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COMMENTARY

HIV-1 *trans-Activating* (Tat) Protein

BOTH A TARGET AND A TOOL IN THERAPEUTIC APPROACHES

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ABSTRACT. Tat proteins (*trans*-activating proteins) are present in all known lentiviruses and are early RNA binding proteins that regulate transcription. Tat from the human immunodeficiency virus type-1 is a protein comprising 86 amino acids and encoded by 2 exons. The first 72 amino acids are encoded by exon 1 and exhibit full *trans*-activating activity. The second exon encodes a 14-amino-acid C-terminal sequence that is not required for *trans*-activation but does contain an RGD motif, which is important in binding to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. Tat has an unusual property for a transcription factor; it can be released and enter cells freely, yet still retain its activity, enabling it to up-regulate a number of genes. Tat also has an angiogenic effect; it is a potent growth factor for Kaposi sarcoma-derived spindle cells, and, separately, it has been shown to bind to a specific receptor, Flk-1/KDR, on vascular smooth muscle cells, as well as to integrin-like receptors present on rat skeletal muscle cells and the lymphocyte cell line H9. It appears that the basic domain of tat is important, not only for translocation but also for nuclear localisation and *trans*-activation of cellular genes. As such, targeting of tat protein or, more simply, the basic domain provides great scope for therapeutic intervention in HIV-1 infection. There is also opportunity for tat to be used as a molecular tool; the protein can be manipulated to deliver non-permeable compounds into cells, an approach that already has been employed using ovalbumin, β-galactosidase, horseradish peroxidase, and caspase-3. BIOCHEM PHARMACOL **58**;10:1521–1528, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. tat; basic domain; *trans*-activation; translocation; angiogenesis

This commentary will not dwell on the intricacies of HIV-1† infection, replication, and pathogenesis since more comprehensive reviews are available in this field [1–4]. Instead, we will discuss the properties of the HIV-1 regulatory protein tat. We will address briefly its role in HIV-1 infection but will concentrate mainly on how the properties of tat can be manipulated so that it can be used as a molecular tool. Tat has a remarkable property; it can leave cells from which it is synthesised and cross the membrane of adjacent cells, where it localises in the nucleus. Importantly, tat maintains its activity and, once inside the nucleus, is able to *trans*-activate a number of genes, especially those relating to cytokine production, and thus can modulate certain cellular activities. The ability of tat to translocate across the cell membrane is of major importance, particularly since the cell membrane can be a formidable obstacle for macromolecules to negotiate; therefore, by manipulating this property of tat, non-permeable molecules can be introduced into the cell.

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† *Abbreviations*: HIV-1, human immunodeficiency virus type-1; tat, *trans*-activating protein; LTR, long terminal repeat; TAR, tat activating response element; AIDS, acquired immunodeficiency syndrome; and KS, Kaposi sarcoma.

PROCESSING AND STRUCTURE OF TAT

Depending upon the pattern of splicing, the HIV-1 genome can be transcribed to produce three different sizes of mRNA: the 9-kb full-length transcript, the partially spliced 4- to 5-kb transcript, and the multiply spliced 2-kb transcripts. The identification of a number of donor and acceptor splice sites has revealed that HIV-1 has the potential to produce more than 30 differentially spliced mRNAs [5]. Different mRNAs predominate at different times during the replication cycle [6]; early in infection the multiply spliced 2-kb mRNAs predominate, and high levels of tat as well as nef (negative factor) and rev (regulator of viral protein expression) are produced. Nef protein is synthesised at all stages of the virus replication cycle and functions to enhance virion infectivity and to regulate T-cell functions [4], whereas the role of rev is to regulate viral mRNA expression. In the later stages of infection rev can down-regulate its own production as well as that of other proteins such as tat and nef, thus limiting virus replication [3]. HIV-1 tat is a polypeptide comprising 86 amino acids and is encoded by 2 exons. The first 72 amino acids are encoded by the first exon and are organised into three major domains. The 14 carboxy terminal amino acids are encoded by the second exon and contain an RGD motif; although this region is not required for *trans-*

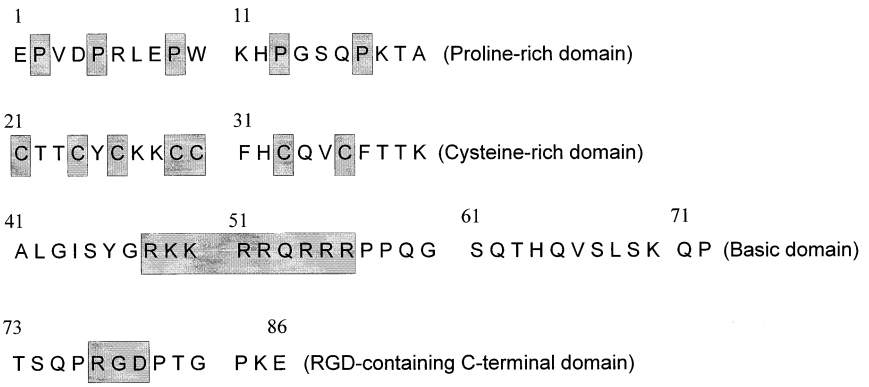

FIG. 1. Structure of tat. The complete amino acid sequence [1–86] of the tat polypeptide is shown, and the four major domains are indicated. These are termed the proline-rich, the cysteine-rich, the basic, and the RGD-containing domains. The residues that characterise each domain are shaded.

activation, it is important in binding to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [7] (Fig. 1).

TRANSCRIPTIONAL ACTIVITY OF TAT

Tat is a potent *trans*-activator of HIV-1 and is essential for viral replication. It is also able to *trans*-activate other cellular genes. The cysteine-rich region is essential for tat function [8, 9] and mediates the formation of metal-linked dimers *in vitro* [10]. The basic region is important for nuclear localisation [11, 12] and *trans*-activation [13]. Indeed, the product of the first exon possesses full *trans*-activating activity [8]. In the absence of virally encoded tat there is little or no gene expression directed by the proviral LTR [14, 15]. *trans*-Activation by tat is entirely dependent upon the presence of TAR RNA, a transcriptional control element that appears to be unique to lentiviruses [4]. TAR is found at a region near the start of transcription in the HIV-LTR, and TAR RNA forms a stable stem–loop structure [16] that is important for the tat response [17–20]. Although it is specifically the basic region that interacts with the TAR RNA, the precise amino acid sequence necessary for specific RNA recognition is surprisingly flexible. If a peptide using the amino acids in the basic region is synthesised in the opposite direction, i.e. from carboxyl to amino terminus, or scrambled sequences are used, binding to TAR RNA occurs with a similar affinity to that of the wild-type peptide [13]. However, the overall charge does appear to be important, since substituting a single arginine residue with alanine reduced binding by 2-fold. In the absence of tat, only low levels of mRNA transcripts are found. However, as RNA species detected are relatively short, this suggests that initiation does occur, but without tat transcriptional elongation does not. Elongation is restored if tat is re-introduced, and thus it appears that tat increases the processivity of RNA polymerase rather than simply initiating transcription [21].

PARACRINE EFFECTS OF TAT

Tat has an unusual property for a transcription factor. It can be released from cells and enter other cells, where it translocates to the nucleus in an active form [22]. In infected but quiescent cells, it can stimulate the transcriptional activity of the HIV-LTR [23, 24]. In uninfected cells, it can *trans*-activate cellular genes such as tumour necrosis factor, interleukin-2, and interleukin-6 [25–27]. It is conceivable that the up-regulation of cytokine production in uninfected cells primes them for later infection with HIV-1; in essence, circulating tat perpetuates virus entry and productive infection. Tat also regulates collagen expression in glioblastoma cells [28] and cell survival-related proteins including p53 and Bcl-2 [29, 30]. Interestingly, tat activates B lymphocytes, and it is possible that tat may contribute to B cell hyperactivation during the early stage of HIV-1 infection and to activation-induced B cell death mediated by Fas during the late stage of infection [31].

STIMULATION OF CELL GROWTH BY TAT

Tat has potent angiogenic activity. Tat induces the migration and invasion of cytokine-stimulated endothelial cells and stabilises capillary-like networks formed by endothelial cells on a matrix support [32]. The angiogenic activity of tat *in vivo* is strongly potentiated by heparin [33], although it also appears that high concentrations of heparin inhibit tat activity. Many potent angiogenic factors are heparin binding proteins, including fibroblast growth factors and vascular endothelial growth factor, and these, in turn, are dependent on heparin or heparan sulphate for their activity [34–36]. Common to these growth factors is a basic domain: Albini *et al.* [37] demonstrated that tat bound strongly to heparin and the putative heparin binding region was highly active in growth and migration assays. Interestingly, heparin discloses the angiogenic activity of tat at concentrations where tat alone is ineffective, suggesting that like basic fibroblast growth factor [38], tat binds tightly to heparin or extracellular heparan sulphate, and this protects it from proteolytic degradation. This suggests that tat can be stored in heparan sulphate-rich extracellular matrices. In our laboratory, using a solid-phase binding assay, we investigated the binding of the sulphated polysaccharide dextrin 2-sulphate to full-length recombinant tat and overlapping peptides spanning the entire sequence of the polypeptide. Dextrin 2-sulphate bound strongly to recombinant tat, and the interaction appeared to be medi-

ated predominantly through a hydrophobic core region adjacent to the basic domain of tat [39]. This hydrophobic region is thought to be important in presenting the adjacent basic amino acids in the correct orientation to the TAR RNA. Evidence for this was reported by Kashanchi *et al.* [40], who found that mutations in the hydrophobic domain abolished the *trans*-activating activity of tat.

Among the complications of HIV infection and AIDS is KS, a highly vascularised lesion often found in homosexual males [41–43]. The aetiology of KS is still unclear, but it has been proposed that high levels of basic fibroblast growth factor may contribute to the development of KS [44], although a herpes-like virus has been found in KS lesions, which matches well with the epidemiology of AIDS-associated KS [45]. Tat appears to be a cofactor in AIDS-associated KS; expression of the *tat* gene in transgenic mice induces KS-like lesions [46, 47], and hyperblastic lesions have been found in baboons infected with HIV-2 [48]. Indeed, tat is a growth factor for human KS spindle cells and for cytokine-stimulated endothelial cells [49].

TARGETING TAT FOR THERAPY

As tat is essential for HIV-1 replication, it is an obvious target for therapeutic intervention. During the initial phases of infection, large amounts of tat, together with other regulatory proteins, are synthesised and drive HIV-1 replication. Suitable therapeutic agents can conceivably work in one of two ways: either by binding to extracellular tat and stopping it from entering other cells or by delivering a therapeutic agent into infected cells to inhibit tat activity or synthesis.

Binding to extracellular tat and inhibiting its activity can be achieved in one of two ways: either by generating antibodies or by administering compounds that are able to bind and sequester extracellular tat. One of the major goals for HIV-1 researchers is to produce a vaccine that will either stop infection or reduce the pathogenicity of an underlying virus load. Antibodies to tat protein have been produced in animals and shown to block uptake of tat *in vitro*. Antibodies to tat added to culture medium inhibit HIV-1 infectivity *in vitro* [50, 51], suggesting that if this regulatory protein is targeted, HIV-1 infection can be diminished. There is some evidence that patients who developed antibodies to tat survive longer. Re *et al.* [52] demonstrated that haemophiliac patients with higher titres of anti-tat antibodies had lower levels of p24 antigen, which is a recognised surrogate marker of HIV-1 replication, and appeared to survive longer. One of the major concerns regarding the use of antibodies against HIV-1 proteins such as tat is the problem of mutation and immune escape; however, it may be possible to reduce the chances of this occurring. Tat appears to be highly conserved throughout the various quasi-species of HIV-1, and certain domains are essential for activity. In addition, there appear to be three immunodominant regions in tat corresponding to amino acids 17–32, 33–48, and 65–80 [53]. If an immunisation strategy is adopted using peptides directed against both the conserved regions and the immunodominant domains of tat, the polyclonal response in vaccinated individuals should ensure that antibodies are produced that yield a variety of binding patterns. Therefore, any subsequent mutations in tat could be accommodated by the immune system.

The use of drugs to inhibit tat is also possible. The initial step in HIV-1 infection is thought to involve the interaction of virus gp120 with the T cell receptor CD4 [54, 55], although the subsequent discovery of alternative receptors suggests that other mechanisms also may be involved [56–58]. Sulphated polysaccharides are thought to inhibit HIV-1 *in vitro* by disrupting the gp120–CD4 interaction, by binding to either gp120 [59, 60] or to the cellular receptor CD4 [61, 62]. However, it is quite clear that these acidic compounds can interact with tat, and for heparin, it is believed to be via an ionic interaction with the basic domain [63]. Several sulphated polysaccharides including dextran sulphate, heparin, fucoidan, and dextrin 2-sulphate have been shown to have potent anti-HIV-1 activity *in vitro* [64–67] as well as binding to full-length tat [39]. Since it is unlikely that sulphated polysaccharides readily enter cells due to their charge, it is likely that such compounds act by sequestering extracellular tat. This mechanism suggests an alternative explanation for the anti-HIV-1 activity of sulphated polysaccharides.

Targeting intracellular tat is a more difficult problem. Some workers have attempted to introduce antibodies into infected cells in an attempt to inhibit the activity of HIV-1. Cruikshank *et al.* [68] lipidated an anti-tat antibody and demonstrated with confocal microscopy that it is able to enter cells and inhibit HIV-1 replication of various cell lines by up to 85%, whereas the non-lipidated antibody does not. An alternative strategy was employed by Mhashilkar *et al.* [69], who transfected COS-1 cells with various single-chain anti-tat "intrabodies." One "intrabody" was generated against the first exon of tat and modified with a C-terminal C_κ domain to increase cytoplasmic stability. This "intrabody" was able to inhibit intracellular transport of tat to the nucleus and inhibit tat *trans*-activation. Importantly, when these antibodies were transfected into $CD4^+$ SupT-1 cells, HIV-1 infection was blocked completely. Indeed, it appeared that the cells were resistant to infection throughout the 36-day experiment. Evidence to support the validity of "intrabodies" as potential therapeutic tools was reported by Pilkington *et al.* [70], who used a Fab phage display library derived from the peripheral blood lymphocytes of an HIV-1 asymptomatic individual to produce recombinant human Fab antibody fragments against HIV-1 tat and rev proteins. Using peptide mapping experiments, the binding of anti-tat Fab was found to be directed against an epitope situated within a broad linear region comprising amino acids 22–54. Importantly, this region contains the *trans*-activation domain, and, theoretically, if these Fab fragments were transfected into HIV-1-

susceptible cells, then the intracellular anti-tat antibodies could inhibit viral replication.

Another possible gene therapy approach is to insert a tat-inhibitory gene (*antitat*) into the cell. Such a gene has been produced by Lisziewicz *et al.* [71] using a dual-function inhibitory molecule comprising polymeric TAR and antisense tat. Tat synthesised by the provirus or taken up by the cell extracellularly activates the transcription of antitat RNA. Antitat decreases the amount of cellular tat protein by inhibiting the translation of tat mRNA (antisense tat) and also by blocking the function of polymeric TAR. Another strategy taken by Zhou *et al.* [72] uses hammerhead ribozymes. These are RNA molecules that are capable of catalytic cleavage of complementary RNA molecules. These workers used ribozymes directed against two portions of the HIV-1 genome that are designed to cleave RNA in the *tat* gene or in a common exon for tat and rev. In this way they were able to demonstrate inhibition of virus replication *in vitro*.

The approaches outlined above were relatively successful *in vitro*, but whether this technology can be applied *in vivo* is uncertain since there are two major problems. First, it will be necessary to introduce DNA or ribozymes into every infected cell, as it only requires one infected cell to drive replication and disease, and second, the specificity of the therapy is essential because it is important that only tat is affected and not other cellular proteins or genes.

INTERNALISATION OF TAT

Tat is efficiently taken up by cells. Frankel and Pabo [24] have shown that as little as 100 ng of tat is required to *trans*-activate HeLa cells transfected with HIV LTR linked to a reporter gene. In the presence of lysosomotropic agents such as chloroquine, *trans*-activation can be achieved with just 1 nM of exogenous tat protein. It is the region centred on the basic domain of tat that is believed to be responsible for translocation. Physiochemical studies indicate that the region spanning amino acids 38–49 of tat adopts an α-helical structure with amphipathic characteristics [73]. This suggests that the α-helical structure adopted by this region is crucial in uptake. However, structural studies undertaken on the HIV-1 tat protein using two-dimensional nuclear magnetic resonance and molecular dynamics calculations revealed that tat has two highly flexible domains corresponding to the cysteine-rich and basic domains but that there was no evidence of an α-helical structure [74]. The basic domain comprising residues 48–58 does not overlap the amphipathic domain; however, this region comprises a nuclear localisation signal, GRKKR. This sequence has been added to the amino terminus of β-galactosidase, and was shown to allow accumulation of the reporter protein in the nucleus [9]. In a later study, Vives *et al.* [75] used truncated peptides to delineate the domains needed to allow internalisation and concluded that the basic domain is essential for internalisation, whereas the amphipathic region is not required. In fact, the complete basic region is required, since if the three arginine residues at the C-terminal end of the peptide are removed, then no internalisation occurs, even at high concentrations.

The mechanism by which tat enters cells is still not fully understood. Mann and Frankel [76] have suggested that tat accumulates on the cell surface and enters through a passive process termed adsorptive endocytosis. However, in studies using vascular endothelial cells, Albini *et al.* [77] reported that tat bound specifically, with a K_d of 73 pM, to the receptor Flk-1/KDR, which is found only on vascular endothelial cells. The natural ligand for this receptor is vascular endothelial growth factor, and this protein, unlike basic fibroblast factor, is able to compete with tat for binding to Flk-1/KDR. More importantly, tat is able to activate the Flk-1/KDR tyrosine kinase and induce a rapid, specific, and concentration-dependent tyrosine phosphorylation of a 205-kDa protein. It is highly likely that similar specific tat receptors exist on other cell types, since Vogel *et al.* [78] demonstrated that tat bound to $\alpha_v\beta_5$ integrin present on rat skeletal muscle cells and Weeks *et al.* [79] reported that tat bound to a 90-kDa receptor present on the surface of the lymphocytic cell line H9. Interestingly, both research groups found that tat peptides containing the basic domain also were able to bind to the receptors. A third potential mechanism for tat entry into cells was described by Vives *et al.* [75], who showed that tat peptides corresponding to amino acids 37–60 can translocate across the cell membrane rapidly, even at 4°, indicating that the uptake mechanism is unlikely to be receptor-mediated endocytosis. However, the amphipathic region of tat extending from amino acids 37 to 47 is not required for tat uptake; only peptides containing the basic domain were internalised by the cells. These authors suggest that since the basic region is essential for translocation, it is likely that a tight ionic interaction occurs between the basic groups of the peptide side chain and the negative charges of the phospholipid heads in the cell membrane bilayer, thus inducing a local invagination of the plasma membrane. The local reorganisation of the bilayer would result in the formation of inverted micelles with tat enclosed in the hydrophilic core, culminating with the release of tat in the cytoplasm. Additionally, the presence of the basic region and the nuclear localisation sequence (GRKKR) would result in speedy translocation to the nucleus. The accumulation of tat in the nucleus is rapid, occurring after a few minutes. The addition of hydrophobic groups to tat was shown to increase the uptake of both full-length protein and peptides comprising amino acids 37–58. Chen *et al.* [80] showed that when tat was coupled through a cysteine thiol to the spacer arm of a biotin analogue, there was a 6-fold increase in tat uptake and subsequent *trans*-activation. However, uptake and *trans*-activation were increased only when reducible spacer arm groups were used; conjugating tat to biotin linked through non-cleavable spacer arms inhibited *trans*-activation. This also indicates that cysteine residues in tat are important for *trans*-activation activity [8, 81].

EXPLOITATION OF TAT TRANS-ACTIVATION

Most molecules such as oligonucleotides, genes, peptides, or proteins are taken up poorly by cells, since they do not efficiently cross the lipid bilayer of the plasma membrane or of the endocytic vesicles. This is a major limitation with respect to their *ex vivo* or *in vivo* therapeutic use, but the ability of tat to cross cell membranes may be exploited to enable the intracellular delivery of non-permeable molecules. Anderson *et al.* [82] reported that conjugation of a Fab antibody fragment to a tat peptide comprising amino acids 37–62 enhanced its *in vitro* cell surface association and internalisation. In studies on antigen processing, Kim *et al.* [83] demonstrated that ovalbumin conjugated to a tat peptide comprising amino acids 49–57 entered T cells and was processed by the MHC class I processing pathway. In contrast, coupling ovalbumin to a peptide comprising 9 lysine residues did not stimulate T cells, suggesting that translocation was not simply due to the charge of the peptide. In earlier work, Fawell *et al.* [84] showed that β-galactosidase, horseradish peroxidase, RNase A, or domain III of *Pseudomonas* exotoxin coupled to full-length tat or peptides comprising amino acids 1–72 or 37–72 could enter various cell types, and there was also evidence of selectivity towards certain organs. Mice treated with β-galactosidase coupled to these tat peptides resulted in delivery to several tissues, with high levels evident in the heart, liver, and spleen, whereas none was detected in kidney or brain [84].

Ironically, the properties of HIV-1 tat may be of use in treating HIV-1 infection. In a recently published study [85], HIV-1 tat was coupled to caspase-3. This protein has been shown to be important in apoptosis because it cleaves the inhibitor of caspase-activated DNase, resulting in its activation and ultimately precipitating cell death via apoptosis [86–89]. More exquisite, however, was the modification of the caspase-3 protein by deleting two endogenous caspase cleavage sites and inserting an HIV-1 gag cleavage site. This modification rendered only HIV-1 infected cells susceptible to apoptosis by this pathway, thus reducing the risk of killing healthy cells and further compromising the immune system.

These examples underline the potential for using tat to introduce biologically relevant conjugates into the cell, and this property of tat probably will be exploited further in the foreseeable future.

CONCLUSIONS

It is apparent that tat is a very important protein, not only in terms of HIV-1 infection, but also with regard to its use in other aspects of biology. It is unusual, in that it is able to leave the cell and enter adjacent cells without loss of its transcriptional activity. It is a potent *trans*-activator not only of HIV-1 but also of certain cytokines, and is also able to influence cell survival genes and stimulate growth. Importantly, all of these properties can be utilised in other research; for example, taking advantage of the ability of tat to translocate across the cell membrane allows other less permeable molecules to enter the cell and, where required, localise to the nucleus, since tat also contains a nuclear localisation sequence. Utilising tat offers a unique opportunity to undertake some good basic cellular research and opens the door to understanding more about cell permeability, intracellular localisation, and *trans*-activation of genes, as well as, of course, HIV.

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