} \rightarrow ADP + P_i + 3Na_{out}^{+} + 2K_{in}^{+}}$$

(where Na_{in}^{+} and K_{in}^{+} are intracellular ions and Na_{out}^{+} and K_{out}^{+} are extracellular ions)

Thus, Na^+,K^+-ATPase, which is present in all animal cells, performs the electrogenic exchange of intracellular Na^+ for extracellular K^+.

The Na^+,K^+-ATPase is a member of the family of P-type ATPases; other ion pumps that transport biologically significant cations (gastric mucosa H, K-ATPase, Ca-ATPases of plasma membrane and sarco (endo) plasmic reticulum (SERCA ATPases)), H-ATPases from fungi and plants, and some ATPases of prokaryotes are also members of the P-type ATPase family (Moller *et al.* 1996).

Discovered more than 40 years ago, the Na^+,K^+-ATPase continues to attract the attention of the researchers today. However, whereas soon after the discovery of this enzyme most efforts were directed toward the study of the structure and function of the enzyme, more attention is now given to the problem of its regulation.

Na^+,K^+-ATPase structure

The Na^+,K^+-ATPase consists of two polypeptide chains: the catalytic alpha-subunit and the beta-subunit, which is a glycoprotein that does not participate in the catalysis directly. The molecular masses of the alpha- and beta-subunit are 110 and 55 kD, respectively. Both subunits are integral membrane proteins and are bound to each other by noncovalent interactions: in purified preparations of Na^+,K^+-ATPase they are present in molar ratio 1 : 1.

The crystal structure of the pig renal Na^+,K^+-ATPase has been recently elucidated (Morth *et al.* 2007). The polypeptide chain of the Na^+,K^+-ATPase alpha-subunit is packed into the lipid bilayer forming 10 transmembrane

alpha-helical segments; the N- and C-termini of the chain are located in the cytosol. Most amino acid residues are also located inside the cell forming a large cytoplasmic domain; significantly less of the alpha-subunit polypeptide chain is exposed outside the cell (**Fig. 1.31**).

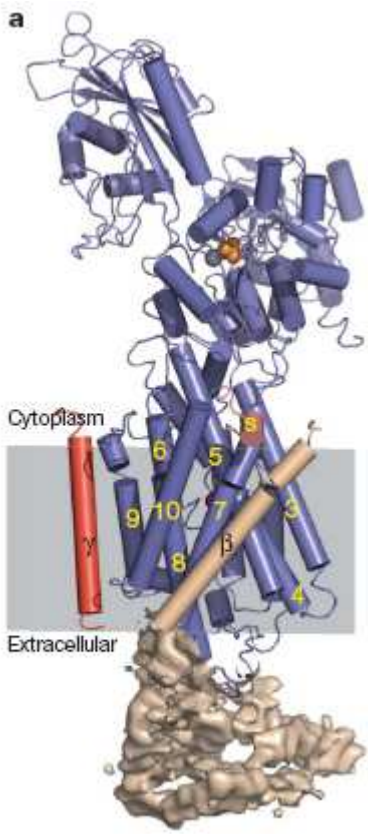

Fig. 1.31: proposed 3D-structure and membrane topology of the plasmalemmal Na^+,K^+-ATPase, deducted from X-ray analysis (Morth *et al.* 2007)

The transmembrane segments embedded in the membrane are in a close proximity and appear to participate in the formation of the channel through which cations cross the membrane. High resolution X-ray analysis of Na^+,K^+-

ATPase disclosed a structure very similar to the sarcoplasmic Ca-ATPase (Toyoshima *et al.* 2000), a protein closely related to the Na^+,K^+-ATPase alpha-subunit (the homology in amino acid sequence is only about 20%, but hydropathy profiles and apparent three-dimensional structure are very similar).

The polypeptide chain of the beta-subunit crosses the membrane only once (Kirley 1989); a small N-terminal segment of the chain is located in the cytoplasm, whereas most of the subunit is located outside the cell (see review (Therien and Blostein 2000)). Amino acid residues located on the extracellular part of the protein in close proximity to the C-terminal fragment of the beta-subunit appear to participate in the association of the beta-subunit with the alpha-subunit (Beggah *et al.* 1993), as does its transmembrane fragment, which interacts with transmembrane fragments 9-10 of the alpha-subunit (Sarvazyan *et al.* 1995).

There are numerous experimental data demonstrating that membrane Na^+,K^+-ATPase is an oligomer (most likely an asymmetric tetramer (Askari 2000)) consisting of 2-4 alphabeta-protomers. Both negative (Askari 2000) and positive (Therien and Blostein 2000) cooperative interactions are characteristic for this structure.

The formation of oligomeric complexes of Na^+,K^+-ATPase is due to the interaction between the alpha-subunits of the enzyme. A peptide containing about 150 amino acid residues and located within the large cytoplasmic domain (Pro561-Gln709) appears to play a significant role in the process of association of alpha-subunits (Koster *et al.* 1995). Solubilized non-denatured Na^+,K^+-ATPase is a mixture of associating-dissociating protomers, the relative amount of which depends on protein and ligand concentrations. However, there is evidence showing that the lipid bilayer in which enzyme is embedded and the interaction with protein-partners control the interactions between Na^+,K^+-ATPase protomers (Askari 2000).

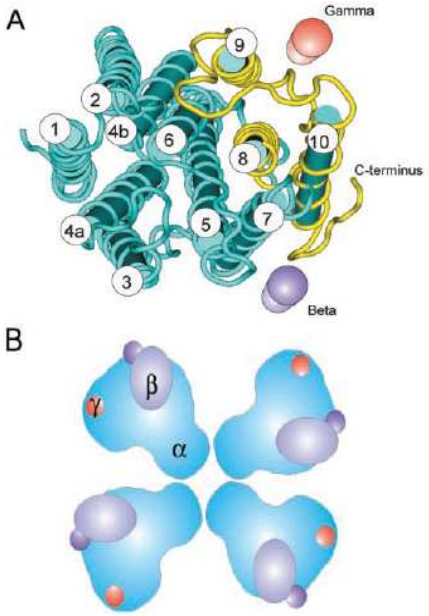

Fig. 1.32: proposed arrangement of the different subunit in the Na^+,K^+-ATPase oligomers (Donnet *et al.* 2001)

Na^+,K^+-ATPase functions and regulation

The regulation of Na^+,K^+-ATPase attracts attention because of the significant and various roles played by the pump in the functioning of different tissues. Na^+,K^+-ATPase creates a gradient of Na^+ and K^+ concentrations that is used in different ways in different tissues. The density of Na^+,K^+-ATPase in the cellular plasma membrane differs in different tissues: it varies from several molecules per square micrometer in erythrocytes to several thousands molecules per square micrometer in nephron epithelium or in the cells of excitable tissues. In all animal cells, Na^+,K^+-ATPase participates in the creation of resting membrane potential and in the maintenance and regulation of cell volume (Kabakov 1994). However, Na^+,K^+-ATPase also performs specific functions that are characteristic for particular tissues.

Na^+,K^+-ATPase plays a significant role in the maintenance of ion conductivity in excitable tissues (neurons and muscle tissues), while a Na-gradient is used in many tissues to provide the activity of different ion-exchange transport systems. The increase of intracellular Na^+ concentration results in activation of Na^+/Ca^{2+} exchanger (NCX) that is embedded in the plasma membrane of different cells including neurons and cardiac and smooth muscle (Moore *et al.* 1993).

In the kidney, Na^+,K^+-ATPase is located in the basolateral membrane of collecting duct epithelial cells and accomplishes Na^+ transports from the cell to the extracellular medium (Nelson and Veshnock 1987).

Na^+ concentration gradient is used in other tissues to accomplish transport of some metabolites (sugars, amino acids) into the cell. Inhibition of Na^+,K^+-ATPase and dissipation of the Na^+ concentration gradient results in a decreased rate of amino acid transport into the cell, and this in turn may lead to inhibition of cell growth.

The idea of the existence of different functions of Na^+,K^+-ATPase depending on tissue type is supported by the discovery of Na^+,K^+-ATPase isoenzymes. Four isoforms of the alpha-subunit (Alpha1, alpha2, alpha3, and alpha4) and three isoforms of the beta-subunit (beta1, beta2, and beta3) encoded by different genes are found in vertebrates; these subunits may associate into alphabeta-dimers in different combinations, resulting in isoenzymes with different kinetic properties and different patterns of distribution in various tissues and within each tissue during ontogenesis (Blanco *et al.* 1995; Mobasheri *et al.* 2000). The expression of the various isoforms is under the control of various hormones (Mobasheri *et al.* 2000).

The Alpha1-isoform of Na^+,K^+-ATPase is the predominant form in kidney, where especially fine regulation of Na^+,K^+-ATPase activity is required. This isoform, also present in other tissues including heart, skeletal muscle, and neurons, can be phosphorylated by cAMP-dependent (PKA) (Kurihara *et al.* 2000; Murtazina *et al.* 2001), cGMP-dependent (PKG) protein kinases (Fotis

et al. 1999), Ca-phospholipid-dependent (PKC) protein kinase (Yudowski *et al.* 2000), and by tyrosine kinases of the Src family (Feraille *et al.* 1999).

Numerous attempts to find how PKA-mediated phosphorylation of Na^+,K^+-ATPase in vitro affects the enzyme activity gave controversial results: most probably, the effect of Na^+,K^+-ATPase phosphorylation by PKA depends on the type of alpha-isoform that is phosphorylated.

When Na^+,K^+-ATPase is phosphorylated by tyrosine kinases Pi is bound to Tyr10 of rat Na^+,K^+-ATPase alpha-subunit (Feraille *et al.* 1999). Phosphorylation of Tyr10 in Na^+,K^+-ATPase alpha-subunit in kidney nephrons is induced by insulin and is accompanied by an increase of Na^+,K^+-ATPase activity.

PKC phosphorylates Na^+,K^+-ATPase too, Pi being bound to two serine residues located in the N-terminal part of the Alpha1-subunit: Ser11 and Ser18 (Feschenko and Sweadner 1995; Feschenko and Sweadner 1997).

The phosphorylation of Na^+,K^+-ATPase by PKC may be a signal for endo- and exocytosis: the phosphorylation of Ser18 leads in some species to endocytosis and combined phosphorylation of Ser11 and Ser18 to exocytosis.

The phosphorylation of Na^+,K^+-ATPase by PKC demonstrates that Na^+,K^+-ATPase is able to perceive signals that are usually transmitted in the cell with the participation of cell proteins containing different conserved domains like the SH2- or SH3-domain, the plekstrin domain, and some others. Such interactions of proteins inside cells are now considered one of the important routes of signal transduction (Pawson 1995).

Na^+,K^+-ATPase interaction with other proteins

Regulation of Na^+,K^+-ATPase via interactions with different protein-partners is still not well enough studied; the growing list of proteins for which interactions with the catalytic alpha-subunit of Na^+,K^+-ATPase has been

described includes also the beta- and gamma-subunits of Na^+,K^+-ATPase and the cytoskeleton protein ankyrin.

A beta subunit is part of the Na^+, K^+- and H^+,K^+-ATPases but is not included in other P-type ATPases, for example, in Ca-ATPase of both plasma membrane and sarcoplasmic reticulum. The sensitivity of Na^+,K^+-ATPase to the cations Na^+ and K^+ is known to change depending on the isoform of the beta-subunit included in the alpha-beta complex, because the beta-subunit affects the activation of Na^+,K^+-ATPase by extracellular K^+ (Jaisser *et al.* 1992). This subunit does not participate in the process of catalysis directly, nevertheless is tightly bound to the alpha-subunit and is required for the insertion of the catalytic subunit into the membrane and the delivery of both subunits to the plasma membrane (Lutsenko and Kaplan 1992). However, it appears that the role of the beta-subunit is not limited to these functions.

A known partner for the Na^+,K^+-ATPase alpha-subunit is ankyrin (Devarajan *et al.* 1994). Ankyrins compose a family of proteins that are linkers between the integral membrane proteins and the proteins of cytoskeleton (spectrin and fodrin fibrils). Different isoforms of ankyrins are present in different tissues. They differ also depending on the type of membrane to which the cytoskeleton is attached. One domain binds ankyrin to integral membrane proteins, another to spectrin (fodrin), and the third domain is a regulatory domain.

Melittin, a bee venom peptide consisting of 26 amino acid residues and forming amphipatic alpha-helix with positive charge concentrated on the C-terminal region also interacts with the Na^+,K^+-ATPase, in addition to numerous other proteins, and with negatively charged lipid bilayers. The interaction between melittin and the ATPase involves sites located in the catalytic subunit of the enzyme (Cuppoletti and Malinowska 1992). This suggests that the site of melittin binding is a binding site of some intracellular proteins, and melittin imitates a module of these proteins that participate in binding.

Na^+,K^+-ATPase and cardiac glycosides

Cardiac glycosides, specific inhibitors of Na^+,K^+-ATPase, are steroid O-glycosides containing in position 17 an unsaturated ring: gamma-lactone with 5 carbon atoms (cardenolides) or sigma-lactone with 6 carbon atoms (bufadienolides). A hydrocarbon chain located in 3beta-position usually consists of 1-5 monosaccharides. Digoxin and ouabain, substances of plant origin, are well known members of this group. These compounds were first used for treatment of cardiac failure more than 200 years ago because at low (nontoxic) concentrations they improve the function of cardiac muscle (so-called positive inotropic effect).

According to modern understanding, the mechanism of the effect of cardiac glycosides on cardiac muscle contraction is the following: partial inhibition of Na^+,K^+-ATPase by these compounds leads to some increase of intracellular Na^+ concentration that in turn activates Na^+/Ca^{2+} exchanger. The consequence of this is a significant increase of intracellular Ca^{2+} concentration and increase of cardiac muscle contractility.

The site of cardiac glycoside binding is located on the extracellular part of the Na^+,K^+-ATPase alpha-subunit (Lingrel *et al.* 1994). Sensitivity to these inhibitors differs depending on animal species (minimal sensitivity is characteristic for the enzyme from rat (Emanuel *et al.* 1988)) and on the type of alpha-subunit isoform that is present in the Na^+,K^+-ATPase (for the enzyme obtained from one animal species inhibition of Na^+,K^+-ATPase containing Alpha1-isoform is 500-1000-fold higher than for the enzyme containing alpha2-isoform). This is mainly connected with the substitution of certain amino acids in the ouabain-binding site of the Na^+,K^+-ATPase alpha-subunit.

The study of these substances of plant origin specifically affecting the activity of an important animal cell enzyme lead to the discovery that animals posses endogenous digitalis (ouabain)-like factors that can specifically regulate the activity of this enzyme (Wang *et al.* 2004). Digitalis-

like factors identified as isomers of ouabain were found in the hypothalamus and adrenocortical glands (Doris 1994).

Na^+,K^+-ATPase signaling functions

It is nowadays accepted that, in addition to pumping ions, Na^+-K^+-ATPase is engaged in assembly of multiple protein complexes that transmit signals to different intracellular compartments (Xie and Askari 2002).

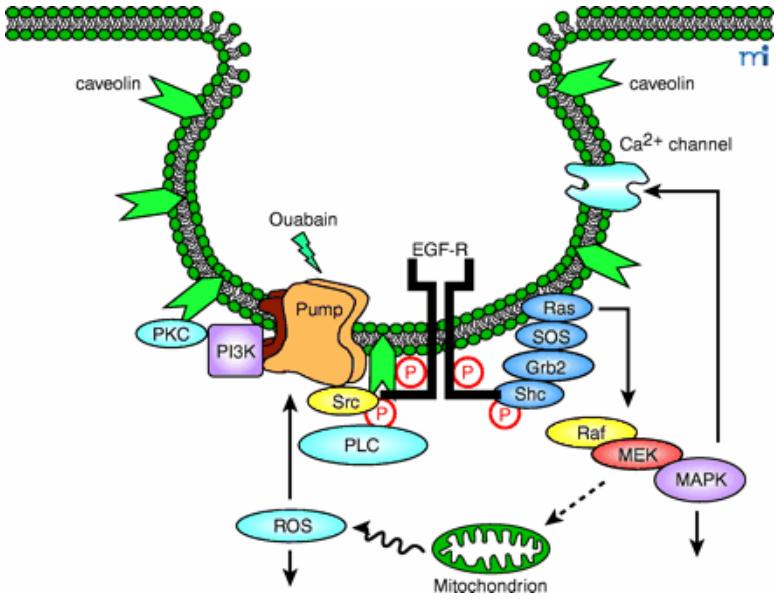

Fig. 1.33 : the "signalosome" assembled at the sites of ouabain-Alpha1 interaction (Xie and Cai 2003)

In some cell types, the signaling Na^+,K^+-ATPase and its protein partners are compartmentalized in coated pits (i.e., caveolae) in the plasma membrane, where the clustering of the pump facilitates ouabain-activated signal transduction (Xie and Askari 2002; Liu *et al.* 2003; Liu *et al.* 2004b). Cholesterol depletion leads to a reduction of ouabain-induced recruitment of Src to the ATPase signaling complex, leading to a repression of ouabain

induced activation of ERKs. Clearly, the caveolar Na^+,K^+-ATPase represents the signaling pool of the pump that transmits ouabain hormonal signals (Wang *et al.* 2004).

Binding of ouabain to the signaling Na^+,K^+-ATPase activates the cytoplasmic tyrosine kinase Src, resulting in the formation of an active "binary receptor" that phosphorylates and assembles other proteins into different signaling modules. This in turn activates multiple protein kinase cascades including mitogen-activated protein kinases and protein kinase C isozymes in a cell-specific manner. It also increases mitochondrial production of reactive oxygen species (ROS) and regulates intracellular calcium concentration. Crosstalk among the activated pathways eventually results in changes in the expression of a number of genes. Although ouabain stimulates hypertrophic growth in cardiac myocytes and proliferation in smooth muscle cells, it also induces apoptosis in many malignant cells. The signaling function of the enzyme is also pivotal to ouabain-induced nongenomic effects on cardiac myocytes.

Finally, it has been demonstrated that ouabain induces endocytosis of plasmallemal Na, K-ATP ase by a clathrin dependent mechanism that translocates it to intracellular compartments (Liu *et al.* 2004a), thus suggesting a potential role of endocytosis in ouabain-induced signal transduction.

Chapter 2

Materials and Methods

MATERIALS AND METHODS

Plasmid constructs

Constructs expressing HSV-TK, alone or as a fusion protein with Tat11 (Tat11-TK) or Tat86 (Tat86-TK) in the eukaryotic expression vector pCDNA3 (Invitrogen, Carlsbad, CA) were a kind gift of Dr. E. Tasciotti (Tasciotti *et al.* 2003); the starting construct was the prokaryotic vector pGEX-2T (Pharmacia, Uppsala, Sweden) containing Tat as a fusion protein with the glutathione-S-transferase (GST) and the Green Fluorescent Protein (GFP) (GST-Tat-GFP), with Tat inserted between BamHI and HindIII sites, and GFP inserted between HindIII and EcoRI. From this plasmid, GFP was replaced with TK obtained by PCR amplification with primers containing BamHI and EcoRI sites at their extremities, producing pGEX-Tat86-TK. In this plasmid, Tat86 was replaced with Tat11 (aa sequence YGRKKRRQRRR, preceded by an initiator methionine) produced as a pair of complementary oligonucleotides with flanking BamHI and HindIII sites. Plasmid pGEX-TK was obtained by substituting the Tat86-GFP cassette with a PCR product corresponding to TK with flanking HindIII and EcoRI sites. To obtain the pCDNA3 versions of these two constructs, the Tat11-TK and TK cassettes were excised with BamHI and EcoRI and cloned into the respective sites of the vector. Tat11-CRE expressing vector was a gift of Dr. E. Tasciotti (ICGEB, Trieste).

The rat Alpha1 subunit cds was obtained by PCR amplification from a construct expressing the above cds in the eukaryotic vector pCB6 (Brewer and Roth 1991) (a gift from Prof. Levenson, Penn State College of Medicine, Hershey, PA); briefly, the upper primer, spanning the ATG of the protein, contained a BamHI restriction site, while the lower, spanning the stop codon, contained a site for HindIII; the amplicon was then cloned in pCDNA3 expression vector and sequenced.

A rat Alpha1 point mutant (D716N), lacking catalytic activity, had been already described (Lane *et al.* 1993); briefly, the point missense mutation (G→A) at nucleotide position 2447 (leading to the substitution of the Asp at position 716 with Asn, in the third, large cytoplasmic loop of the protein) was introduced by two-step PCR mediated mutagenesis, using PCR primers carrying the designed substitution; the second step was performed using the same primers described for the deletion mutants. The amplicon was then digested with HindIII and NotI, and cloned in pCDNA3. Alpha1 ΔCTERM lacks the fragment spanning aminoacids 784-3040 (comprising the last two C-terminal cytoplasmic fragments as well as a transmembrane region between them); lower primer was designed containing a nonsense mutation (i.e. a STOP codon) at aminoacid position 784 (correspondent to nucleotide 2352) and the restriction site for NotI. The truncated cds of rat Alpha1 was amplified from the pCDNA3-Alpha1 vector with these primers and upper primer, containing the site for HindIII.

Alpha1 deletion mutant was cloned in pCDNA3 vector between HindIII and NotI sites.

FLAG epitope was inserted as follows: complementary oligonucleotides encoding the FLAG sequence lacking the ATG were designed, annealed, and the resulting dsDNA was cloned into the Alpha1 mutant vectors (as well as the pCDNA3 vector encoding the wild type Alpha1) between the NotI and BamHI sites, resulting in vectors expressing wild type, catalytic mutant or deletion mutant Alpha1 proteins tagged with the FLAG epitope at the C-terminus (named respectively pCDNA3-Alpha1-FLAG, pCDNA3-Alpha1 ΔCTERM FLAG, and pCDNA3-Alpha1 D716N-FLAG).

Constructs expressing either Tat86- or Tat11-GFP in pCDNA3 have been described elsewhere (Fittipaldi *et al.* 2003); a construct expressing Tat86 protein mutated in its basic region, referred to as Tat86 Basic Mutant, in the eukaryotic vector pCDNA3, was a gift from M.I. Gutierrez (Mitola *et al.* 2000); the three constructs, expressing the larger cytoplasmic regions of rat

Alpha1 subunit of Na^+,K^+-ATPase, as fusion proteins with GST in the prokaryotic vector pGEX1T (Pharmacia, Uppsala, Sweden) were a gift from Prof. P. Devarajan (Devarajan *et al.* 1994); the construct encoding a single chain Ig tagged with the SV5 peptide (Sc-VHD16-SV5), in the eukaryotic vector pCDNA3, was a kind gift of Prof. O. Burrone (Sepulveda *et al.* 2003);
The pAlpha1-CFP construct (where Alpha1 is fused to Cyan Fluorescent Protein, CFP) was obtained as follows: the Alpha1 CDS was amplified from the pCDNA3-Alpha1 construct and cloned in pECFP-N1 vector (Clontech, Mountain View, CA) between BamHI and EcoRI sites; pYFP-Tat11 construct (where Tat11 is fused to Yellow Fluorescent Protein, YFP) was obtained by annealing of two oligonucleotides encoding for the Tat11 sequence, followed by their cloning into the pEYP-C1 vector (Clontech, Mountain View, CA) between the BamHI and the EcoRI sites; pCDNA3-Tat86-YFP construct was obtained from pCDNA3-Tat86-GFP construct, by substitution of the EGFP cassette in the starting pGEX-Tat86-GFP construct with the YFP form the pEYP-N1 vector (Clontech, Mountain View, CA); the resulting Tat86-YFP cassette was then cloned in pCDNA3 between BamHI and EcoRI sites.
A sequence corresponding to the fusion of the three C-terminal cytoplasmic peptides of the Alpha1 subunit, linked by a glycine residue, named P1-P2-P3, was obtained by annealing of two oligos collectively encompassing the whole region, followed by PCR amplification of the fragments using external primers containing restriction sites in order to allow cloning.
The P1-P2-P3 fragment was then cloned in pFLAG-CMV2 vector, between NotI and EcoRI sites, obtaining the pFLAG-P1-P2-P3 construct, and in the pGEX-2T vector, between HindIII and EcoRI sites, obtaining the pGEX-P1-P2-P3 construct.

Recombinant fusion proteins

The recombinant fusion protein representing GST-Tat, GST, and respectively Alpha1 cytoplasmic fragment 1, 2 and 3, and P1-P2-P3 fused to GST were

produced and purified from BL21 bacteria transformed with the corresponding vectors. 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 OD600 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 seconds each. Bacterial lysates were mixed with 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 loaded on an empty plastic column (Bio-Rad, Richmond, CA), letting the unbound proteins pass through, and the beads were washed with 400 bed volumes of lysis buffer. The fusion proteins bound to GST agarose beads were then resuspended in an adequate amount of lysis buffer and stored at -80°C prior their use. The integrity and purity of the proteins were assessed by sodium-dodecyl-sulphate gel electrophoresis (SDS-PAGE) followed by coomassie staining.

The pCDNA3-Tat86 and the pCDNA3-Tat86BM (tat86 Basic mutant) were used as templates to produce the *in vitro* [^{35}S] Tat86 and Tat86 BM proteins for *in vitro* binding by using the TNT Reticulocyte Lysate System (Promega, Madison, WI).

Synthetic peptides

Synthetic peptides corresponding to residues 824-843, 939-951 a 1007-1023 of Alpha1 C-terminus (thereafter named P1, P2 and P3, respectively) were obtained from Peptide Synthesis Facility at ICGEB – Trieste. Peptides were synthesized on solid phase (preloaded NovaSyn TGT, Novabiochem) by Fmoc/t-Bu chemistry using a home-built automatic synthesizer based on a Gilson Aspec XL SPE system. The peptide-resin were cleaved/deprotected using a modified Reagent H mixture (trifluoroacetic acid 80%, phenol 3%, thioanisole 3%, 3,6-dioxa-1,8-octanedithiol 8%, water 2.5%,

methylethylsulphide 2%, hydroiodic acid 1.5% w/w) for 3 h. The peptides were then precipitated by diethylether, washed and freeze-dried. The crude peptides were purified by preparative RP-HPLC on a 25x300 mm column (Load&Lock system, Varian) packed with VariTide RPC resin (Polymer Laboratories - Varian) using a gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile. The purified fractions were checked by ESI-MS, pooled and freeze-dried.

For all the three peptides, an N-Biotinylated, an N-Fluoresceinated and a non-conjugated form were synthesized.

As negative controls, we used a biotinylated MPO Mimicking Peptide (Biotin-Ala-RNLKLARKLMEQYGT) corresponding to aa 622-637 of Human Myeloperoxidase (ACCESSION AAI30477), synthesized by Espichem (Firenze) (a gift of Dr. Lorena Tedeschi) and a synthetic FLAG peptide (Sigma Aldrich).

***In vitro* GST-pull down assays**

Binding of GST-fused Alpha1 fragments and P1-P2-P3 to [^{35}S]-Tat86 and [^{35}S]-Tat86 BM was performed as follows: briefly, 1 µg of recombinant proteins, after pretreatment in a solution containing DNase I 0.25 U/µl and RNase 0.2 µg/µl to remove contaminant bacterial nucleic acids, were incubated with 600 c.p.m. of *in vitro* translated Tat in a solution containing 0.2 mg/ml ethidium bromide. Following extensive washes, the reaction mixture was resolved by SDS–PAGE electrophoresis and analyzed by PhosphoImager.

Binding and washes were performed in NETN buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA pH 8.0, 0.5% Nonidet P40, 1 mM DTT, 1 mM PMSF).

***In vitro* biotin pull down assays**

Synthetic biotinylated peptides, corresponding to aminoacids 824-843, 939-951 and 1007-1023 of Na^+,K^+-ATPase Alpha1 subunit, were used for binding assays as follows: 25 μl of streptavidin resin (UltraLink Plus streptavidin beads, Pierce,), were pretreated with 3 mg of BSA for 10' at room temperature, and subsequently incubated with 50 μg of peptide at 22°C for 1 hour; after 3 washing with NETN buffer, the peptide-coated beads were incubated with either 600 c.p.m. of in vitro radiolabeled translated Tat as previously described, or with 250 ng of recombinant GST-Tat, for 2 hours at 4°C. Following extensive washes, the reaction mixture was resolved by SDS–PAGE electrophoresis and analyzed either by PhosphoImager, or blotted and analyzed by western analysis using an anti-GST antibody.

Antibodies and reagents

Polyclonal antibody against HSV-TK was purchased from William C. Summers Laboratory (Yale University, US); monoclonal antibody against tubulin was from Sigma; monoclonal antibody against SV5 peptide was a kind gift from Prof. Oscar Burrone (ICGEB, Trieste, Italy); Monoclonal antibody against HIV-1 Tat (5A5.3) was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH from Dr. Jon Karn.); monoclonal antibody against Na^+,K^+-ATPase subunit was from Upstate (Lake Placid, NY); monoclonal antibody against GM-130 was from Transduction Laboratories (Lexington, KY); monoclonal anti-FLAG antibody and anti-FLAG conjugated agarose were from Sigma; rabbit polyclonal anti-GST antibody was from Santa Cruz biotechnology. Horseradish peroxidase (HRP)-conjugated secondary antibodies for western blotting were from Santa Cruz biotechnology and Alexa 488-coupled secondary antibodies used for confocal microscopy came from Molecular Probes Inc. (Eugene, OR). The protease inhibitor cocktail was from Roche

(Strasbourg, France). All other reagents were from Sigma unless otherwise specified.

Fluorescence microscopy

For FRET experiments, U2OS cells were grown on four-well glass chamber slides (LabTek II-Nalge Nunc) and transfected as described below; upon expression of the transfected proteins, cells were fixed in PFA 2% and mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA).

For immunofluorescence analysis, U2OS cells were grown on chamber slides, fixed, and incubated twice for 10 min with PBS 0,1% Triton X-100 to permeabilize cell membranes; after extensive wash with PBS+ (0.1% glycine, 0.5% BSA), cells were incubated with primary antibody (either anti-GM130 or anti-FLAG, 1:500) for 1h at 37°C. Cells are then washed again in PBS+ and incubated with secondary antibody (goat anti-mouse, Alexa488 conjugated, 1:2000) for 1 hour at 37°C. Following extensive wash with PBS+, slides are mounted with Vectashield and analyzed. All antibodies were diluted in PBS+.

Fluorescent images were acquired using a TCS-SL Leica confocal microscope.

FRET analysis

Fluorescent images of samples fixed with 2% paraformaldehyde were acquired using a TCS-SL Leica confocal microscopy. The quantitative FRET analysis was performed using the ImageJ software that elaborates the FRET efficiency by applying the formula:

$$\% \text{ FRET efficiency} = (1 - Fda/Fd) \times 100$$

where Fda stands for the fluorescence emitted by the donor fluorophore before bleaching and Fd for the emission of the donor fluorophore after bleaching (Karpova *et al.* 2003).
(Fda and Fd were calculated by subtracting the background fluorescence from the effective fluorescence of the selected ROI).

Cell cultures and transfections

CHO K1 and PsgA-745 (Rostand and Esko 1997) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in Kaighn's modification of Ham's F12 medium. HEK 293T and U2OS (obtained from the ATCC) and HL3T1 cells (a HeLa cells derivative stably transfected with a silent LTR-CAT cassette), a kind gift of B. Felber (Felber and Pavlakis 1988), were maintained in DMEM; human osteogenic sarcoma (HOS) $CD4^+$ $CCR5^+$ cell line was maintained in DMEM (Gibco, UK); human acute lymphoblastic T-cell leukaemia (CEM) $CD4^+$ cell line was maintained in 1640 RPMI medium (Gibco, UK).
All culture media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamicin.
U2OS cells (5×10^4) were seeded into four-well glass chamber slides (LabTek II-Nalge Nunc) and transfected with the Polyfect transfection kit (Qiagen) for 24 h; in all transfections, an adequate quantity of empty vector *pcDNA3* was used to achieve a similar DNA amount.
HEK 293T cells were transfected using the classical Calcium-Phosphate protocol (Molecular Cloning: a laboratory manual – Eds. Sambrook J. & Russell D.V., 16.14-16.20, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2001) incubated for 36 hours, and then processed for secretion or co-immunoprecipitation experiments.

Drug treatment

Cells were pretreated with the different drugs (10 μM BFA, 25 μM ouabain, 1 mM methylamine, 50 μM curcumin, 20μM EIPA, 10 μM glyburide, and 20 μg/ml heparin) for 30 min in OPTIMEM, and then fresh OPTIMEM containing the same drugs was added to the cell cultures. After the time of incubation (4 hours), the medium was collected and the cells were harvested and processed for western blotting.

Secretion assay

HEK 293T or CHO A-745 cells were seeded in 6-cm plates (10^6) one day prior transfection; cells were transiently transfected with either Tat11-TK, Tat86-TK or TK construct and with Sc-VHD16-SV5 construct as a positive control of classical secretion. The CaP-DNA complexes were incubated for 12 hours, then the medium was replaced with fresh DMEM, and the cells were incubated for 24 more hours. The secretion assays were performed by washing the cells (3 washes of 10 minutes) with Optimem containing 20 μg/ml heparin to prevent secreted tat protein binding to the extracellular heparan sulfate proteoglycans upon transfection (Tyagi *et al.* 2001), and incubating the cells with 2 ml of Optimem + heparin for the needed time lapses. Following incubation, cell culture supernatants were collected and concentrated using Amicon Ultra 10 concentrator (Millipore) according to the manufacturer's instructions; the concentrated fraction was then processed for western blotting. As a control of protein expression, the cellular fraction was collected, lysed in NHEN buffer (20 mM Hepes pH 7.5, 300 mM NaCl, 0.5% NP-40, 20% glycerol, 1 mM EDTA) containing protease inhibitors (Roche), the total protein concentration was assessed by the Bradford assay (BioRad), and 30 μg of each samples were processed for Western Blotting.

Flow cytometry

For the fluorescent peptides uptake experiment, synthetic biotinylated peptides, corresponding to aminoacids 824-843, 939-951 and 1007-1023 of Na, K ATPase Alpha1 subunit, and conjugated to fluorescein, were diluted in OPTIMEM and incubated with cells for 4 hours at 37°C. Cells were then trypsinized, washed with DMEM and twice with PBS, and processed for flow cytometry.

For the internalization experiment in presence of ouabain, cells were plated in 6-well plated to about 60% confluence, pretreated with 25 μM ouabain for 30 min, and incubated with either recombinant GFP fusion proteins or TRITC-labeled transferrin plus 25 μM ouabain for 4 hours. 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). For the surface staining experiment with anti TK antibody, cells were detached from the plates with PBS, washed twice in PBS containing 1% FCS, then incubated in anti-TK antibody for 30 min on ice; cells were then washed again twice with PBS 1% FCS, and incubated with secondary antibody (goat anti-rabbit conjugated with AlexaFluor 488) for 30 min on ice; cells were washed twice in PBS 1% FCS, fixed in PFA 2% and analyzed by flow cytometry. The antibodies were both diluted in PBS 1% FCS. A total of 10,000 cells per sample were considered.

Immunoprecipitation

For immunoprecipitation, cell pellets were lysed in a NHEN buffer (20 mM Hepes pH 7.5, 300 mM NaCl, 0.5% Nonidet P40, 20% glycerol, 1mM EDTA, 1 mM PMSF) containing protease inhibitors (Roche). The protein concentration of the extracts was determined by the Bradford assay (BioRad). Anti-TK and anti-Alpha1 antibodies were incubated overnight at

4°C with cell extracts (2-3 mg); following incubation, Protein-A Trisacryl beads (Pierce Technologies Inc., Rockfors, IL) was used to bind the immunocomplexes, according to the manufactor's instructions. After incubation, the beads were washed 5 times with NHEN buffer, and then processed for SDS-PAGE and Western Blotting using appropriate antibodies.
Anti-FLAG M2-Agarose beads (Sigma) were incubated overnight at 4°C with cell extracts (2.5–4.5 mg). After incubation, the beads were washed 5 times with NHEN buffer, and then the immunocomplexes were analyzed by Western blotting using the appropriate antibodies.

CAT ELISA

For testing the biological activity of secreted Tat protein, 293T cells were transfected with a pCDNA3 construct expressing Tat86 and a pCDNA3 expressing either wild type or deleted mutant of Alpha1; after 24 hours of incubation, cells were washed and the medium substituted with fresh medium plus heparin 20 μg/ml and, for the secretion inhibition experiments, supplemented with 25 μM ouabain; after 4 hours, the surnatant was collected and added to HL3T1 cells with the addition of 100 μM chloroquine; as HL3T1 cells stably express a bacterial chloramphenicol- acetyltransferase gene (CAT) fused to HIV LTR, after 24 hours of incubation, Tat-driven transactivation was assayed by inducing long terminal repeat (LTR)-dependent chloramphenicol acetyltransferase (CAT) production and quantifying the levels of CAT protein by using a CAT ELISA kit (Roche Diagnostics, Meylan, France).

Virus preparation

Viral stocks of the Bal-luciferase vectors were prepared by the standard calcium-phosphate method of transfection in HEK 293T cells using pNL4.3-luciferase plasmid (Connor *et al.* 1995; He and Landau 1995) and the M-

tropic Bal envelope encoding plasmid at a ratio 3:1. The supernatant containing virions were collected 48 hours after transfection, centrifuged 5 minutes at 1500 rpm and filtered with 45 μm Millipore filter. Cells were infected for 4 hours.

Viral stocks of the viral clones HIV-1_{BRU} (Petit *et al.* 1999) were prepared by the standard calcium-phosphate method of transfection in HEK293T cells. The supernatant containing virions were collected 48 hours after transfection, centrifuged 5 minutes at 1500 rpm and filtered with 45 μm Millipore filter. Before infection viral stocks were treated with DnaseI (Invitrogen) 40U/ml for 1 hour at room temperature. Cells were infected for 4 hours.

Infections

For single-round infections, HOS $CCR5^+$ cells were infected with pNL4.3-luciferase pseudotyped with the M-tropic Bal envelope. Prior infection, the virus was pre-incubated with an equimolar mix of P1, P2 and P3 peptides (Pmix) in a 15 μg/ml concentration, at 37°C for 1 hour; cells were incubated with the virus for 4 hours, washed, and fresh medium containing 15 μg/ml Pmix was added; after 24 hours, the cells were harvested and the expression level of luciferase was tested with DualGlo Luciferase assay kit (Promega, Madison, WI) according to manufactor's instructions. Luciferase activity was calculated after normalization against the activity of the co-transfected Renella luciferase.

For infections with wild type virus, for each time point, 10^6 CEM cells were infected with an equal amount of HIV-1_{BRU} for 4 hours; where indicated, the infection was performed in presence of 10 μg/ml of Pmix, or of FLAG peptide, as a negative control. After the infection, the medium containing the virus was washed and substituted with fresh medium, containing the corresponding peptide preparation. At t=0 and subsequently, every 3 days until the 12^{th} day, the supernatants were collected tested, while cells were

diluted 1:2 and fresh peptides were added to the culture media. The amount of virus was evaluated by measuring the RT activity in the supernatants following standard protocols.

Luciferase assay

HeLa P4 cells, stably expressing a copy of LTR-driven luciferase gene, were transfected with pCDNA3 Tat and a vector expressing Renella luciferase under the control of CMV promoter for normalization purposes; after 48 hours of incubation, cells were lysed and luciferase assays were carried out with DualGlo Luciferase assay kit (Promega, Madison, WI) according to manufactor's instructions. Luciferase activity was calculated after normalization against the activity of the co-transfected Renella luciferase.

Statistical analysis

Significativity of data was assessed by calculating P values from two-tailed Student's t test.

Chapter 3

Results

RESULTS

Heterologous proteins fused to HIV-1 Tat are released from the expressing cells

One of the peculiar features of HIV-1 Tat, which is very unusual for a transcription factor, is the characteristics of being released by the expressing cells in the absence of cell lysis (Ensoli *et al.* 1993; Chang *et al.* 1997) and being able to enter neighboring cells, where it is transported to the nucleus in a transcriptionally active form (Tyagi *et al.* 2001). Of notice, the fusion of heterologous proteins to Tat also imparts these proteins the intercellular trafficking capacity of wild type Tat (Tyagi *et al.* 2001; Krosl *et al.* 2003; Tasciotti and Giacca 2005). We have previously observed that the minimal region of Tat capable of mediating both cellular release and internalization of heterologous proteins corresponds to amino acids 48-59 (hereafter named Tat11), encompassing the 9-aa-long, basic region of the protein (Tyagi *et al.* 2001; Tasciotti *et al.* 2003); **Fig. 3.1A**.

To start investigating the mechanisms involved in the release of Tat-fusion proteins, we transfected HEK293T cells with constructs expressing the TK reporter protein of the herpes simplex virus type 1 (HSV-1) carrying an N-terminal extension corresponding to Tat11 or to the 86-aa-long, full length Tat protein of the widely investigated HIV-1 laboratory strain HX2B (Tat86); we have previously shown that both these constructs are biologically active both *in vitro* (Tasciotti *et al.* 2003) and *in vivo* (Tasciotti and Giacca 2005). At 36 h after transfection, a considerable portion of both fusion proteins (~2-10%) was found in the cell culture supernatant in the absence of detectable cell lysis, as concluded by the lack of cellular tubulin protein in the same supernatants (**Fig. 3.1B**).

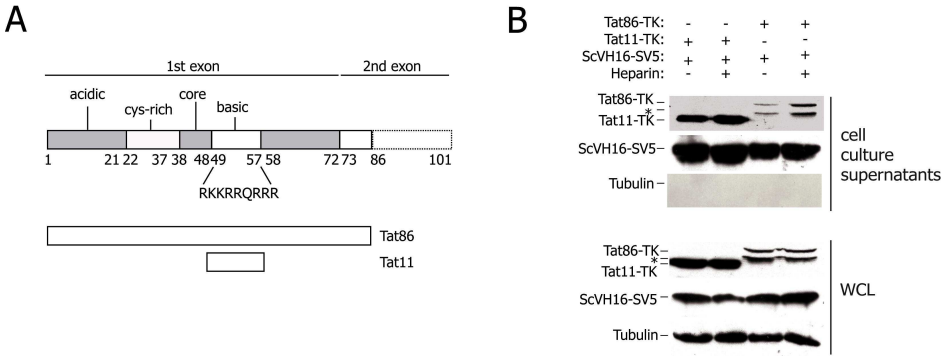

Fig. 3.1: Release of Tat86- and Tat11-fusion proteins from the expressing cells

(**A**) Schematic representation of the major functional domains of HIV-1 Tat. Acidic, acidic domain; Cys-rich, cysteine-rich domain; core, core domain; basic, basic domain. Tat has 101 aa in several clinical isolates and 86 aa in the laboratory strain HX2B. The amino acid sequence of the basic domain of the protein, which imparts the protein intercellular trafficking capability, is indicated. The lower part of the panel shows a schematic representation of the two Tat proteins used in this study (Tat11, corresponding to the Tat basic domain plus two additional amino acids at both extremities, and Tat86).

(**B**) Tat86-TK and Tat11-TK are released from the expressing cells and bind extracellular HSPG upon secretion. The immunoblots in the upper panel show the amount of proteins released in the cell culture supernatants of cells transfected with Tat86-TK, Tat11-TK or scVH16-SV5, treated or untreated with 25 μM soluble heparin. The immunoblots in the lower part show the levels of intracellular protein expression in the same samples. WCL: whole cell lysates. The asterisk indicates an additional band present in the Tat86-TK immunoblots, probably corresponding to a degradation product. The absence of immunoreactivity for tubulin in the supernatants indicates the absence of appreciable cell lysis under the adopted experimental conditions.

Of interest, the amount of Tat-TK proteins free in the supernatant was increased by cell treatment with soluble heparin, consistent with the notion that this polyanion acts as a competitive inhibitor of cell surface HSPGs, which sequester soluble Tat (Chang *et al.* 1997), and that heparinase III releases the attached protein (Chang *et al.* 1997). For comparison, cells were also transfected with a plasmid expressing a single-chain Fv antibody (scFv) tagged with the SV5 epitope (ScVH16-SV5). This protein, which contains a signal peptide and is thus secreted through the ER-Golgi compartments, also accumulated in the cell culture supernatant, but its levels were unmodified by cell treatment with heparin. The lower part of

Fig. 3.1B shows the intracellular levels of expression of the three investigated proteins.

Kinetics of Tat-fusion protein release

Next we wanted to investigate the kinetics of Tat-fusion protein release. HEK293T cells were co-transfected to express both Tat86-TK and ScVH16-SV5; after 36 h, cells were extensively washed with fresh medium containing heparin, to remove HSPG-bound proteins, and fresh medium containing heparin was added. The scFV and Tat86-TK proteins were expressed at comparable levels by the cells, however the kinetics of their appearance in the cell culture supernatants was drastically different. As shown in the upper part and quantified in the lower part of **Fig. 3.2A**, the levels of the extracellular Tat-fusion protein peaked as early as 30 min after the addition of fresh medium and slightly decreased in the subsequent 2 h. In contrast, secreted scFv appeared at later times and its levels progressively increased. Analogous findings were also detected when cells were co-transfected with Tat11-TK and ScVH16-SV5, thus indicating that the release kinetics is a property imparted by the Tat basic domain (**Fig. 3.2B**). As compared to Tat86-TK, the overall efficiency of Tat11-TK release was significantly higher.

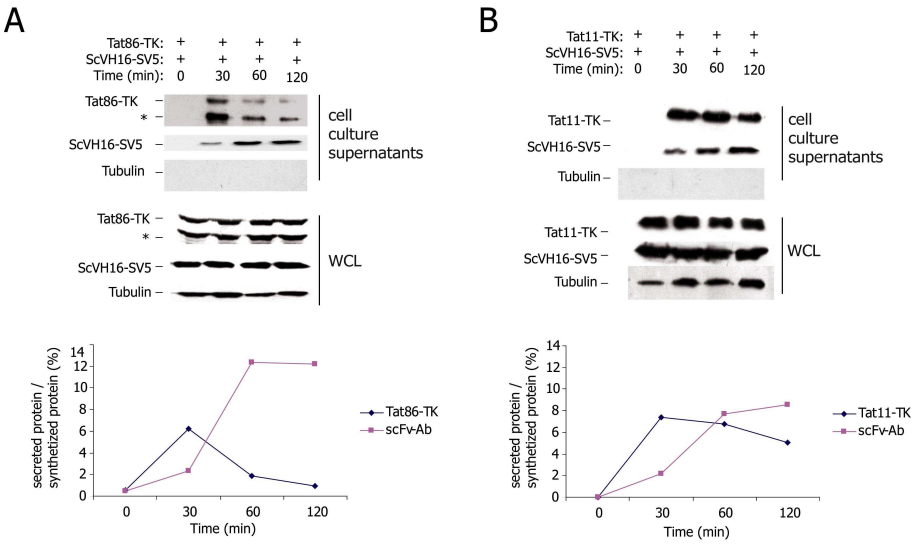

Fig. 3.2: Kinetics of Tat-fusion protein release
(**A**) Kinetics of extracellular accumulation of Tat86-TK and the scFv antibody ScVH16-SV5, which is secreted through the ER-Golgi, in cells co-transfected to express both proteins. The immunoblots in the upper panel show the levels of the two proteins in the cell culture supernatants at different time points. In the lower panel, the immunoblots show the levels of Tat86-TK, ScVH16-SV5 and tubulin in whole cell lysates (WCL) from the same cells as a control of protein expression. The asterisk indicates unspecific bands recognized by the primary antibody.
(**B**) Same as in panel (**A**) in cells transfected with Tat11-TK and ScVH16-SV5.

Tat secretion is independent from HSPG binding

Finally, we also ascertained that proteins other than TK had the same behavior when fused to Tat. Indeed, under similar experimental conditions, both Tat11-GFP (not shown) and Tat11-Cre (**Fig. 3.3A**) were found to behave similar to the two TK fusion proteins.

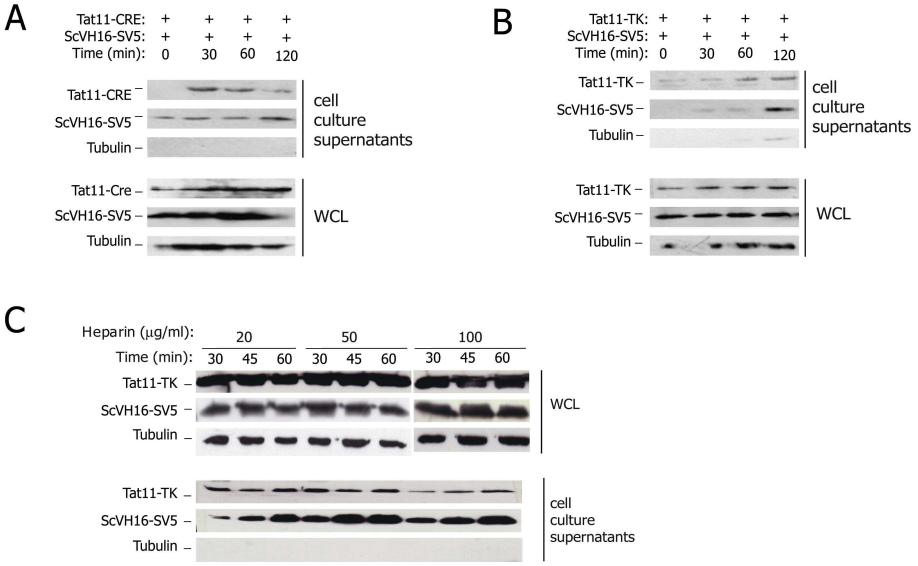

Fig. 3.3: Tat secretion is independent from HSPG binding
(**A**) Kinetics of extracellular accumulation of Tat11-Cre and the scFv antibody ScVH16-SV5, which is secreted through the ER-Golgi, in cells co-transfected to express both proteins. The immunoblots in the upper panel show the levels of the two proteins in the cell culture supernatants at different time points. In the lower panel, the immunoblots show the levels of Tat11-Cre, ScVH16-SV5 and tubulin in whole cell lysates (WCL) from the same cells as a control of protein expression.
(**B**) Tat11-TK secretion is independent from HSPG binding. The kinetics of Tat11-TK and ScVH16-SV5 accumulation was assessed by immunoblotting on supernatants of CHO-A745 cells, genetically defective for HSPG synthesis, transfected to express both proteins. The levels of intracellular protein expression were assessed by western blotting on whole cell lysates (WCL).
(**C**) Tat11-TK binds to HSPG upon secretion. The amount of secreted protein was assessed in presence of growing amounts of soluble heparin in the cell culture supernatants. The levels of intracellular protein expression were assessed by western blotting on whole cell lysates (WCL).

The apparent lack of accumulation of Tat-fusion proteins in the supernatant over time likely reflects a steady-state equilibrium between the amount of released protein that remains bound to extracellular HSPGs despite the presence of soluble heparin inhibitor, and that undergoing internalization through the interaction with cell membrane-associated HSPGs (Tyagi *et al.* 2001; Fittipaldi *et al.* 2003). Consistent with this possibility, we observed that CHO A745 cells, which do not express detectable amount of

proteoglycans due to a genetic defect in their biosynthetic pathway (Rostand and Esko 1997), release progressively increasing amounts of Tat11-TK in their supernatants (**Fig. 3.3B**). Moreover, when we evaluated Tat11-TK secretion in the presence of growing quantities of soluble heparin in the cell culture media, the amount of secreted protein showed a progressive increase (**Fig. 3.3C**).

Collectively, these results show that heterologous proteins containing Tat or the Tat basic domain possess the property of being progressively released from the producing cells, independent from the production of HSPGs.

Tat secreted from HEK293Tcells is able to transactivate LTR-CAT genes in HL3T1 cells

In co-cultures experiments employing Tat-GFP-expressing cells together with LTR-CAT-expressing cells, the Tat-GFP protein was shown to traffic from the producing cells to the responding cells, in which it determined a detectable level of CAT activation; these results demonstrated that Tat is secreted from the expressing cells in a biological active form (Tyagi *et al.* 2001).

In order to study Tat secretion *in vivo*, we first wanted to test whether transiently transfected HEK293Tcells were able to secrete Tat and this protein could be easily detected in the extracellular medium, without employing co-culture techniques. Moreover, as unconventional secretion has been shown to be a way of disposal of the excess of unfolded, overexpressed proteins in transfected cells (Tanudji *et al.* 2002), we wanted to test the functionality of secreted Tat. For this reason, we developed an ELISA-CAT assay, using HL3T1 cells (HeLa cells stably expressing an integrated LTR-CAT gene) as a recipient for the cell culture medium previously incubated with Tat- expressing cells. Briefly, HEK293Tcells were transfected with an expressing vector encoding for Tat, and after 24 hours of incubation the supernatants was transferred to HL3T1 cells. Then, the amount of secreted protein was measured by detecting the amount of CAT

protein resulting from the transactivation of the LTR (scheme of the experiment in **Fig. 3.4A**).

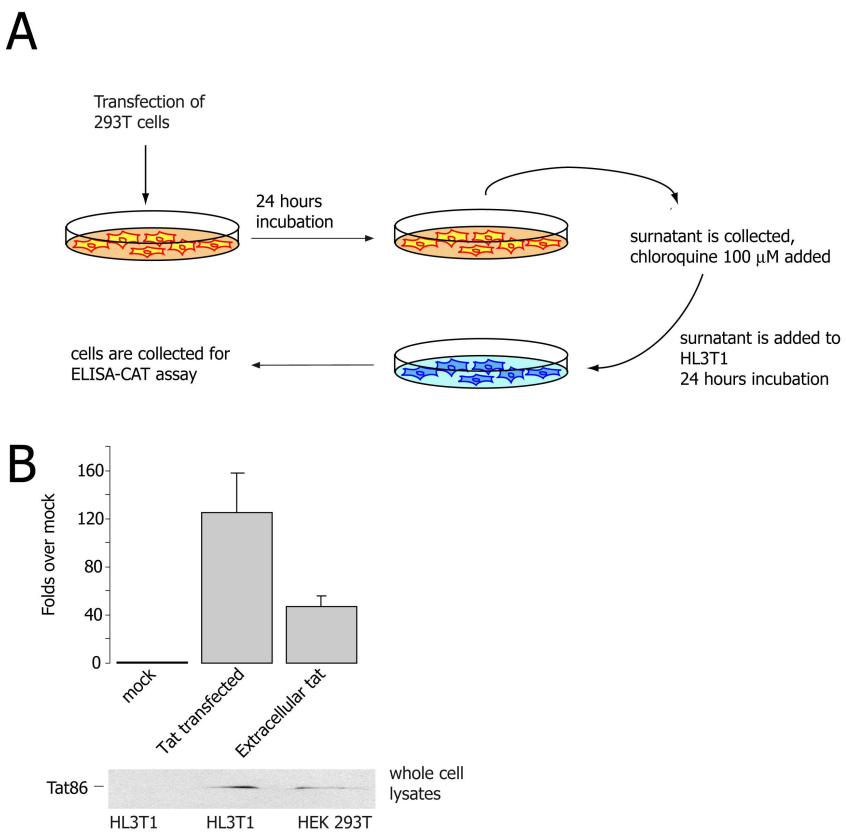

Fig. 3.4: Tat secreted from HEK293T is able to transactivate LTR-CAT genes in HL3T1 cells

(**A**) Scheme of the experimental procedure followed for the secretion assay employing HL3T1 as a reporter of Tat transactivation activity. HEK293T cells were transfected with a Tat expressing vector. After 24 hours, the medium was collected and transferred to HL3T1 cells in the presence of choroquine, in order to enhance protein delivery. After 24 hours, cells were collected, and the lysates used for CAT-ELISA assay in order to determine the amount of CAT enzyme. As a positive control, HL3T1 cells transfected with the same Tat expressing vector were lysed and processed for CAT-ELISA assay.

(**B**) Level of LTR transactivation, quantified as fold of CAT activation over mock. The first column is the level relative to mock-treated HL3T1 cells; the second is relative to HL3T1 transfected cells; the third represents the amount of CAT enzyme in HL3T1 cells treated with the supernatant of Tat-transfected HEK293T cells. Western blot using an anti-Tat antibody, showing the levels of Tat protein expression in HEK293Tand HL3T1 transfected cells, is presented below.

As a positive control we used HL3T1 cells transfected with a Tat- expressing vector (*pcDNA3*-Tat, (Fittipaldi *et al.* 2003)). The amount of CAT protein was determined by an ELISA assay against the CAT antigen.

The results are shown in **Fig. 3.4B**. They clearly indicate that secreted Tat is present in the cell culture medium of Tat-transfected cells, and is able to cross the cell membrane of HL3T1 cells, where it is targeted to the nucleus and transactivates the LTR-driven reporter gene. The lower level of transactivation obtained in comparison to the positive control was expected, as only a small portion of expressed Tat is released by HEK293T cells and therefore is available for CAT transactivation. The western blot performed on whole cell lysates with an anti-Tat antibody proves the expression of Tat protein in transfected cells.

Tat is excluded from the Golgi apparatus

In order to confirm previous data indicating that Tat secretion proceeds through a pathway which is independent from the classical pathway of protein secretion, we wanted to analyze the intracellular localization of transfected Tat in respect to the Golgi apparatus, which is the final compartment where proteins targeted for secretion are stored. For this purpose, HeLa cells were transfected with a Tat11-GFP fusion protein expressing vector, fixed, and analyzed by immunofluorescence using an antibody against GM-130, a resident glycoprotein of the Golgi apparatus. In contrast to GFP (**Fig. 3.5B**), Tat-GFP showed a preminent nuclear-nucleolar localization (**Fig. 3.5A**). No co-localization of Tat-GFP with GM-130 was detected (**Fig. 3.5A**).

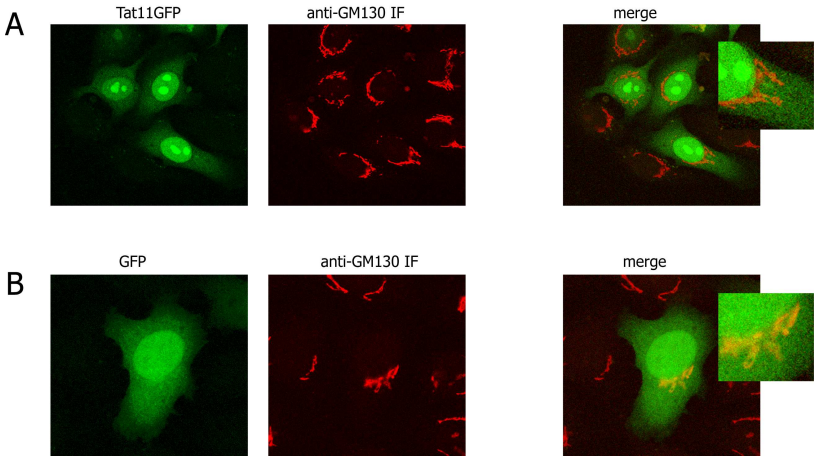

Fig. 3.5: Tat is excluded from the Golgi compartment in transfected cells
HeLa cells transfected with either Tat11-GFP- (**A**) or GFP- (**B**) expression vectors were fixed, processed for immunofluorescence with an Alexa488-labeled anti-GM130 antibody, and then analyzed by confocal microscopy for co-localization of GFP and Alexa488-derived fluorescence.

These results confirm that Tat is not found inside the Golgi apparatus even when overexpressed; thus the protein must otherwise be secreted through a different route.

Tat binds heparan sulfate proteoglycans at the cell surface

In order to characterize the process of Tat secretion from HEK293T cells, we took advantage of recombinant proteins containing Tat86 or Tat11 fused to HSV-TK. As characterized previously (Tasciotti *et al.* 2003), these proteins are secreted from transfected cells and retain the ability to cross cell membranes, thus spreading among cells. We decided to use TK as a reporter gene due to the availability of an anti-TK antibody that readily detects the fusion proteins with high specificity and sensitivity.
In a set of initial experiments, we wanted to confirm previous findings (Chang *et al.* 1997; Tyagi *et al.* 2001) on the binding of secreted Tat to cell surface heparan sulfate proteolglycans (HSPGS).

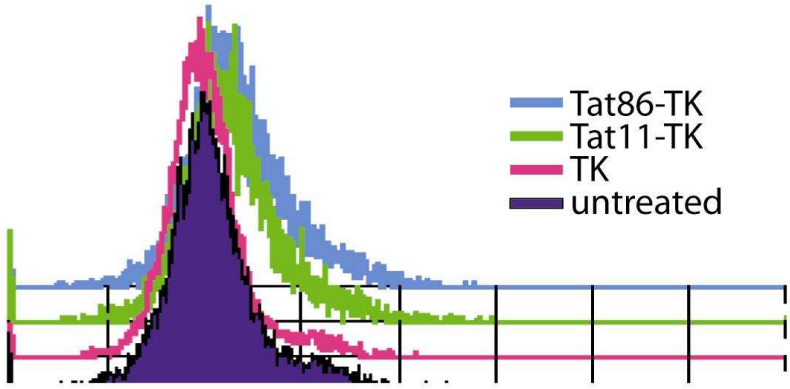

Fig. 3.6: Secreted Tat binds cell surface HSPGs
HEK293T cells were transfected with Tat86-TK, Tat11-TK or TK expressing vectors, and subsequently processed as follows: cells were non-enzymatically detached from plates, incubated with an anti-TK antibody, washed and incubated with an alexa488-conjugated secondary antibody. Cells were then processed by flow cytometry to assess differences in fluorescence between Tat86-TK, Tat11-TK and TK expressing cells.

HEK293T cells, transfected with Tat86-TK, Tat11-TK or TK expression vectors, were treated with anti-TK primary antibody and Alexa-488 conjugated anti-rabbit secondary antibody, and assayed by flow cytometry for surface staining. The results, shown in **Fig. 3.6**, indicate that, while TK expressing cells showed no increase in fluorescence compared to control, non transfected cells, fluorescence of Tat86-TK and Tat11-TK expressing cells was increased, implying the presence of TK- fusion proteins bound to the cell surface.

The cellular Na^+,K^+-ATPase inhibitor ouabain blocks extracellular release of Tat86 and Tat11 fusion proteins

To assess the involvement of distinct cellular machineries in the release of Tat, a panel of metabolic drugs, interfering with trafficking of proteins, organelles and cytoplasmic vesicles, were tested for their effect on Tat-

fusion protein release in HEK293T transfected with Tat11-TK or Tat86-TK together with the scFv antibody (**Figs. 3.7A** and **3.7C** respectively). Glyburide (GLY) and methylamine (METH), which interfere with the non-classical secretion of other proteins (Rubartelli *et al.* 1990; Hamon *et al.* 1997; Zhou *et al.* 2002), did not affect either the export of Tat11-TK or the secretion of the antibody. No effect was either detected by 5-(N-ethyl-N-isopropyl) amiloride (EIPA), a drug interfering with macropinocytosis (West *et al.* 1989) and known to limit HIV-1 replication in cultured cells (Ewart *et al.* 2004). In contrast, BFA (BFA), which interferes with the Golgi vesicular system and inhibits the ER-Golgi secretion pathway (Misumi *et al.* 1986), severely impaired the scFv antibody secretion, but did not exert any detectable effect on Tat11 and Tat86 fusion protein release.

Most notably, cell treatment with the cardiac glycoside ouabain (OUA) at 25 μm, a concentration sufficient to abrogate the function of the cell membrane Na^+,K^+-ATPase and to block the non-classical secretion of FGF-2 (Dahl *et al.* 2000), blocked (>8 fold inhibition) Tat11-TK release, while leaving scFv antibody secretion unaffected (**Fig. 3.7A** for blots and **Fig. 3.7B** for quantification). Similar results were also observed when using Tat86-TK (**Figs. 3.7C** and **3.7D**). The effect of ouabain appeared specific for the Na^+,K^+-ATPase, since cell treatment with curcumin, a broad inhibitor of P-type ATPases, had no apparent consequence.

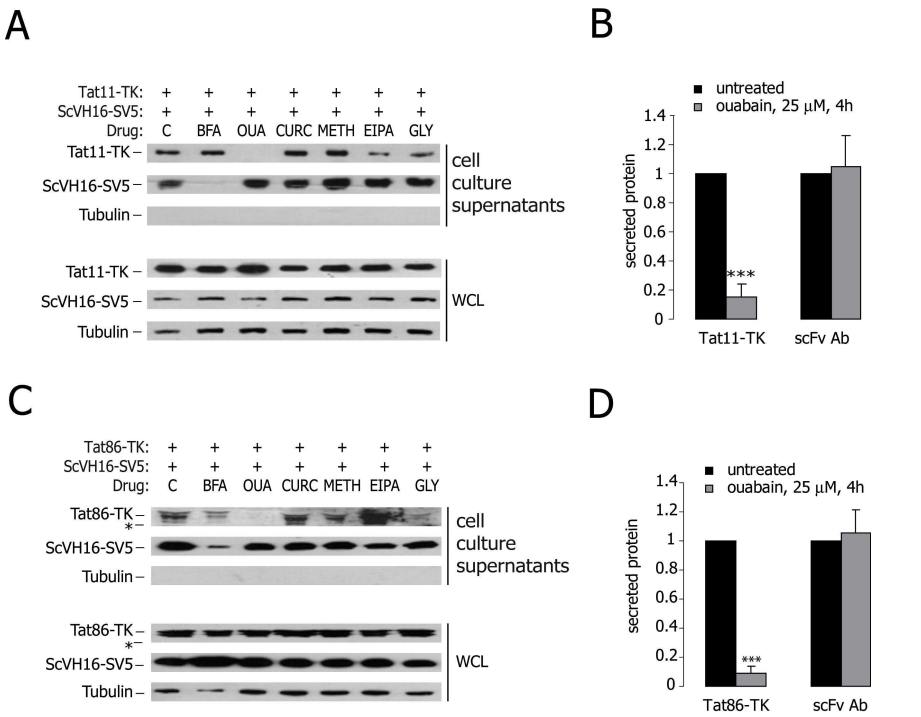

Fig. 3.7: The cellular Na^+,K^+-ATPase inhibitor ouabain blocks extracellular release of Tat86 and Tat11 fusion proteins

(**A**) Sensitivity of Tat11-TK release from expressing cells treated with several drugs known to interfere with vesicular transport. The amount of secreted protein was assessed by western blot on cell culture supernatants, while protein expression and loading was checked on whole lysates.

(**B**) Ouabain treatment significantly decreases Tat11-TK release from expressing cells; the amount of secreted Tat11-TK, normalized over the amount of expressed protein, was assessed in untreated and ouabain-treated cells, after 4 h incubation. Non-parametric T-student test was run on the result of three independent experiments.

(**C**) Sensitivity of Tat86-TK release from expressing cells treated with several drugs known to interfere with vesicular transport. The amount of secreted protein was assessed by western blot on cell culture supernatants, while protein expression and loading was checked on whole lysates. The histograms report the result of three independent experiments.

(**D**) Ouabain treatment significantly decreases Tat11-TK release from expressing cells; the amount of secreted Tat11-TK, normalized over the amount of expressed protein, was assessed in untreated and ouabain-treated cells, after 4 h incubation.

Ouabain-sensitive interaction of Tat with the Alpha1 subunit of the cellular Na^+,K^+-ATPase

The specific cellular target of ouabain is the Alpha1 subunit of the Na^+,K^+-ATPase, which has been also previously involved in the unconventional release of FGF-2 (Dahl *et al.* 2000). Thus, we wanted to investigate whether Tat might directly interact with this protein and whether this interaction might be involved in extracellular Tat release. To test this hypothesis, we performed co-immunoprecipitation experiments between Tat86-TK and the endogenous Na^+,K^+-ATPase Alpha1 subunit in HEK293T cells expressing Tat86-TK.

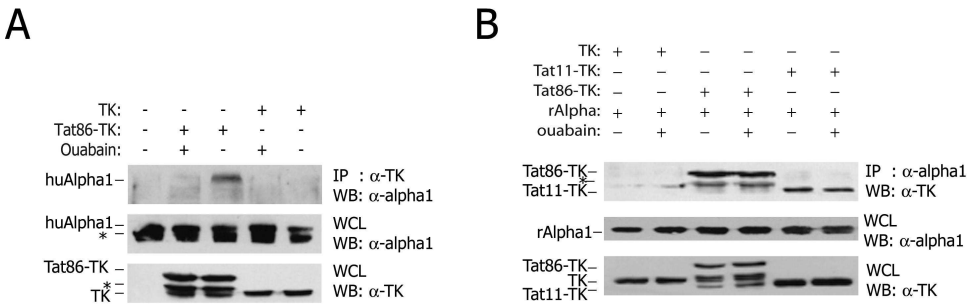

Fig. 3.8: Ouabain-sensitive interaction of Tat with the Alpha1 subunit of the cellular Na^+,K^+-ATPase

(**A**) Tat co-immunoprecipitates with endogenous Alpha1 subunit of Na^+,K^+-ATPase, and their binding is sensitive to ouabain. The antibodies used for immunoprecipitation and subsequent western blots are indicated on the right side. Expression of proteins was assayed on whole lysates. The cells used were HEK293T cells transfected with Tat86-TK, either treated or not with ouabain. The bands marked with (*) are degradation products of either proteins, recognized by the specific antibodies.

(**B**) Tat co-immunoprecipitates with the rat isoform of Alpha1, and the binding is not sensitive to ouabain. The antibodies used for immunoprecipitation and subsequent western blots are indicated on the right. Expression of proteins was assayed on whole lysates. The cells used were HEK293T cells co-transfected with TK, Tat11-TK or Tat86-TK, and rAlpha1. The bands marked with (*) are degradation products of either proteins, recognized by the specific antibodies.

Indeed, we found that Alpha1 was present in the anti-Tat86-TK immunoprecipitate and, most notably, that cell treatment with 25 μM ouabain for 4h prior of harvesting the cells for immunoprecipitation completely abolished the interaction between the two proteins (**Fig. 3.8A**). Thus, Tat interacts with the Na^+,K^+-ATPase Alpha1 subunit and the cardiac glycoside ouabain, which specifically inhibits the release of Tat into the extracellular compartment, disrupts this interaction.

The results so far obtained indicate that ouabain both inhibits the extracellular release of Tat and prevents the interaction between Tat and the Alpha1 subunit in human cells. Since the rat Na^+,K^+-ATPase is know to be insensitive to ouabain treatment, due to the presence of natural amino acid variations in its Alpha1 subunit (Emanuel *et al.* 1988), we wondered whether this protein still interacted with Tat and whether its expression in human cells might overcome the inhibition that ouabain imparts on Tat release. Human HEK293T cells were transfected with vectors expressing rat Alpha1 together with either Tat11-TK or Tat86-TK. When Alpha1 was then immunoprecipitated with a specific antibody, both Tat-fusion proteins were also present in the immunoprecipitates. Of notice, however, and in contrast with human Alpha1 (**Fig. 3.8A**), the addition of ouabain did not modify the interaction between rat Alpha1 and the Tat proteins (**Fig. 3.8B**).

Ouabain treatment does not affect Alpha1 or Tat intracellular localization

To rule out the possibility that the ouabain effect might be due to a modification of the intracellular localization of either Alpha1 subunit or Tat induced by the drug, we wanted to study the intracellular localization of both Tat and Alpha1 proteins after ouabain treatment.

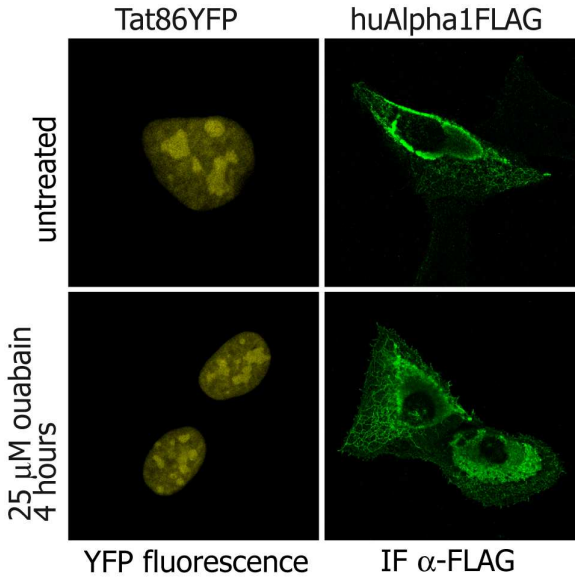

Fig. 3.9: Ouabain treatment does not affect Alpha1 or Tat intracellular localization
Left: intracellular localization of Tat-YFP. U2OS cells were transfected with a vector encoding for a Tat-YFP fusion protein, fixed in 2% PFA and analyzed by confocal microscopy. Where indicated, cells were treated with 25 μM ouabain for 4 h before fixation (lower panel).
Right: intracellular localization of human Alpha1 subunit. The cDNA encoding for the human apha1 subunit cds was cloned and expressed in U2OS cells as a FLAG-tagged protein. Cells were then fixed in 2% PFA and subjected to anti-FLAG immunofluorescence, prior their examination by confocal microscopy. In the lower panel, cells treated with ouabain 25 μM for 4 h prior fixation.

We used U2OS cells, transiently transfected with a YFP-fused Tat protein expressing vector, and treated them with ouabain. Upon treatment, the slides were analyzed by confocal microscopy after fixation (**Fig. 3.9, left**).
To study the localization of the human Alpha1, the cDNA encoding for the complete coding sequence of the protein was amplified from HEK293 total RNA, cloned as a FLAG tagged protein, and overexpressed in U2OS cells, which were subsequently treated with ouabain. After fixation, cells were subjected to immunofluorescence against the FLAG epitope, and then analyzed by confocal microscopy (**Fig. 3.9, right**). As a result, no relevant

change in distribution was observed for either protein, leading to the conclusion that the ouabain inhibiting effect on Tat-Alpha1 interaction is not due to the relocalization of either of the two protein partners.

Ouabain does not interfere with Tat internalization

In order to further explore the effect of ouabain on the biological activity of Tat, we tested its effect on the internalization pathway of a recombinant GST-Tat11-GFP protein, which had been previously shown to be internalized by cells through caveolar endocytosis (Ferrari *et al.* 2003; Fittipaldi *et al.* 2003). Cells were co-incubated for 4 h with the recombinant protein and ouabain, and then trypsinized, washed and analyzed by flow cytometry to determine the amount of internalized fluorescent protein. As a control of membrane integrity, we used a GST-GFP recombinant protein which is not internalized and therefore does not confer fluorescence to treated cells. TRITC-labeled transferrin was used to evaluate the effect of ouabain treatment on clathrin-mediated endocytosis.

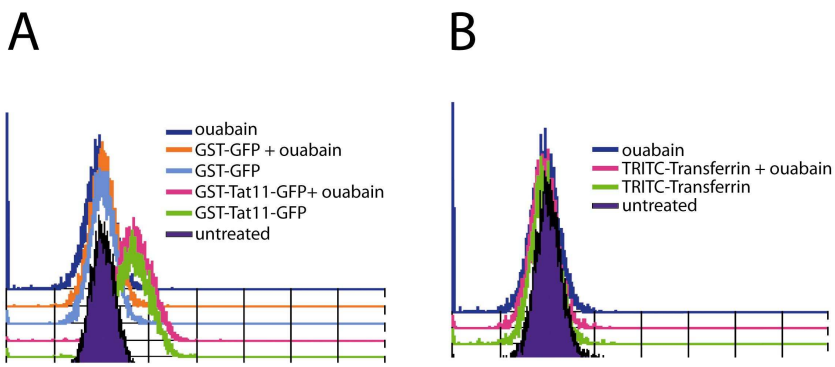

Fig. 3.10: Ouabain does not interfere with Tat internalization
(**A**) HEK293T cells were incubated for 4 h with 25 μM ouabain and GST-Tat11-GFP or GST-GFP; after incubation, cells were tryspinized and washed in order to detach any recombinant protein bound to the cell surface, and analyzed by flow cytometry in order to determine the amount of fluorescence. Untreated HEK293T cells were used as a control.
(**B**) Same experiments were conducted with TRITC-Transferrin as a control of ouabain effect on clathrin mediated endocytosis.

As a result, no effect whatsoever was observed on the internalization of either Tat11-GFP or Transferrin when cells were treated with ouabain (**Fig. 3.10**), showing that the effect of ouabain on Tat release was specific and not due to some disturbance of the overall cell membrane permeability.

Visualization of the interaction of Tat with the Na^+,K^+-ATPase Alpha1 subunit by fluorescence resonance energy transfer (FRET)

FRET imaging using GFP spectral mutants provides the ability to localize and monitor ion binding and molecular protein–protein interactions in living cells (Sekar and Periasamy 2003). An appealing approach to detect FRET is acceptor photobleaching (Bastiaens and Jovin 1996; Bastiaens *et al.* 1996; Wouters *et al.* 1998; Kenworthy and Edidin 1999; Kenworthy 2001). This technique is based on the principle that energy transfer is reduced or eliminated when the acceptor is bleached, thereby yielding an increase in donor fluorescence (**Fig. 3.11**, **A, B and C**). Such an increase in fluorescence following bleaching is particularly diagnostic of FRET, because, in most circumstances, fluorescence normally decreases following a bleach.

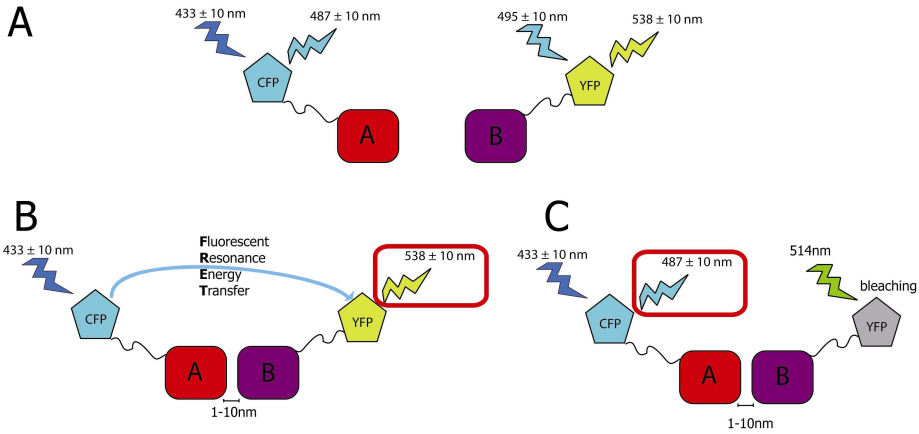

Fig. 3.11: FRET detection by acceptor photobleaching
(**A, B, C**): Scheme of FRET experiment with the YFP-CFP pair, using acceptor photobleaching. Putatively interacting proteins are fused to YFP and CFP; if interaction takes place, excitation of CFP results in FRET and subsequent excitation of YFP; as a consequence, YFP bleaching will result in an increase of CFP fluorescent emission.

In order to confirm the interaction between Tat and the rat Alpha1 subunit *in vivo*, we performed FRET experiments to determine whether we could detect a physical contact between the two proteins in transfected cells.

For this purpose, we cloned Tat and Tat11 as fusions to YFP, and Alpha1 fused to CFP, and transfected these proteins into U2OS cells. After 24 h, the cells were fixed, and the quantitative FRET analysis was performed by confocal microscopy evaluating CFP fluorescence after YFP photobleaching.

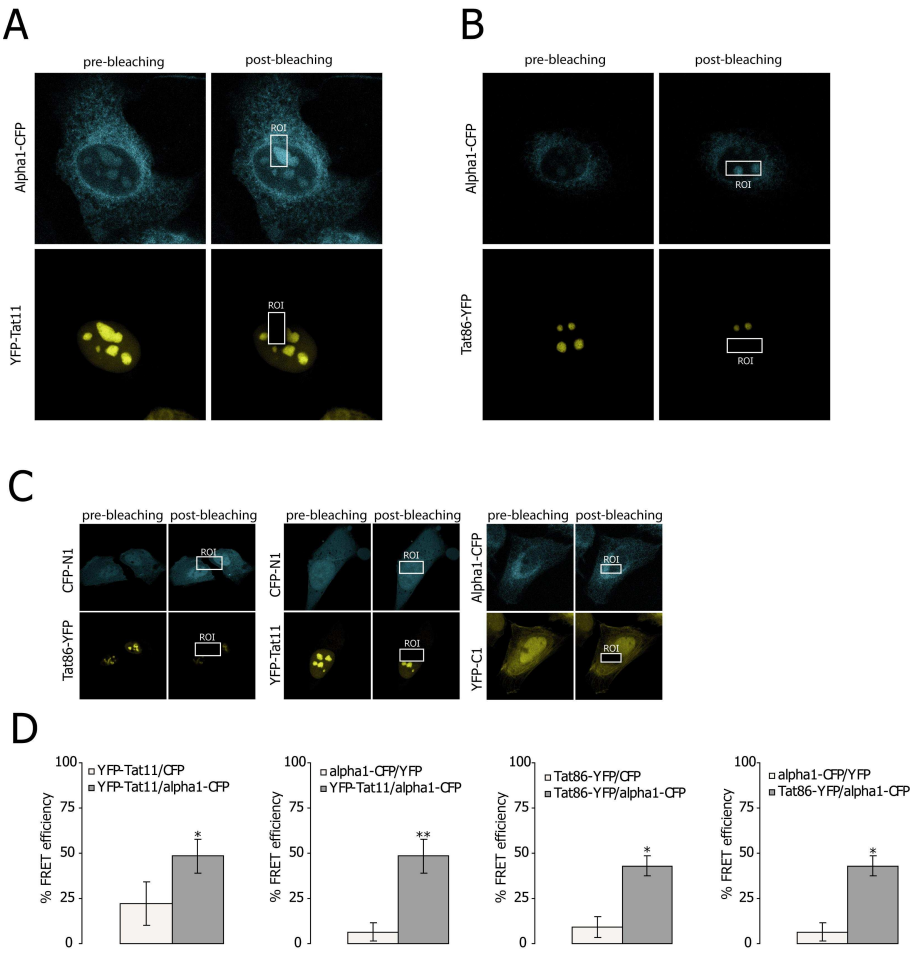

Fig. 3.12: Tat interacts with rat Alpha1 subunit *in vivo*
U2OS cells were transfected with the indicated plasmids, then, after 24 h, cells were fixed and analysed by confocal microscopy.
(**A**) Cells transfected with YFP-Tat11 and Alpha1-CFP. The upper panel shows cyan fluorescence, and the lower yellow fluorescence. The pre- and post-bleaching status is indicated.
(**B**) Cells transfected with Tat86-YFP and Alpha1-CFP. The upper panel shows cyan fluorescence, and the lower yellow fluorescence. The pre- and post-bleaching status is indicated.
(**C**) From the left, cells transfected with either YFP-Tat11 or Tat86-YFP, and CFP-N1, and cells transfected with Alpha1-CFP and YFP-C1.
(**D**) Quantification of FRET efficiency for each experimental condition.

Both Tat and Tat11 showed a strong interaction, localized in the nucleolar region (**Fig. 3.12A and B,** and **3.12D** for quantification), while no FRET was measured when CFP-N1 or YFP-C1 were transfected (**Fig. 3.12C** and **3.12D** for quantification). Thus, an *in vivo* physical interaction between the two proteins appears to exist, at least after protein overexpression by transfection. In evaluating these results, however, it needs to be observed that the localization of transfected Alpha1, which is nuclear, appears different from the physiological localization of the protein at the cell membrane.

Alpha1 catalytic function is not necessary for Tat release

The Alpha1 subunit of the Na^+,K^+-ATPase contains an ATP binding site, a phosphorylation site, and the amino acids essential for the binding of cations and cardiac glycosides (Blanco *et al.* 1994; Devarajan *et al.* 1994; Lingrel *et al.* 1994; Koster *et al.* 1995; Fotis *et al.* 1999; Yudowski *et al.* 2000; Murtazina *et al.* 2001) (**Fig. 3.13A**). An Asp to Asn mutation in the ATP binding site (mutant D716N) impairs the catalytic activity of the enzyme, blocking its ability to hydrolyze ATP, while still permitting the correct localization of the Alpha1 subunit onto the cellular membranes (Lane *et al.* 1993). Mutant D716N was still found able to bind both Tat86-TK and Tat11-TK, regardless of ouabain treatment (**Fig. 3.13B**). This result clearly

indicates that the interaction between Tat and Alpha1 occurs independent of the enzymatic activity of the Na^+,K^+-ATPase.

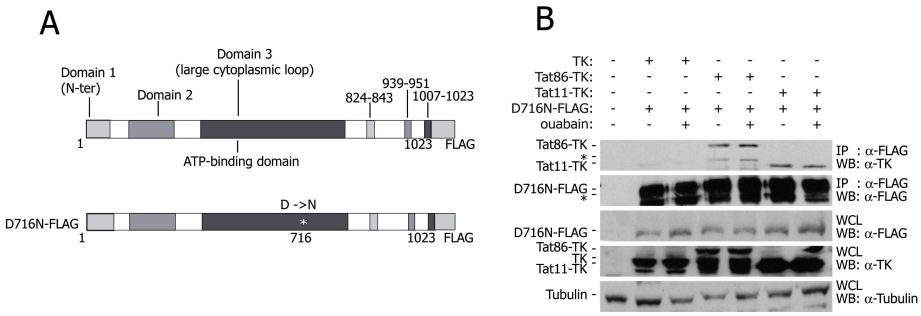

Fig. 3.13: Alpha1 catalytic function is not necessary for Tat release
(**A**) Schematic representation of the rat Alpha1 D716N construct, highlighting the residue mutated in order to inactivate the catalytic site.
(**B**) Tat co-immunoprecipitates with D716N mutant and their binding is not sensitive to ouabain. The antibodies used for immunoprecipitation and subsequent western blots are indicated on the right side. Expression of proteins was assayed on whole lysates. The cells used were HEK293T cells co-transfected with TK, Tat11-TK or Tat86-TK, and rAlpha1 D716N-FLAG. Cells were treated or not with ouabain as indicated. The bands marked with (*) are degradation products of either proteins, recognized by the specific antibodies.

Tat-Alpha1 interaction is crucial for Tat secretion

Since rat Alpha1 binds Tat in a ouabain-insensitive manner, we wondered whether its overexpression might rescue the inhibition that ouabain imposes on the secretion of Tat-fusion proteins in human cells. To ascertain this possibility, HEK293T cells were transfected with Tat86-TK or Tat11-TK, together with the rat Alpha1 subunit. Indeed, the overexpression of this protein restored the cellular release of the Tat proteins in cells treated with ouabain (**Fig. 3.14A** and **3.14B** for Tat11-TK and Tat86-TK respectively). Of notice, the D716N Alpha1 mutant was equally effective as the wild type protein in rescuing ouabain inhibition, again indicating that the effect of Alpha1 on Tat release is independent from its enzymatic activity. Secretion of the ScVH16-SV5 was left unaffected by any of these treatments.

Superimposable results were also obtained when studying the release of Tat86-TK under exactly the same experimental conditions (**Fig. 3.14B**).

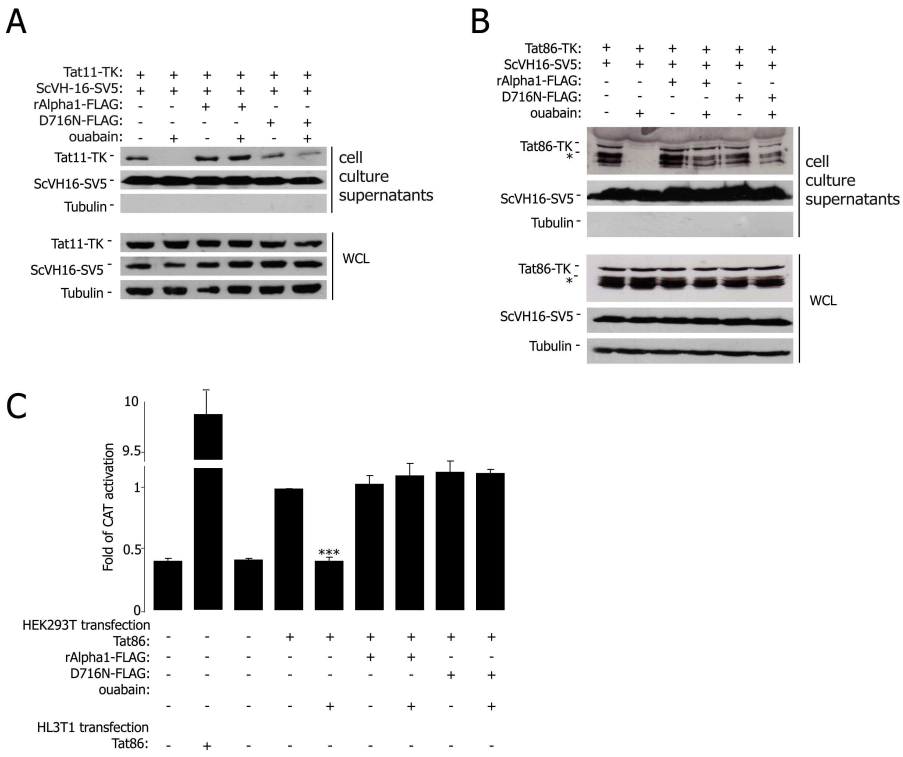

Fig. 3.14: Tat-Alpha1 interaction is crucial for Tat secretion

(**A**) D716N catalytic mutant is able to rescue ouabain-blocked secretion of Tat11-TK. Cells were transfected with Tat11-TK and either rAlpha1-FLAG or rAlpha1 D716N-FLAG, then treated with ouabain; the amount of secreted protein was assessed by immunoblot on cell culture supernatants, while protein expression was assessed on whole lysates.

(**B**) D716N catalytic mutant is able to rescue ouabain-blocked secretion of Tat86-TK. Cells were transfected with Tat86-TK and either rAlpha1-FLAG or rAlpha1 D716N-FLAG, then treated with ouabain; the amount of secreted protein was assessed by immunoblot on cell culture supernatants, while the protein expression was assessed on whole lysates.

(**C**) D716N catalytic mutant is able to rescue ouabain-blocked secretion of Tat86. HL3T1 cells were treated with the surnatants of HEK293T cells, transfected with Tat alone, or cotransfected with either rAlpha1-FLAG or rAlpha1 D716N-FLAG; cells were treated with ouabain, and their supernatant incubated with HL3T1 cells. The levels of LTR activation were assessed by ELISA assay on CAT levels. As a control of LTR activation, HL3T1 cells were transfected with Tat.

Finally, we also wanted to verify whether the marked inhibition that ouabain exerts on the release of Tat-fusion proteins also impairs the transcellular transactivation properties of wild type Tat. For this purpose, we transiently transfected HEK293T cells with an expression vector for the 86 aa form of Tat (Tat86); 24 h after transfection, cells were either left untreated or treated with 25 μM ouabain for 4 h, and the resulting cell conditioned media were collected and added to human HL3T1 cells, a HeLa cell derivative cell line carrying a silent HIV-1 LTR-CAT construct (Felber and Pavlakis 1988). Conditioned medium from cells expressing Tat86 activated LTR transcription in the reporter cell line, as assessed by CAT quantification, while ouabain treatment completely abrogated such effect (**Fig. 3.14C**). This result is consistent with the conclusion that transfected HEK293T might secrete transcriptionally active Tat, and that such release is blocked by ouabain treatment. No decrease of CAT activity in the reporter cell line after ouabain treatment was observed when the Tat86-expressing cells were co-transfected with a vector encoding rat Alpha1, either wild type of catalytically inactive, thus confirming that the ouabain-sensitive interaction between Tat and endogenous Alpha1 is crucial for the process of Tat release (**Fig. 3.14C**).

Collectively, the results so far obtained are consistent with the conclusions that the Tat basic domain specifically recognizes the Na^+,K^+-ATPase Alpha1 subunit in a ouabain-sensitive manner and that this interaction, rather than the catalytic activity of the enzyme, is essential to mediate the release of active Tat or Tat-fusion proteins in the extracellular environment.

The Tat basic region binds the Alpha1 C-terminal domain

Next we wanted to better define the regions of Tat and Na^+,K^+-ATPase Alpha1 subunit involved in the interaction between the two proteins. Alpha1 is a large, integral membrane protein with 10 transmembrane-spanning domains plus cytoplasmic N- and C-terminal regions (Lingrel *et al.* 1994;

Morth *et al.* 2007). We focused our attention on the cytoplasmic domains of the protein which were the most likely candidates for Tat binding, as the rest of the protein is either embedded in the phospholipidic bilayer, or protruding into the extracellular space (**Fig. 3.15A**).

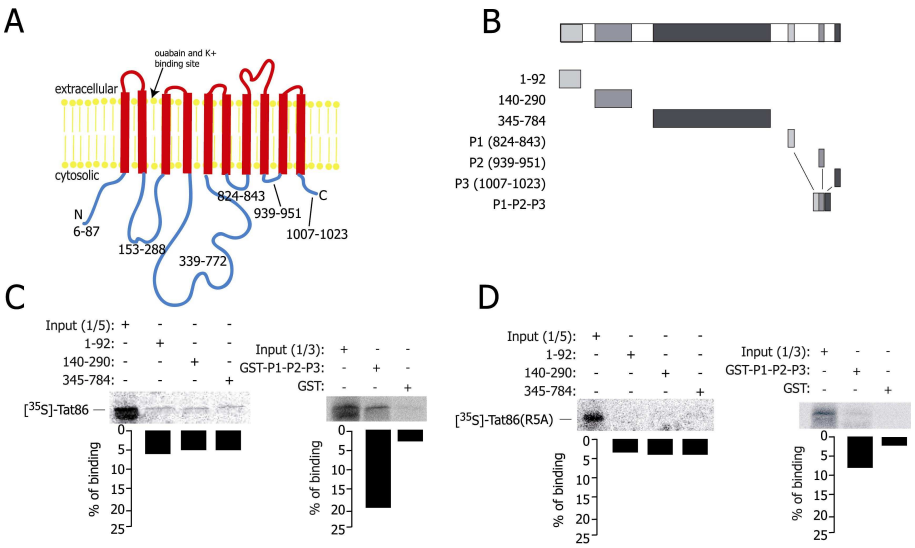

Fig. 3.15: Tat binds rat Na^+,K^+-ATPase Alpha1 subunit *in vitro* – Mapping of the interacting regions
(**A**) Scheme of the aplha1 subunit structure and membrane topology; the cytoplasmic domains are indicated.
(**B**) Schematic representation of the rAlpha1 fragments used for the pull-down assay showed in (C) and (D).
(**C**) Tat binds the C-terminal cytoplasmic region of the rat Alpha1 subunit *in vitro*. rAlpha1-GST fragments or GST alone were incubated with [^{35}S]-Tat86, extensively washed, and then resolved by SDS-PAGE. The panel shows the gel exposed to a phosphoimager. The graph shows the amount of bound proteins expressed as percentages of radiolabeled input.
(**D**) Tat binds the C-terminal cytoplasmic fragments of the rat Alpha1 subunit. rAlpha1-GST fragments or GST alone were incubated with [^{35}S]-Tat86 basic mutant, extensively washed, and then resolved by SDS-PAGE. The panel shows the gel exposed to a phosphoimager. The graph shows the amount of bound proteins expressed as percentages of radiolabeled input.

GST-fused Alpha1 fragments corresponding to the three main cytoplasmic regions (Devarajan *et al.* 1994), as well a fusion of the three C terminal

short cytoplasmic peptides (P1-P2-P3) of the rat apha1 subunit (**Fig. 3.15B**) were used in GST-pull down experiments using *in vitro* translated [^{35}S]- labeled Tat, or an [^{35}S]-Tat basic mutant, in which the basic residues of the Tat11 peptide had been replaced by alanines (Demarchi *et al.* 1999) (**Fig. 3.15C**).

Labeled Tat showed a stronger binding with the C-terminal Alpha1 cytoplasmic fragment (**Fig. 3.15C**); in contrast, the Tat basic mutant showed barely detectable interaction with any of the Alpha1 fragments, with a binding signal comparable to background (**Fig. 3.15D**).

These experiments clearly indicate that Tat interacts *in vitro* with the C-terminal region of the rat Alpha1 subunit, and that the basic region of the protein is responsible for this interaction, consistent with all the binding and protein export data presented so far.

Interaction between Tat and the cytoplasmic portion of Alpha1 C-terminal domain

To further characterize the region of binding between Tat and the C-terminus of the Alpha1 subunit, we analyzed in vitro the affinity between Tat and three synthetic peptides, corresponding to the three small cytoplasmic stretches (residues 824-843, 939-951 and 1007-1023, named P1, P2 and P3, respectively) residing in the C-terminal domain of the Alpha1 protein, as an interaction preceding the release of Tat is most likely to take place in the cytosolic environment. To perform the binding assays, synthetic biotinylated peptides were bound to streptavidin beads and subsequent pull down assays were performed employing both radiolabeled, in vitro translated Tat, and recombinant GST-Tat produced in *E. coli*.

Following incubation and extensive washes, Tat protein showed an increased affinity towards the peptide-coated beads, if compared to beads alone, or beads coated with an unspecific biotinylated peptide (**3.16A** and **3.16B**).

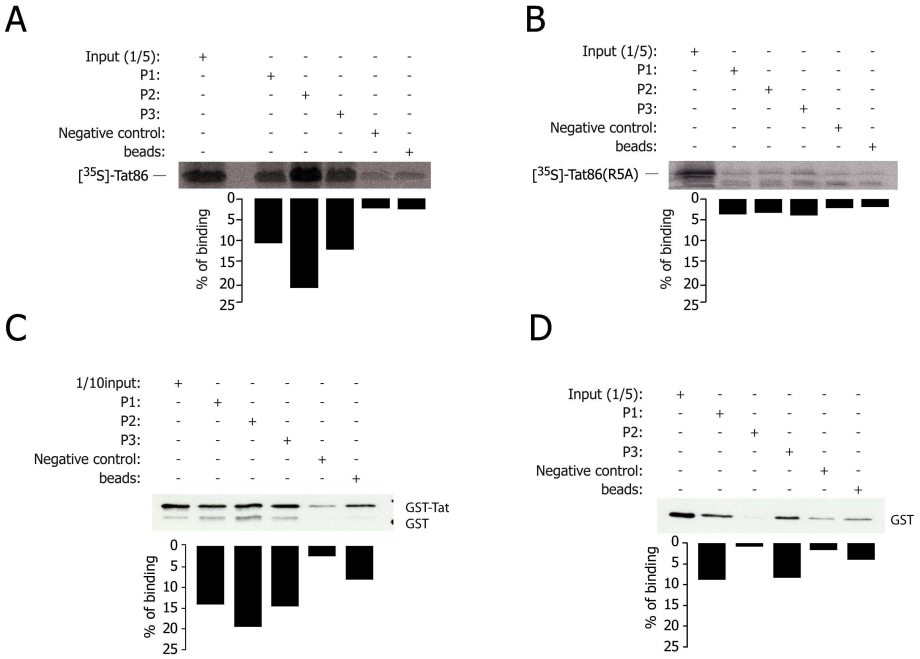

Fig. 3.16: Interaction between Tat and the cytoplasmic portion of the Alpha1 C-terminal domain

(**A**) Tat binds the three cytoplasmic peptides in the C-terminus of the Alpha1 protein. Biotinylated peptides were bound to streptavidin beads, incubated with [^{35}S]-Tat86, extensively washed, and then resolved by SDS-PAGE. The panel shows the gel exposed to a phosphoimager. The graph shows the amount of bound proteins expressed as percentages of radiolabeled input.

(**B**) Tat binds the three cytoplasmic peptides in the C-terminus of the Alpha1 protein through its basic domain. Biotinylated peptides were bound to streptavidin beads, incubated with [^{35}S]-Tat86 basic mutant, extensively washed, and then resolved by SDS-PAGE. The panel shows the gel exposed to a phosphoimager. The graph shows the amount of bound proteins expressed as percentages of radiolabeled input.

(**C**) Tat binds the three cytoplasmic peptides in the C-terminus of the Alpha1 protein. Biotinylated peptides were bound to streptavidin beads, incubated with GST-Tat, extensively washed, resolved by SDS-PAGE and blotted. The panel shows the results of a western blot against GST. The graph shows the amount of bound proteins expressed as percentages of loaded input.

(**D**) GST does not bind the three cytoplasmic peptides in the C-terminus of the Alpha1 protein. Biotinylated peptides were bound to streptavidin beads, incubated with GST, extensively washed, resolved by SDS-PAGE and blotted. The panel shows the results of a western blot against GST. The graph shows the amount of bound proteins expressed as percentages of loaded input.

Moreover, binding was shown to be specific for Tat, as a GST recombinant protein, used as a negative control, showed low or no affinity for the peptides (**Fig. 3.16D**).

Finally, as a mutant version of Tat, lacking the basic stretch of amino acids (previously described and named Tat Basic Mutant – mutant R5A (Demarchi *et al.* 1999)), showed reduced affinity for the peptide-bound beads (**Fig. 3.16B**), we could conclude that the binding between Tat and the cytoplasmic portion of the C-terminus of Alpha1 is mediated by its basic region.

In all different assays, a slightly increased affinity for Tat was shown by peptide P2; however, as no dramatic differences were revealed among the affinity of the three peptides (no more than 10% difference in all assays), we conclude that all three peptides are capable of independent binding to Tat.

3D modeling of C-terminal peptides in Alpha1 subunit tertiary structure

The 3D structure of the C-terminal peptides with respect of the tertiary structure of the Alpha1 protein was obtained by loading the PDB file corresponding to the crystal structure of the pig Na^+,K^+-ATPase recently published (Morth *et al.* 2007)(PDB entry # 3b8e) on Protein Workshop Java Applet, a tool for molecular modeling available at the online RCSB Protein Data Bank (www.rcsb.org).

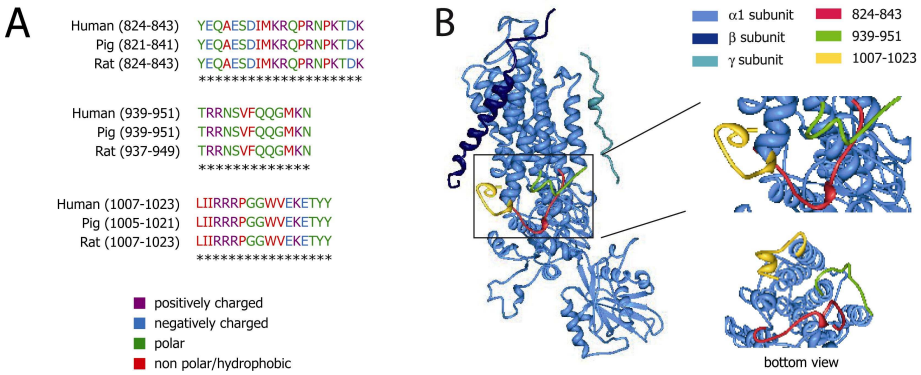

Fig. 3.17: Spatial arrangement of Alpha1 C-terminal cytoplasmic peptides
(**A**) Multiple alignment sequences corresponding to Alpha1 C-terminal cytoplasmic peptides in Human, Pig and Rat ortholog of Alpha1 subunit.
(**B**) 3D modeling of pig Alpha1 tertiary structure, as resolved by X-ray crystallography (Morth *et al.* 2007). The regions corresponding to the three C-terminal cytoplasmic peptides are highlighted; a bottom view of the C-terminal region is shown.

As shown in **Fig. 3.17**, the three small cytoplasmic coils are in close proximity one to another, and therefore it is plausible to hypothesize that they could form an interacting "surface" for Tat; in this scenario, the binding of Tat to only one of the three peptides could be less efficient than the binding to the whole native region, as found in the Alpha1 subunit embedded in the cellular membrane.

Tat fails to co-immunoprecipitate an Alpha1 mutant lacking the C-terminus

Once the binding of Tat to a specific region of the Alpha1 subunit was demonstrated *in vitro*, we wanted to understand whether the same region was also responsible of Tat-Alpha1 interaction in vivo. For this purpose, we obtained a deletion mutant lacking the C terminus (**Fig. 3.18A**) tagged with a FLAG epitope. The levels of expression and subcellular localization of this mutant was first analyzed, along with those of the flagged wild type protein, after transient transfection followed by immunofluorescence using and anti-

FLAG antibody. All the proteins were found to be expressed at comparable levels and to localize at the cell membrane (**3.18B**).

When co-transfected with Tat11-TK, the C-terminus deleted mutant was not able to co-immunoprecipitate the Tat fusion protein, unlike wild type Alpha1, thus confirming that, also inside the cells, the interaction of Alpha1 with the Tat basic domain requires the integrity of the C-terminal region of the protein, similar to the in vitro results (**Fig. 3.18C**). Identical results were also obtained using the Tat86-TK protein (**Fig. 3.18D**).

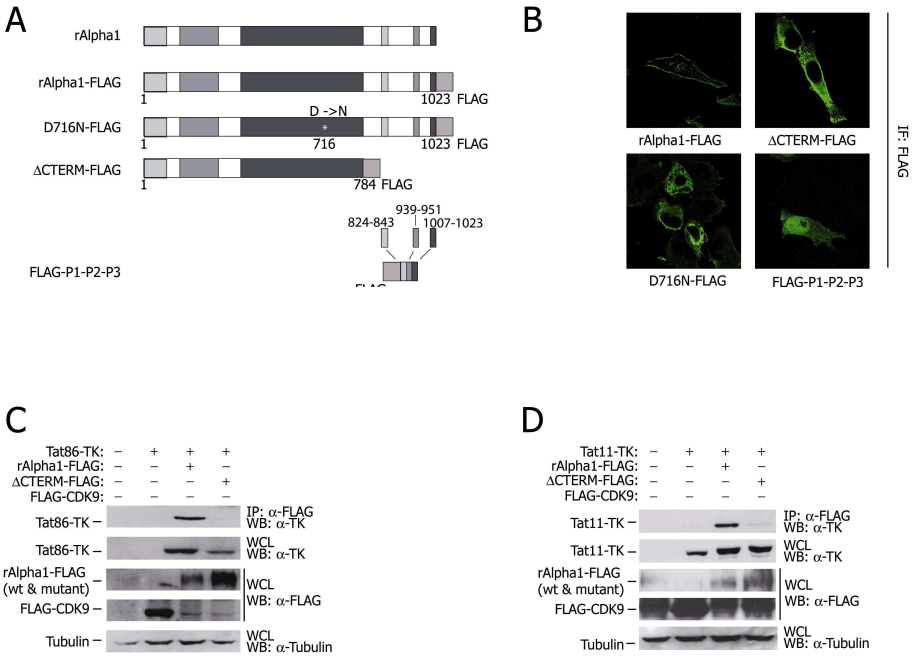

Fig. 3.18: Tat fails to co-immunoprecipitate an Alpha1 mutant lacking the C-terminus

(**A**) Schematic representation of the constructs; the deletion mutant of rat Alpha1, the wild type, as well as the catalytic mutant and the P1-P2-P3 fusion protein, fused to FLAG epitope. The residues at which deletions, fusion or mutations were designed are highlighted.

(**B**) Intracellular localization of rat Alpha1 mutant, wild type and fusion protein. Upon transfection, U2OS cells were fixed and subjected to IF against the FLAG epitope; the slides were analyzed by confocal microscopy.

(**C**) rAlpha1 C-terminal deletion mutants fail to co-immunoprecipitate Tat86-TK. The antibodies used for immunoprecipitation and subsequent western blots are indicated on the right side. Expression of proteins was assayed on whole lysates. The cells used were HEK293T cells co-transfected with Tat86-TK and either full-length rAlpha1-FLAG or the truncated mutant. FLAG-CDK9 was used as a control of FLAG immunoprecipitation.

(**D**) rAlpha1 C-terminal deletion mutant fails to co-immunoprecipitate Tat11-TK. The antibodies used for immunoprecipitation and subsequent western blots are indicated on the right side. Expression of proteins was assayed on whole lysates. The cells used were HEK293T cells co-transfected with Tat11-TK, and either full-length rAlpha1-FLAG or the truncated mutant. FLAG-CDK9 was used as a control of FLAG immunoprecipitation.

Alpha1 deletion mutant fails to rescue Tat86 secretion in ouabain treated cells

Next we wondered whether the rat deletion mutant, when expressed in human cells, was still able to rescue the inhibition that ouabain imposes on Tat release. After transfection of HEK293T cells with either wild type rat Alpha1 or mutant together with Tat11-TK, no release of the Tat fusion protein was observed in the cell culture supernatants in the presence of ouabain in the cells expressing the mutant, unlike the wild type protein (**Fig. 3.19A**). Consistent with our previous observations, secretion of the scFv antibody was unaffected by these treatments.

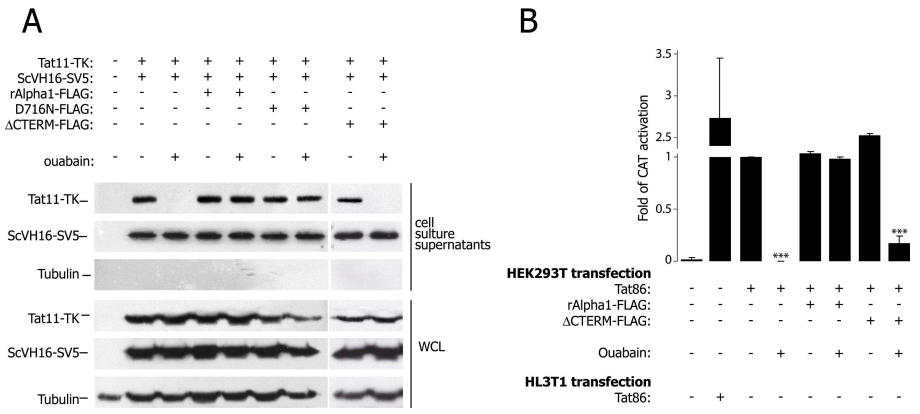

Fig. 3.19: Alpha1 deletion mutant fails to rescue Tat86 secretion in ouabain treated cells

(**A**) rAlpha1 C-terminal deletion mutant fails to rescue Tat11-TK secretion in ouabain treated cells. Cells were transfected with Tat11-TK and either rAlpha1-FLAG or the truncated mutant, then treated with ouabain; the amount of secreted protein was assessed by immunoblot on cell culture supernatants, while the protein expression was assessed on whole lysates. The bands marked with (*) represent degradation products.

(**B**) rAlpha1 C-terminal deletion mutants fail to rescue Tat86 secretion in ouabain treated cells. HL3T1 cells were treated with the supernatants of HEK293T cells, transfected with Tat alone, or cotransfected with either rAlpha1-FLAG or the deletion mutant; cells were treated with ouabain, and their supernatant incubated with HL3T1 cells. The levels of LTR activation were assessed by ELISA assay on CAT levels. As a control of LTR activation, HL3T1 cells were transfected with Tat.

Thus, binding the C terminal region of Na^+,K^+-ATPase Alpha1 subunit is an essential requisite for Tat-fusion proteins to be released extracellularly.

Finally, we performed a transcellular transactivation experiment in which the supernatants of HEK293T cells, transfected with wild type Tat86 and either the wild type or the mutant form of Alpha1 and treated with ouabain, were subsequently incubated for 24 h with reporter HL3T1 cells (**Fig. 3.19B**). The lysates of HL3T1 were used to perform an ELISA-CAT assay to quantify the levels of transactivation, compared to cells incubated with the supernatants of untransfected cells. HL3T1 cells transfected with a plasmid expressing Tat86 were used as a positive transactivation control. The results obtained showed that the supernatants of HEK293T cells transfected with wild type

Alpha1 were able to maintain levels of CAT activation in the presence of ouabain comparable with those obtained with the supernatants of ouabain-untreated cells. When the mutant was transfected, however, the supernatants possessed virtually no LTR transactivation activity.
Thus, the Tat-rat Alpha1 interaction, mediated by the Alpha1 C-terminus, is necessary for the secretion of wild type Tat86 when the interaction of Tat with endogenous human Alpha1 is disrupted by ouabain treatment.

A fusion protein corresponding to the Na^+,K^+-ATPase Alpha1 C-terminal loops binds Tat and impairs transactivation

To test binding of Tat to the three C-terminal, short cytoplasmic loops of the Na^+,K^+-ATPase Alpha1 subunit in vivo, a plasmid expression vector was obtained, encoding for the P1-P2-P3 fusion protein, corresponding to the Na^+,K^+-ATPase Alpha1 C-terminal loops linked by a glycine residue, under the control of the constitutive CMV promoter, and fused to FLAG epitope.
Expression of the P1-P2-P3 encoding plasmid was verified upon expression in U2OS cells.

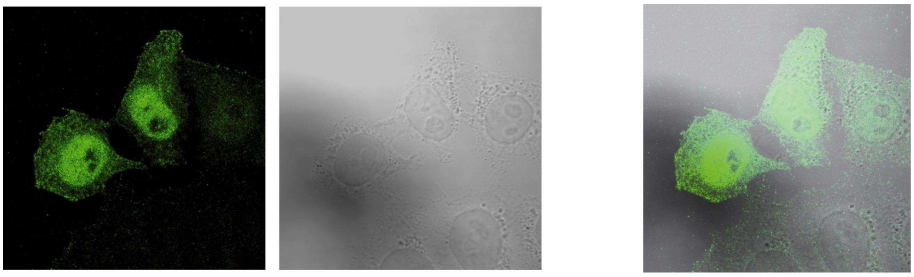

Fig. 3.20: Intracellular localization of FLAG-P1-P2-P3 fusion protein.
U2OS cells were transfected with the FLAG-P1-P2-P3 construct and, after 24 hours, fixed and subjected to immunofluorescence against the FLAG epitope. A wide field image of cells is also shown.

As shown in **Fig. 3.20**, immunofluorescence against the FLAG epitope showed a diffuse localization of the protein, which was present in both the cytoplasmic and the nuclear compartments of the cells, with the exclusion of the nucleoli.

The FLAG-P1-P2-P3 construct was then co-expressed in HEK293T cells with TK, Tat11-TK or Tat86-TK, and the lysates were used immunoprecipitation experiments using an anti-FLAG antibody. Western blot using an anti-TK antibody showed that co-immunoprecipitation with P1-P2-P3 occurred when using both Tat11-TK and Tat86-TK, but not TK (**Fig. 3.21**).

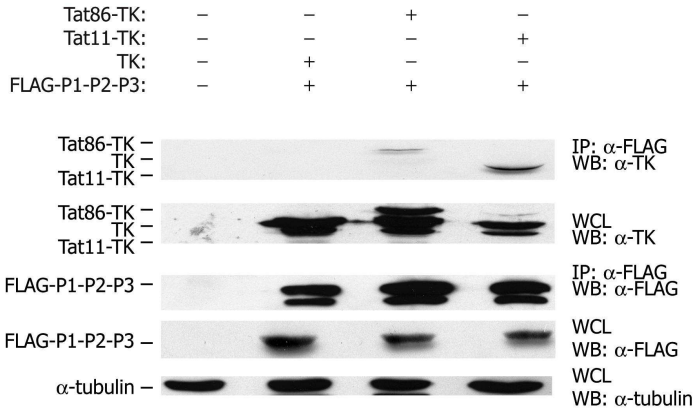

Fig. 3.21: FLAG-P1-P2-P3 fusion protein interacts with Tat

P1-P2-P3 fusion protein binds Tat86-TK and Tat11-TK *in vivo*. The antibodies used for immunoprecipitation and subsequent western blotting are indicated on the right side. Expression of proteins was assayed on whole lysates. The cells used were HEK293T cells co-transfected with either Tat86-TK, Tat11-TK or TK, and FLAG-P1-P2-P3.

Since these co-immunoprecipitation experiments indicated that the P1-P2-P3 fusion protein, once expressed intracellularly, was able to bind the basic region of Tat, we wondered whether the overexpression of this protein might also affect Tat transactivation. To test this possibility, HeLa cells expressing an LTR-driven luciferase gene were transfected with different amounts of both a Tat-expressing plasmid (1, 5 and 25 ng) and a plasmid

expressing the P1-P2-P3 protein (300 and 600 ng). These results revealed that, at any of the three Tat plasmid concentrations, the co-expression of the higher dose of the P1-P2-P3 plasmid significantly inhibited the transcriptional activation of the LTR-reporter construct (**Fig. 3.22**). Cell viability was unaffected by P1-P2-P3 transfection at the highest concentration (not shown).

In particular, when Luciferase activity (normalized over Renilla) was taken into account, the co-transfection of 600 ng P1-P2-P3 significantly reduced Tat transactivation (>50% reduction).

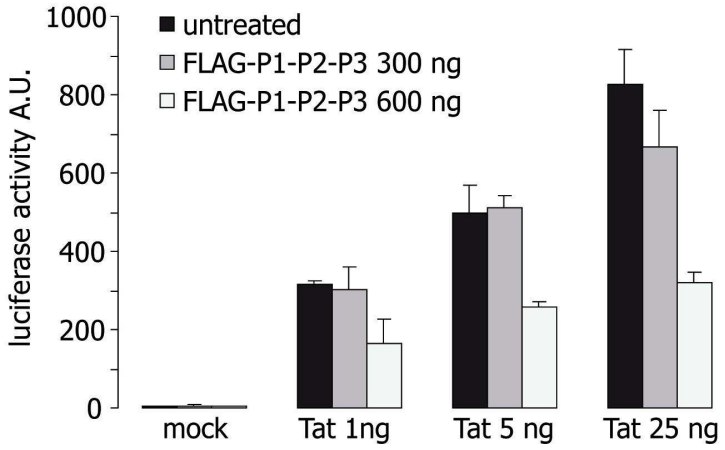

Fig. 3.22: Expression of FLAG-P1-P2-P3 fusion protein interferes with Tat transcriptional activity

C-Terminal cytoplasmic region of Na^+,K^+-ATPase interferes with transactivation. HeLa cells expressing an LTR-driven luciferase gene were transfected with different amounts of both Tat and FLAG-P1-P2-P3, in order to assess the possible effect of an interaction between the two proteins in the transactivating activity of Tat; a plasmid expressing the Renilla Luciferase gene under the control of a CMV promoter was co-transfected. The measured activities were standardized by the expression of Renilla; the histograms show the mean values of three independent experiments, together with the standard error.

These results support the general conclusion that binding between Tat and the tripartite P1-P2-P3 protein occurs inside the cells, and is consistent with

the possibility that the overexpression of the fusion protein diverts Tat from its transactivation function at the HIV-1 LTR promoter, thus acting as a competitive inhibitor.

Internalization of fluorescent peptides

The observation that the fusion protein P1-P2-P3 was able to decrease Tat transactivation, albeit at a high molar ratio to Tat, prompted us to wonder what the effect of short peptides corresponding the Alpha1 C-terminal loops might be on Tat extracellular release. At difference with the endogenously expressed P1-P2-P3 protein, peptides can be administered exogenously at high concentration and their binding to Tat should not be subject to possible structural constrains imposed by their fusion in the P1-P2-P3 construct. In addition, our in vitro binding data (**Fig. 3.16 C**) showed that any of the three C-terminal intracytoplasmic loops of the Alpha1 subunit was able to independently bind Tat.

To text the efficacy of intracellular internalization of the individual P1, P2 and P3 peptides, these were synthesized conjugated to fluorescein and administered to HEK293T cells at the concentrations of 2, 5 or 10 μg/ml. After 4 h, cells were submitted to prolonged trypsinization and extensively washed to remove extracellular fluorescence, and then analyzed by flow cytometry. All three peptides showed a remarkable capacity of dose-dependent cell internalization (**Fig. 3.23**).

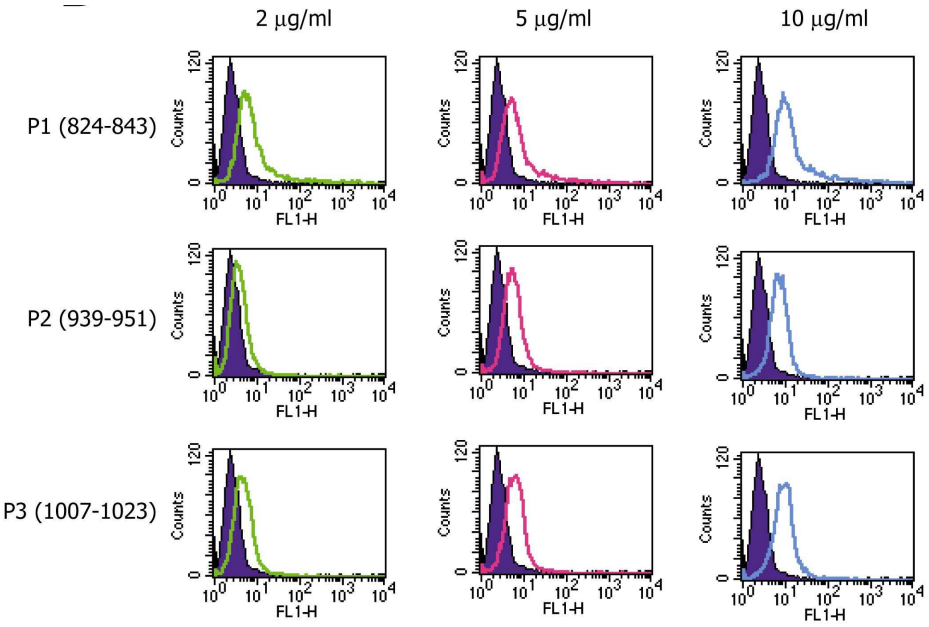

Fig. 3.23: Internalization of fluoresceinated Alpha1 peptides
Cell internalization was analyzed for each single peptide at different concentrations; the FACS analysis for three different concentrations is shown.

Intracellular cell fluorescence after 4 h treatment with an equimolar mixture of the P1, P2 and P3 peptides (hereafter named Pmix) is shown in **Fig. 3.24**. Of notice, the flow cytometry profiles show increase of fluorescence in the vast majority of cells, indicating that internalization of Pmix is a general cell property.

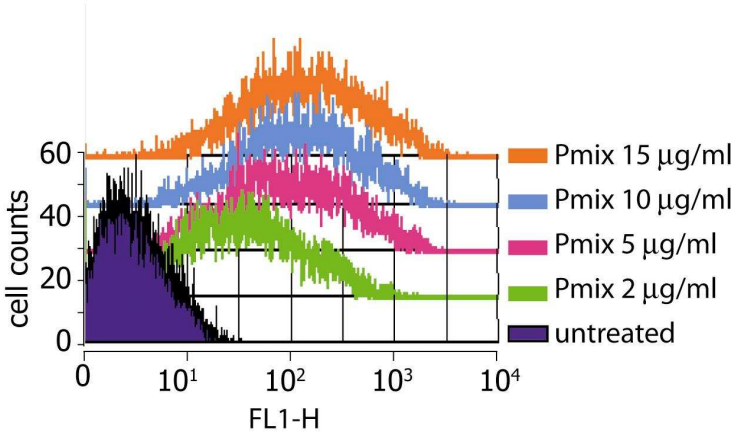

Fig. 3.24: A mixture of the three Alpha1 peptides (Pmix) is highly effective in being internalized by recipient cells
A mixture of the three Alpha1 C-terminal peptides, conjugated to fluorescein, is efficiently internalized upon addition to the cell culture medium. An equimolar mix of the three peptides, at the indicated concentrations, was added to Optimem medium, and incubated with HEK293T cells for 4 hours. Cells were then trypsinized, extensively washed, and analyzed by flow cytometry.

Synthetic peptides corresponding to the Na^+, K^+-ATPase Alpha1 C-terminal loops block Tat export

As all Alpha1 C-terminal peptides showed high cell permeability, next we tested the effects of cell treatment with the individual peptides or with Pmix on the release of Tat. Cells were transfected with Tat11-TK together with the single chain antibody ScVH16-SV5 and treated with 5 or 10 µg/ml of the P1, P2 and P3 peptides. Cell treatment with any of the three peptides, at the higher concentration, showed marked inhibition of extracellular Tat-fusion protein release, while leaving the secretion of the control scFv antibody undisturbed; an almost complete block was observed when using Pmix at the lower concentration (**Fig. 3.25**).

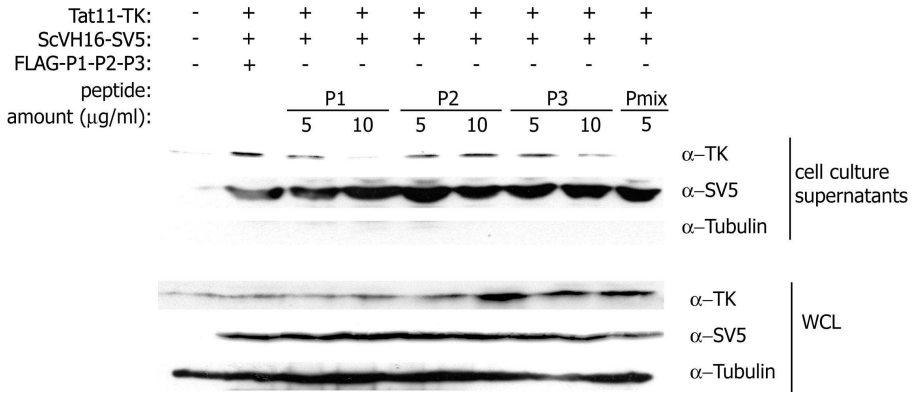

Fig. 3.25: Cell treatment with Alpha1 peptides inhibits Tat release.
HEK293T cells expressing Tat11-TK and the ScVH16-SV5 scFc antibody were treated with the individual Alpha1 C-terminus P1, P2 and P3 peptides, or with a mixture of the three (Pmix), at the indicated concentrations. The amount of secreted proteins was measured by western blotting in the cell culture supernatants at 4 h after peptide incubation. For each experimental condition, the level of intracellular protein expression was assessed by western blotting on whole cell lysates (WCL).

Based on these results, we tested the effects of a broader Pmix concentration range (from 2 to 15 μg/ml) on the secretion of Tat86-TK. Cell treatment with Pmix inhibited extracellular Tat release in a dose-dependent manner, while canonical secretion of ScVH16-SV5 was unaffected (**Fig. 3.26**). Of notice, transfection of the P1-P2-P3 tripartite fusion protein was ineffective in this assay, consistent with the possibility that the affinity of this protein for the Tat basic domain might be lower than that of the individual peptides.

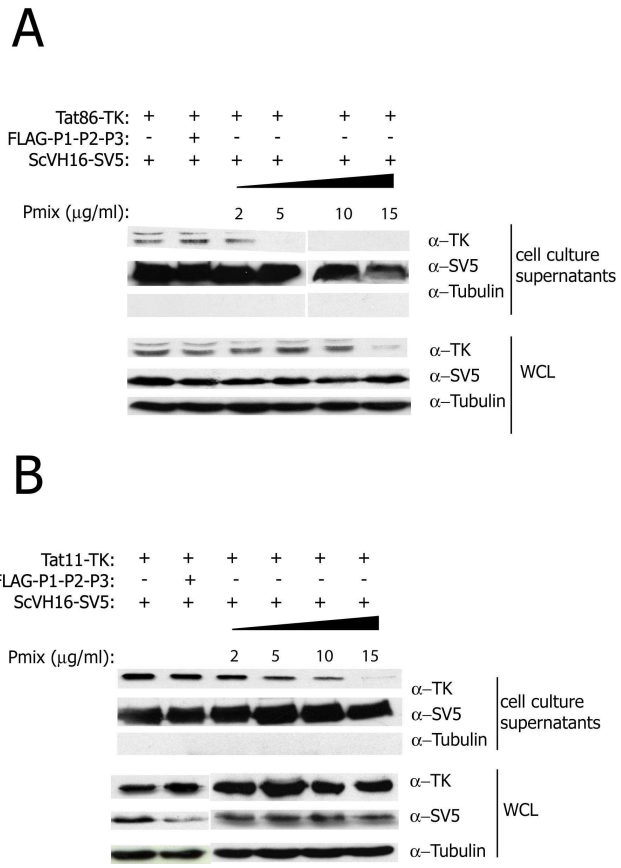

Fig. 3.26: Inhibition of extracellular Tat release by peptides corresponding to the C-terminus of Alpha1.
(**A**) Inhibition of Tat86-TK release. HEK293T cells expressing Tat86-TK and ScVH16-SV5 were incubated with the P1, P2 and P3 peptide mix (Pmix) and, after 4 h, the levels of Tat86-TK protein in the supernatant was analyzed by western blotting. The effect of P1-P2-P3 protein expression was analyzed upon transfection of the encoding plasmid. The levels of intracellular protein expression were assessed by western blotting on whole cell lysates (WCL).
(**B**) Inhibition of Tat11-TK release. The experiment was performed as in panel A by analyzing extracellular release of Tat11-TK.

Synthetic peptides corresponding to the Na^+, K^+-ATPase Alpha1 C-terminal loops inhibit HIV-1 replication

Next we wondered whether the exogenous administration of the Pmix peptides might affect HIV-1 infection. A first set of experiments was performed by a single round infection assay in HOS CCR5 cells using an HIV-1-NL4-Luc virus carrying the M-tropic Bal envelope. When cells were pre-incubated with Pmix, however not with a control FLAG peptide (10 µg/ml in each case), before viral infection a significant decrease in luciferase activity, measured 24 h post-infection, was observed (**Fig. 3.27**).

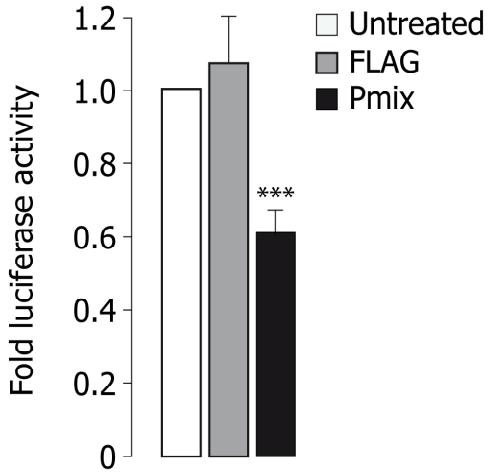

Fig. 3.27: A soluble mix of the three alpha 1 peptides inhibits HIV-1 infection
Addition of Pmix during single-round infection with M-tropic HIV-based vector expressing Luciferase gene impairs viral gene expression. HOS CCR5+ cells were infected with an HIV based vector where Nef is replaced by Luciferase gene (pNL4.3 E-R-), pseudotyped with the M-tropic Bal envelope. Prior to infection, the virus was pre-incubated with an equimolar mix of P1, P2 and P3 peptides (Pmix). Cells were incubated with the virus for 4 h, washed, and fresh medium containing Pmix was added. After 24 hours, cells were harvested and the expression levels of Luciferase were tested.

In a subsequent experiment, we wanted to analyze the effect of the Pmix and FLAG (control) peptides on the infection of CEM CD4+ T cells with wild type HIV-1_{BRU}. Over the course of 12 days after infection, half of the cell medium was replaced every second day with fresh medium containing either Pmix or FLAG peptides at a concentration of 10 µg/ml. Viral infection was monitored by measuring reverse transcriptase activity in the cell culture supernatant. The Pmix peptides were found to markedly suppress viral infection, while the FLAG peptide control had no relevant effect (**Fig. 3.28**).

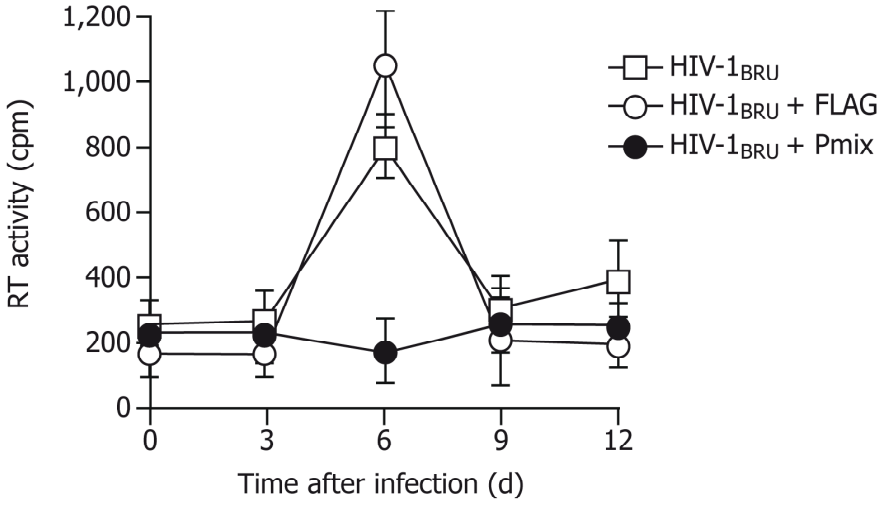

Fig. 3.28: Addition of Pmix during infection with wild type HIV-1_{BRU} impairs viral replication.
CEM cells were infected with HIV_{BRU} for 4 hours; where indicated, the infection was performed in presence of 10 µg/ml of Pmix, or of FLAG peptide, as a negative control. After infection, the medium containing the virus was washed and substituted with fresh medium, containing the corresponding peptide preparation. At t=0 and, subsequently, every 3 days until the 12th day, the supernatants were tested for RT activity, while the cells were diluted 1:2 and fresh peptides were added to the culture media.

These results collectively indicate that the suppression of extracellular Tat release and inhibition of Tat function by peptides derived from the Na^+,K^+-

ATPase Alpha1 subunit C-terminal domain is able to impair HIV-1 infection, thus paving the way to the possibility of developing novel biotherapeutics.

Chapter 4

Discussion

DISCUSSION

HIV-1 Tat protein is a small viral transcriptional activator which binds a *cis*-acting RNA element (TAR) present at the 5'-end of each viral transcript at the LTR promoter. Through this interaction, Tat activates HIV-1 transcription by promoting the assembly of transcriptionally active complexes at the LTR (see Introduction).

In addition to its role in promoting viral RNA transcription, Tat possesses unusual intercellular trafficking properties when expressed in cells (Ensoli *et al.* 1993; Chang *et al.* 1997) or added extracellularly as a recombinant protein (Tyagi *et al.* 2001; Ferrari *et al.* 2003; Fittipaldi *et al.* 2003). This ability to cross cell membranes depends on the integrity of the basic domain of the protein (aa 49-57), and is the basis for the biotechnological utilization of Tat basic region as a "protein transduction domain" (PTD) for the delivery of heterologous proteins, drugs, viral vectors and nanoparticles (Becker-Hapak *et al.* 2001; Tasciotti *et al.* 2003; Fittipaldi and Giacca 2005; Tasciotti and Giacca 2005). Both biochemical and genetic studies have indicated that the internalization of large molecules fused to extracellular Tat involves binding of the Tat basic domain to cell surface heparan sulfate proteoglycan (HSPG) receptors, followed by internalization by different endocytic routes, the most prominent one appearing to be caveolar endocytosis (Ferrari *et al.* 2003; Fittipaldi *et al.* 2003).

As Tat-expressing cells release traceable amounts of biologically active Tat (Ensoli *et al.* 1993; Chang *et al.* 1997; Tyagi *et al.* 2001; Tasciotti *et al.* 2003), an intriguing hypothesis could be that Tat release might be involved in the regulation of HIV-1 infection or even the modulation of HIV-1 pathogenesis *in vivo*.

Tat binds to the heparan sulfate proteoglycans attached to the cell surface or released into the extracellular matrix; upon secretion, HSPG could thus

function as a storage for functional Tat, which, by means of this accumulation, could locally reach high concentrations. As a matter of fact, infected cells presenting Tat bound to HSPGs on their surface, as well as cells treated with recombinant Tat, show increased permissivity to HIV-1 infection, and higher viral replication rates (Marchio *et al.* 2005); this property of extracellular Tat seems to be mediated by its binding to the V1-V2 loop of the gp120 protein.

The mechanisms underlying Tat release from the expressing cells have remained largely elusive so far; as the protein does not contain a N-terminal signal peptide, its secretion is independent from the integrity of the ER-Golgi pathway (Chang *et al.* 1997). Similar to its uptake, Tat release also depends upon the integrity of the basic region of the protein (Tasciotti *et al.* 2003; Tasciotti and Giacca 2005) however is independent of HSPG production and trafficking, since it also occurs in cells genetically defective in proteoglycan biosynthesis (Tyagi *et al.* 2001).

Characterization of the unconventional pathway of Tat secretion

The existence of a non conventional mechanism, independent from the ER-Golgi system, for the export of soluble proteins, has been known for more than 10 years. The molecular machinery underlying this process, however, is still poorly understood, even if it is quite clear that several distinct pathways may be involved for different proteins. For example, while FGF-2 secretion seems to be driven by direct membrane translocation (Schafer *et al.* 2004), the export of Galectin-1 is likely to occur through membrane blebbing (Hughes 1999); IL-1β and FGF-1 secretion, instead, is heat-shock gated, and seems to proceed through a vesicular intermediate (Prudovsky *et al.* 2003).

To investigate the mechanisms involved in the release of Tat-fusion proteins, we started studying the kinetics of release of Tat-fusion proteins from transfected cells (**Fig. 3.1 and 3.2**); a considerable portion of both fusion

proteins (~2-10%) was found in the cell culture supernatants in the absence of detectable cell lysis. Of interest, the amount of Tat-TK proteins free in the supernatant was increased by cell treatment with soluble heparin (**Fig. 3.3C**), consistent with the notion that this polyanion acts as a competitive inhibitor of cell surface and extracellular matrix HSPGs, which sequester soluble Tat (Chang *et al.* 1997), and that heparinase III releases the attached protein (Chang *et al.* 1997).

While a single-chain Fv antibody (scFv) (a positive control for ER-Golgi dependent secretion) and Tat86-TK proteins were expressed at comparable levels by the cells, however, the kinetics of their appearance in the cell culture supernatants was drastically different. The levels of the extracellular Tat-fusion protein peaked as early as 30 min after the addition of fresh medium and slightly decreased in the subsequent 2 h (**Fig. 3.2**). In contrast, secreted scFv appeared at later times and its levels progressively increased. Analogous findings were also detected when cells were co-transfected with Tat11-TK and ScVH16-SV5, thus indicating that the release kinetics is a property imparted by the Tat basic domain. As compared to Tat86-TK, however, the overall efficiency of Tat11-TK release was significantly higher.

Recent evidence (Seelenmeyer *et al.* 2005; Zehe *et al.* 2006) demonstrated that the binding to a cell surface receptor is a necessary step for FGF-2 and Galectin-1 export. The mechanism is not completely clear, however, the authors hypothesize a "molecular trap" (Nickel 2007) capturing the secreted molecules and avoiding their binding to cell surface counter receptors. Moreover, this binding could limit the abundance of free factors in the extracellular milieu and, through the microenvironment of the cell membrane, maintain a gradient leading proteins outside the cells without needing energy supply. While for Tat, however, such dependence from HSPG binding has never been demonstrated, on the contrary, work conducted by different groups has shown that a net export of Tat protein

could be revealed either in presence of heparinase III (which detaches the heparin moiety of HSPGs) (Chang *et al.* 1997) or in cell lines genetically defective in HSPG biosynthesis (Tyagi *et al.* 2001). Thus, in the case of Tat, HSPG binding does not serve to create a positive gradient at the site of export, as the heparin-bound protein can promptly re-enter the cell by caveolar endocytosis (Fittipaldi *et al.* 2003). This feature clearly distinguishes, in the case of Tat, the pathways of release and uptake.

As proteins other than TK had the same behavior when fused to Tat (**Fig. 3.3A**) we concluded that the apparent lack of accumulation of Tat-fusion proteins in the supernatant over time likely reflects a steady-state equilibrium between the amount of released protein that remains bound to extracellular HSPGs despite the presence of soluble heparin inhibitor, and that undergoing internalization through the interaction with cell membrane-associated HSPGs (Tyagi *et al.* 2001; Fittipaldi *et al.* 2003). Consistent with this possibility, we observed that CHO A745 cells, which do not express detectable amount of proteoglycans due to a genetic defect in their biosynthetic pathway (Rostand and Esko 1997), release progressively increasing amounts of Tat11-TK in their supernatants (**Fig. 3.3B**).

Collectively, these results show that heterologous proteins containing Tat or the Tat basic domain possess the property of being progressively released from the producing cells, independent from the production of HSPGs; however, upon secretion, Tat binds to the extracellular surface by means of its association with the HSPGs (as demonstrated by the surface-staining experiments shown in **Fig. 3.6**). There are experimental evidences for the presence of Tat on the envelope of the new virions budding from infected cells (Marchio *et al.* 2005), which could serve several functions in the process of cell infection by HIV-1.

In co-cultures experiments employing Tat-GFP-expressing cells and LTR-CAT-expressing cells, the Tat-GFP protein has been shown to traffic from producing to LTR-CAT-expressing cells, in which it was able to determine a

detectable level of CAT activation, thus demonstrating that Tat is secreted from expressing cells in a biological active form (Tyagi, et al., 2001). Here we extended these experiments by testing the functionality of secreted Tat found in the cell-free supernatants. When HL3T1 cells, stably expressing an LTR-CAT reporter gene, were incubated with the cell culture supernatants of Tat expressing cells, secreted Tat was able to cross cell membranes of HL3T1 cells, where it was targeted to the nucleus and transactivated the LTR-driven reporter gene (**Fig. 3.4**). Collectively, these result confirm that, upon its release, Tat preserves its functionality and biological activity on the LTR promoter.

Since the Tat-GFP fusion protein was excluded from the Golgi apparatus (**Fig. 3.5**), as shown by lack of co-localization in immunofluorescence experiments where an integral Golgi protein (GM130) was labeled, release of the protein had to proceed through a pathway which was independent from the classical, Golgi-dependent, route.

Ouabain blocks extracellular Tat release

We then studied the sensitivity of Tat fusion protein release to some metabolic drugs in order to define similarities and differences between the release of Tat and that of other proteins known to be secreted by unconventional means (**Fig. 3.7**).

Beyond being, as expected, insensitive to BFA, a drug which blocks the classical pathway of secretion disrupting the Golgi apparatus (Misumi *et al.* 1986; Orci *et al.* 1991), Tat secretion was not affected by either methylamine (a drug which blocks the endosomal recycling (Rubartelli *et al.* 1990; Hamon *et al.* 1997; Zhou *et al.* 2002)), or glyburide (a sulfonylurea interfering with an ABC transporter (Hamon *et al.* 1997; Flieger *et al.* 2003)). These data support the notion that several distinct mechanism of non classical export from the cells exist for different proteins. In fact, a

number of publications have described the sensitivity of IL-1β and FGF-2 release to methylamine (Rubartelli *et al.* 1990; Hamon *et al.* 1997; Zhou *et al.* 2002), thus implying a vesicular intermediate in the secretion of these factors, while this drug had no effect in the case of FGF-1 (Shin *et al.* 1996), Galectin-1 (Sato *et al.* 1993; Mehul and Hughes 1997; Hughes 1999) and, now, Tat. On the contrary, Galectin-1 secretion was highly sensitive to glyburide, an inhibitor of the ABC-1 transporter (Flieger *et al.* 2003); this drug, however, had no effect on the release of Tat, further confirming the conclusion that, most probably, the "non classical pathway", unlike the "classical" one, does not rely on a single molecular machinery to drive proteins outside the cells. Quite remarkably, extracellular Tat release showed high sensitivity to ouabain, a specific inhibitor of the Na^+,K^+-ATPase pump; the same drug is also known to inhibit the unconventional release of FGF-2 by a mechanism that so far has remained elusive (Florkiewicz *et al.* 1998; Dahl *et al.* 2000; Trudel *et al.* 2000; Smith *et al.* 2001) (**Fig. 3.7C and D**).

The role of the Na^+,K^+-ATPase in extracellular Tat release

Na^+,K^+-ATPase is an enzyme built into the plasma membrane which catalyzes ATP hydrolysis coupled with Na^+ and K^+ transfer through the membrane against the electrochemical gradient. The site of cardiac glycoside binding is located on the extracellular part of the enzyme alpha-subunit (Lingrel *et al.* 1994) and the sensitivity to these inhibitors differs depending on animal species (minimal sensitivity to ouabain is characteristic for the enzyme from rat).

As we found that Tat secretion is not sensitive to curcumin (**Fig. 3.7**), an inhibitor of all P-type ATPases (also including the Na^+,K^+-ATPase), it can be concluded that the inhibitory effect exerted by ouabain was due to a perturbation of the Tat-Alpha1 interaction, rather than to an involvement of the enzymatic function of the Na^+,K^+-ATPase as a cation pump. This

observation also directed our attention towards a specific role of the Alpha1 subunit, which is bound by ouabain, in the overall process of Tat secretion. This conclusion is also in line with the findings showing no effect on FGF-2 secretion by the obliteration of the transmembrane potential of Na^+ and K^+ ions generated by the Na^+,K^+-ATPase (Florkiewicz *et al.* 1998). This observation once more highlights an analogy in the mechanism of secretion of the two proteins, which share several structural and functional features. Similar to Tat, FGF-2 possesses a basic peptide stretch, binds HSPGs on the extracellular matrix (Zehe *et al.* 2006), is a leaderless secreted protein (Engling *et al.* 2002; Backhaus *et al.* 2004; Schafer *et al.* 2004) and displays angiogenic activity (Nugent and Iozzo 2000).

Next we wanted to investigate the possibility that a physical interaction between Tat and the apha1 protein existed. This was indeed proven by co-immunoprecipitation experiments, which also demonstrated that the Tat and Alpha1 interaction was disrupted by the addition of ouabain to the cells expressing the two proteins (**Fig. 3.8**). When the ouabain-insensitive, rat isoform was used, the interaction between the two protein was unaffected by the addition of the drug.

As ouabain and ouabain-like hormones have been demonstrated to trigger the endocytosis of plasmallemal Na^+,K^+-ATPase by a clathrin dependent mechanism that translocates the enzyme to intracellular compartments (Liu *et al.* 2004a), we investigated whether the inhibiting effect shown on the binding between Tat and Alpha1 could be due to a change of localization of either of the two proteins, which might interfere with their interaction. However, when the localization of either Tat, as a fusion protein with YFP, or a FLAG-tagged version of Alpha1, was studied in the presence of ouabain, no significant modification of their normal localization was revealed (**Fig. 3.9**).

Ouabain did not interfere with Tat endocytosis, either, thus showing that its effect was specific and related to some crucial step in the process of release,

and not to some general, unspecific perturbation of intracellular vesicle trafficking (**Fig. 3.10**).

Finally, FRET experiments also revealed that Tat and rat Alpha1 physically interact *in vivo* (**Fig. 3.12**). In these experiments, however, which were performed by tagging Alpha1 with CFP, localization of Alpha1 was mainly nuclear and nucleolar, and not on the cell membrane. We speculate that a proteolytic degradation of alpha-CFP may occur, leading to the presence of small C-terminal fragments, still fused with CFP, which could still bind Tat and, because of this interaction, be translocated in the nucleus and the nucleolus where overexpressed Tat is known to accumulate.

While searching for the role of ouabain and Alpha1 in the process of Tat release, we found that the expression of a ouabain-resistant Alpha1 isoform was able to rescue Tat secretion in the presence of ouabain, thus pointing out that the effect of the drug was clearly mediated by its interaction with Alpha1 (**Fig. 3.14**). Moreover, a point mutant of rat Alpha1, unable to bind and hydrolyze ATP, thus lacking catalytic activity as a cation pump, was also able to rescue Tat secretion in ouabain treated cells, as well as it was capable to bind Tat, thus confirming that the structure of Alpha1, more than its function as a cation pump, had a crucial role in Tat secretion (**Fig. 3.13** and **Fig. 3.14**).

Taken together, these findings show that a ouabain-sensitive interaction takes place between Tat and the Alpha1 subunit of the Na^+,K^+-ATPase, and that such interaction is critical for the block of Tat secretion observed in ouabain treated cells. The similar effect exerted on FGF-2 secretion by ouabain (Florkiewicz *et al.* 1998), as well as the interaction observed between FGF-2 and the Alpha1 subunit (Dahl *et al.* 2000), suggest that a similar pathway of non classical secretion may be followed by the two proteins, which share several structural features.

Alpha1 is a large, integral membrane protein with transmembrane-spanning domains plus cytoplasmic regions (Morth *et al.* 2007). To map the interaction between the two proteins in a specific region of the apha1 subunit, we focused our attention on the cytoplasmic domains of the protein which were the most likely candidates for Tat binding, as the rest of the protein is either embedded in the phospholipidic bilayer, or protrudes into the extracellular space (**Fig. 3.15**). The GST-pull down assays pointed out binding of three Alpha1 fragments, mapping in the C-terminal region of the protein, with Tat, and specifically with its basic domain; a mutant version of Tat, in which the basic residues of the PTD were mutated to alanines, was unable to bind the alpha-1 cytoplasmic domains *in vitro* (**Fig. 3.15** and **3.16**).

Very recently, when the crystal structure of the Na^+,K^+-ATPase was elucidated, some unique features of the Alpha1 C-terminal region were highlighted (Morth *et al.* 2007). In particular, the existence of a "C-terminal switch", sensitive to membrane potential, and interacting with the Na^+ binding site, was demonstrated, since a protein carrying a deletion of the five most C-terminal residues of the chain (residues 1018-1023) showed remarkably less affinity to Na^+. Can this highly electropositive stretch, whose function is to modulate ion transport, be involved in the binding and release of Tat? An interesting speculation might be that, through its interaction with the C-terminal region, Tat is brought in the proximity of the pump channel, through which it could somehow achieve membrane crossing. The hypothetical mechanism exploiting such ion channel for a larger molecule with the dimension of Tat-fusion proteins however remains obscure.

When an *in vivo* interaction assay was performed (**Fig. 3.18**), an Alpha1 truncation mutant, lacking the C-terminal region was able neither to immunoprecipitate Tat in the lysates of expressing cells, nor to rescue Tat secretion in ouabain treated cells, thus proving that the effect of the drug was to disrupt the interaction taking place in the C terminal region of

Alpha1. In this respect, it is worth noting that, despite having been known as a specific inhibitor of the Na^+,K^+-ATPase for more than 30 years, the molecular mechanism leading to the blockage of ion transport triggered by ouabain is still unclear. The cardiac glycoside (as well as endogenous ouabain-like hormones) binds a specific site on the extracellular side of the pump, located within in the third extracellular hairpin (transmembrane segments 5 and 6), with an involvement also of the first and second transmembrane segments (which do not directly take part in binding themselves however may act by modulating the accessibility of the ouabain-binding site). Little is known, however, on the events leading to the inhibition of the pump function: a possible link between the cardiac glycoside binding site and the cation transport sites of the Na^+,K^+-ATPase, therefore suggesting structural base for cardiac glycoside inhibition of the enzyme, may be such that ouabain binding to the implicated region blocks the movement of the H5 and H6 transmembrane domains which may be required for energy transduction and cation transport (Palasis *et al.* 1996; Lingrel *et al.* 1997; Lingrel *et al.* 1998).

At least two possibilities exist to explain ouabain inhibition of Tat release and Tat-Alpha1 interaction: in a first scenario, ouabain binding imparts some spatial modification in Alpha1 3D structure, thus modifying somehow the affinity of the C-Terminus for Tat. Alternatively, ouabain binding could trigger the detachment of some third part factor or complex of factors (or the recruitment of some inhibitor) which may be necessary for Tat release. Even if our experiments with the D716N catalytic mutant and the peptide-mediated inhibition of Tat release seem to make the first possibility more likely, however, the possible existence of some other factors involved in the process of release (and the presence of which could be somehow regulated by ouabain) cannot be formally excluded and must be taken into account. The experiments entailing mapping of the Tat-Alpha1 interaction domain deserve further comment. Deletion mapping studies clearly indicated that

Tat interacts with the C-terminal intracytoplasmic loops of Alpha1 (**Fig. 3.18**). This notion was later confirmed by co-expressing a FLAG-tagged fusion of the three C-terminal short cytoplasmic stretches, spanning residues 824-843, 939-951, 1007-1023, of the Alpha1 sequence, and showing their co-immunoprecipitation with Tat and Tat11 fusion proteins (**Fig. 3.21**). Most notably, in a model cell line expressing an LTR-controlled reporter gene, co-expression of the P1-P2-P3 fusion protein led to significant reduction of transactivation (**Fig. 3.22**).

Finally, we were also able to demonstrate that cell treatment with synthetic peptides corresponding to the C-terminal region of Alpha1 block extracellular Tat release (**Fig. 3.24**). This remarkable result not only once more highlights the relevance of Alpha1-Tat interaction in extracellular Tat release, but also paves the way to the possibility of pharmacologically inhibiting Tat trafficking. This was best exemplified by the experiments in which cell treatment with the Alpha1 peptide mixture inhibited HIV-1 infection both in a single-round infection assay (**Fig. 3.27**) or during chronic infection of susceptible cells (**Fig. 3.28**). Further experiments are clearly required to understand better the molecular correlates of these observations. In particular, it will be important to understand whether inhibition occurs through diminishing HIV-1 virion infectivity, or by modulating the susceptibility of non-infected cells.

Lately, a novel function has been demonstrated for the Alpha1 subunit as a signal transducer of hormonal stimulation by ouabain-like molecules in different cell types and tissues (Xie and Askari 2002). Of particular interest, ouabain signaling seems to proceeds through a particular pool of Alpha1 which is bound to caveolin-1 through its N-terminus (Wang *et al.* 2004; Cai *et al.* 2008) and therefore, at the cell membrane, resides in lipid rafts domains; of notice, the same domains also mediate Tat endocytosis, and transcytosis (Hansen *et al.* 1999) of extracellular substances across tight-junction lined barriers (such as epithelia and blood-brain barrier). While the

mechanism by which ouabain signaling through caveolar Alpha1 and Tat-mediated trafficking could be linked remains unclear, still the possibility that Alpha1 could supply the link between Tat internalization and release, possibly coordinating its intracellular and extracellular activities, is appealing.

Given the intriguing similarity with the pathway of FGF-2 release as well as with FGF-2 structure, an interesting speculation could be that Tat may hijack the secretion pathway evolved for FGF-2, by means of mimicking its structure, in the same way by which viral components often use the cellular machinery to achieve viral functions.

In recent years, the interaction with acidic domains in the phospholipidic components of the inner membrane leaflet has turned out to be a crucial step that may precede membrane crossing by proteins devoid of a signal peptide. In particular, Temmerman and coworkers have demonstrated that partially folded FGF-1 possesses higher affinity for acidic phospholipids in the inner leaflet of the cell membrane and that this interaction results in more efficient extracellular export of the growth factor (Temmerman *et al.* 2008). In this perspective, the Alpha1 C terminal region might function as a docking site promoting a spatial juxtaposition of Tat to the inner membrane leaflet, which in turn could lead to membrane translocation. Very recently, it has been proposed that, under specific pH conditions, a single tryptophan residue in the sequence of Tat could mediate its insertion into the plasma membrane (Yezid *et al.* 2009). In this context, the involvement of the Alpha1 subunit has great relevance, since it could provide a preferential route through which cytosolic Tat may be targeted to the membrane for its export. Of interest, the properties of Alpha1 C-terminal peptides, which in our assays were able to cross the cell membrane and localize inside the cells (**Figs. 3.23 and 3.24**), could suggest the existence of a sort of "translocable region" in the Alpha1 C-terminus, which could somehow promote Tat membrane translocation.

More investigation is clearly needed to uncover the mechanism by which this transmembrane protein could drive or regulate the membrane crossing of cargo proteins. However, our findings open a totally new chapter in the understanding the mechanisms leading to unconventional protein release, as they reveal an unexpected new role of the Alpha1 subunit of the Na^+,K^+-ATPase, traditionally seen only as a cationic pump essential for the maintenance of transmembrane potential.

Intercellular trafficking and lentiviral biology: Why is Tat secreted?

In recent years, peculiar trafficking properties have been demonstrated for a number of accessory viral proteins of various pathogens, including both viruses and bacteria. Most noticeably, regulatory proteins of all the three families of complex retroviruses (HFV Bet, HTLV-1 Tax and HIV-1 Tat) as well as the HIV-1 accessory proteins Vpr and Nef, have the ability to cross cell membranes and traffic to nearby cells, where they mediate a number of pleiotropic effects not necessarily related to the cytopathic effects of the respective viruses.

While at first these proteins have been regarded as "accessory" or "auxiliary", as they are not necessary for the assembly of mature virions and are often dispensable for virus replication *in vitro*, subsequent *in vivo* studies have made clear that these proteins are instead fundamental players in the onset and development of the infection. Of particular interest are the overall effects on the immune system, where, according to our current understanding, the combined activities of Tat, Vpr and Nef (which can all be released and therefore also act on uninfected, bystander cells), would enhance the survival of infected cells, while at the same time promoting the death of uninfected immune cells, or the anergy of the effectors of the immune CTL response (Li *et al.* 1995; Curtain *et al.* 1997; Collins *et al.* 1998; Poggi *et al.* 1998; Zocchi *et al.* 1998; Bennasser and Bahraoui 2002; Muthumani *et al.* 2005). This combined, synergic effect of immune depletion

and down-modulation could explain both the lack of anti-HIV CTL response and the rapid immune depletion, associated with HIV infection, which can hardly be otherwise explained by only taking into account the virus replication-associated cell death.

Moreover, these small, membrane permeable proteins, also exert cytotoxic effects on different cell types, such as renal cells, spindle and endothelial cells, neurons, and glial cells (reviewed in (Foreman 2001; Jones and Power 2006) (King *et al.* 2006); (Zuo *et al.* 2006) (Kino and Chrousos 2004)). These effects accounts for most of the pathological aspects of the HIV-induced illness which are unrelated to viral propagation.

In the case of Tat, there are evidences (Marchio *et al.* 2005) that extracellular Tat, either in soluble form, or bound to the extracellular matrix of virus-producing cells (and therefore, most likely, also on the surface of budding virions), can enhance virus infectivity and increase the rate of viral replication; in particular, it has been suggested that secreted Tat could bind the HSPGs on uninfected cells, where it could function by promoting virus spread and infection by means of its specific binding with the viral envelope protein gp120 (see also (Nappi *et al.* 2009)).

Moreover, as extracellular Tat is mainly internalized by caveolar endocytosis (Fittipaldi *et al.* 2003), one could hypothesize that the presence of Tat bound to the viral surface could promote virus uptake by the caveolar pathway, and its transmission by transcytosis, a process which starts with caveolar endocytosis. This might constitute a mechanism for the spread of HIV-1 through epithelial and blood-brain barriers despite the presence of tight junctions between lining cells.

Finally, since a possible link between extracellular, HSPG-bound Tat and the extravasation of lymphoid cells has been recently provided (Urbinati *et al.* 2009), we could hypothesize that infected cells release Tat in order to

enhance viral spread and persistence in tissues such as, for example, the CNS.

For all the above mentioned reasons, the Tat protein appears a very appealing target for the development of novel therapeutic strategies aimed at limiting viral spread and replication during the course of HIV-1 infection. In particular, since Tat functions both at the nuclear level (where it drives the transcriptional activation of viral genes) and in the extracellular compartment (where it exerts the pleiotropic activities described above), the development of drugs capable to block Tat, either intracellularly or extracellularly, is highly desirable. Such drugs could stop viral replication on one hand while, on the other hand, might contribute to contrast the pathogenetic mechanisms involved in HIV-1 disease development.

The attempts aimed at designing anti-Tat drugs, however, have led to largely disappointing results so far. The protein has no known enzymatic activity and possesses a highly flexible structure - no Tat crystals have been obtained yet, indicative of its flexible conformation adapting to different cellular protein partners -, which prevents the easy possibility of designing or selecting highly effective drugs. Of the few small compounds that, over the last several years, have been reported to inhibit Tat or block Tat-TAR interaction, none has so far advanced toward clinical experimentation. Use of a decoy RNA sequence, consisting of a multimerized TAR element, has been described as a mean to divert Tat from its natural target at the HIV-1 promoter (Lisziewicz *et al.* 1993). This decoy, however, requires to be expressed intracellularly upon delivery using viral vectors, with obvious limitations in terms of applicability. Finally, anti-Tat vaccination has been proposed as a mean of both preventing HIV-1 replication and targeting extracellular Tat, however the experimentation in this respect (reviewed in (Ensoli and Cafaro 2002; Caputo *et al.* 2004)) has so far generated very poor results.

As we found that a soluble mix of Alpha1 C-terminal peptides, when added to cells, was internalized and was able to block Tat release (**Figs. 3.25 and 3.26**) and to impair HIV-1 infection (**Figs. 3.27 and 3.28**), these peptides could become a useful tool for the treatment of HIV infection and related diseases.

Chapter 5

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GRAZIE A...

Scrivere la pagina dei ringraziamenti in una tesi è sempre un'opera complicata: un elenco di nomi non riesce a catalogare tutte le amicizie che in questi anni ho potuto stringere e, talvolta, purtroppo, ho dovuto sciogliere; tutte le persone che ho incontrato, in certi casi solo per un momento, ma in quel momento hanno risolto qualche mio problema; quelle che mi hanno accompagnato facendo un tratto di questa strada assieme, lavorando, arrabbiandosi e ridendo con me, condividendo gioie e dolori di questa esperienza che è e resterà senz'altro unica, nella mia vita; e quelle che sono entrate a fare parte della mia vita stessa.
Tutti, comunque, si portano dietro un pezzo di me, e io di loro, e di certo posso affermare che in questo lungo percorso io non sia mai stata sola.
Un semplice grazie mi sembra indicibilmente inadeguato per spiegare quanto io sia riconoscente per tutti i momenti in cui qualcuno ha saputo ascoltarmi, aiutarmi, lasciarmi sfogare, o farmi fare una risata che ha reso più piacevole una lunga giornata di lavoro; è grazie a tutti questi momenti che nonostante le difficoltà incontrate, la stanchezza e i momenti di scoraggiamento in cui mi sembrava impossibile riuscire a terminare questo mio percorso, sono sempre riuscita in qualche modo ad andare avanti.
Non riesco nemmeno a immaginare come sarebbe andata la mia vita se dopo la laurea avessi fatto una scelta diversa, e non riesco a immaginare che tipo di persona sarei diventata: di certo posso dire che nonostante abbia pensato mille volte "ma chi me l'ha fatto fare!!!", ho fatto esperienze uniche e ho incontrato persone che sicuramente hanno arricchito la mia vita e solo per questo, ne valeva la pena eccome!

Ringrazio Gabriele che mi ha fatto venire voglia di fare progetti per il futuro, aiutandomi a capire che la mia felicità va oltre la riuscita o meno degli esperimenti; la sua presenza, solida e costante al mio fianco, mi permette di trovare sempre il sentiero giusto anche quando sembra che tutti i segni siano stati cancellati.

Ringrazio la mia famiglia perché mi ha sempre appoggiato in ogni cosa che abbia fatto, senza mai tentare di influenzarmi, sostenendomi in maniera discreta eppure sempre presente. Sembra passato un secolo da quel giorno in cui discutevo con i miei genitori se fosse il caso o meno di tentare il concorso, perché le energie sembravano non bastarmi per studiare tutto quello che avrei dovuto: quante energie ho tirato fuori in questi anni, che non avrei mai pensato di avere!

Un pensiero e un grazie particolare voglio lasciarli alle mie amiche Laura, Alessia e Claudia, che condividono con me gioie e inquietudini della vita, restandomi sempre vicine.

Devo poi ringraziare Mauro Giacca, che credendo nelle mie capacità e affidandomi questo progetto, mi ha dato la possibilità di fare questa esperienza professionale; Arturo Falaschi e Anna Cereseto che mi hanno permesso di far parte del laboratorio di Biologia Molecolare della Scuola Normale Superiore; Pino Rainaldi e tutto il personale LTGM per la loro "ospitalità" all'interno delle loro strutture; Monica, Vania e Antonella che con il loro lavoro insostituibile mi hanno spesso reso le cose molto più semplici; ringrazio inoltre la Scuola Normale Superiore, l'ICGEB di Trieste e l'Istituto di Fisiologia Clinica del CNR di Pisa, per aver reso concretamente possibile lo svolgimento di questo dottorato.

Non saprei da dove cominciare per ringraziare tutti quelli che vorrei, perché c'è un ricordo, una storia, o un'esperienza che mi lega ad ognuna delle persone che ho incontrato o conosciuto, per un motivo o per un altro, dentro o fuori il laboratorio, durante questo lungo e importante periodo della mia vita; mi sembra freddo e privo di significato fare una lista infinita di nomi (rischiando di dimenticare anche qualcuno, perché in sette anni se ne incontrano di persone…) quando quello che io ho in mente e nel cuore è un insieme colorato di facce, sorrisi, risate, frammenti di vite che in qualche modo si sono mescolate alla mia e hanno fatto si, ognuna a suo modo, che io sia diventata chi sono oggi.

GRAZIE A TUTTI, DI CUORE!

Silvia

THANKS TO...

Writing the acknowledgments page of a thesis it's always a complicated matter: a list of names cannot contain all of the friendships I've been able to make and sometimes, unfortunately, I've been forced to break, in all these years; all the people I've met, sometimes only for a moment, but who in that moment have been able to solve a problem of mine; those who went with me for a piece of this path, working, getting angry and laughing along, sharing happiness and pain of this experience which is and will stay unparallelled, in my life; and those who became a part of my own life.
Everybody, however, carry a piece of myself with them, as I carry a piece of them, and I can tell for sure that along this long way I've never been alone.
A simple "thank you" seems unspeakably unadequate to explain how thankful I am for all the moments when someone was able to listen, or help, or let me vent, or make me have a good laugh that made better of a long day of work; thanks to all these moments, in spite of all the hardships that I encountered, of the tiredness and of the moments of discouragement, when reaching the end of this path seemed an impossible task, I've always been able to go on, somehow.
I can't even start to imagine how my life could have been if, after my degree, I had made a different choice, and I can't imagine which kind of person I'd be: I can tell for sure that, in spite of having though "Why in god's name did I do that!" a thousand times, I made unrepeatable experiences and met people who enriched my life for sure, and if for this reason only, it was worth it!

I thank Gabriele who makes me willing to make plans for the future, helping me understand that my happiness goes way beyond how experiments turn out; his presence, strong and unyielding at my side, always makes me find the right path even when the marks along the road seem to have faded.

I want to thank my whole family as they've always stood by me in everything I've done, never trying to influence me, supporting me in an unobtrusive yet always present way. Hundred years seem to have passed since that day when I was discussing with my parents about the opportunity of trying the admission test, as my energy didn't seem enough to study everything I needed to know: how much energy I found along these years, that I would never suspect I've had!

A special thought and thanks go to my friends Laura, Alessia and Claudia, with whom I share good and bad times of our lives, and who have always been standing by me.

I have then to thank Mauro Giacca, who believing in my skills and trusting me with this project, gave me the possibility of living this professional experience; Arturo Falaschi and Anna Cereseto who allowed me to be a part

of the Molecular Biology Laboratory of Scuola Normale Superiore; Pino Rainaldi and all the LTGM people for the "hospitality" in their facilities; Monica, Vania and Antonella who often made things easier with their irreplaceable work; many thanks to Scuola Normale Superiore in Pisa, ICGEB in Trieste and Istituto di Fiosiologia Clinica of CNR in Pisa, for making concretely possible the carrying out of this ph. D.

I wouldn't know where to start to thank everyone I would like to, because there is a memory, a story, or an experience which ties me to each of the people I've met or known, for this or that reason, inside or outside the lab, during this long and important stretch of my life; I feel like it would be cold and devoid of any significance to make an endless list of names (risking to forget someone too, because you can't imagine how many people you meet in seven years...), when all that I see in my mind and in my heart is a multicolored body of faces, smiles, laughs, snippets of lives which somehow mixed with my own, and each of them in its own way, made me become the person I am today.

HEARTFELT THANKS TO ALL OF YOU!

Silvia

"Un ultimo sguardo commosso all'arredamento...

E chi s'è visto s'è visto!"