INTERACTIONS OF

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

PROTEINS WITH ASTROCYTES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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I. ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) infection frequently affects central nervous system (CNS). The most common CNS complication of HIV-1 is the AIDS dementia complex, also known as HIV-1 encephalopathy. The mechanism underlying this illness remains unknown. HIV-1 is known to infect and replicate in microglia and astrocytes with the CNS. Astrocytes are the most abundant cells within the central nervous system. They serve a multiplicity of important functions that contribute to the process of neural development, as well as to normal brain function. Therefore, astrocyte dysfunction may adversely affect neurons and neuronal functions. The astrocytes appear to undergo functional changes either upon infection or interactions with HIV-1 proteins such as gp120 and Tat.

To understand the interaction between gp120 and astrocytes, which may be the initial event for HIV-1 infecton to astrocytes or gp120 effects on astrocytes, we characterized, kinetically and biochemically, the binding sites for HIV-1 gp120 on human fetal astrocytes. A single binding site was observed with values for Kd of 26 nM and Bmax of 29.9 fmoles/$4x10^4$ cells. Specific receptors for gp120 such as CD4 on lymphocytes and galactocerebroside (GalC) on SK-N-MC cells have been identified. In this study, neither a polyclonal antibody against GalC (anti-GalC) nor a monoclonal antibody against the gp120 binding domain of CD4 (OKT4a) affected the ^{125}I-gp120 binding to astrocytes. However, binding of gp120 was inhibited by 60% with OKT4a on HeLa CD4 cells and approximately 70% by anti-GalC on SK-N-MC cells. Furthermore, neither CD4 nor GalC were detectable on astrocytes using flow cytometry or

immunocytochemistry. The mass of the gp120 binding molecule as determined by cell surface labelling and immunoprecipitation was approximately 260 kDa. Thus, specific binding sites for gp120 are present on human fetal astrocytes and these sites appear to be distinct from both CD4 and GalC.

HIV-1 Tat has a number of effects on brain cells including astrocytes. It is released from infected cells and subsequently taken up by several cell types where it may transactivate viral or host genome. Tat is encoded by two exons. The first exon, coding for the N-terminal 72 amino acids, is sufficient for Tat transactivation. The function of the Tat second exon, coding for C-terminal 14 to 32 amino acids, remains unknown. Tat1-72 (1-72 amino acids from first exon) and Tat1-86 (full length Tat) were labelled with ^{125}I and incubated with human fetal astrocytes and human fetal neurons. We demonstrated that the uptake of Tat1-72 without the second exon was much lower than that of Tat1-86. This suggests an important role for the C-terminal region of Tat for its cellular uptake and ~90% of the internalized Tat was localized in the nuclei. Uptake of ^{125}I-Tat1-72 could be inhibited by dextran sulfate and competitively by unlabelled Tat1-72 but not by overlapping 15 mer Tat peptides, suggesting that Tat internalization is charge and conformation dependent. Interestingly, one 15 mer peptide, Tat28-42, greatly enhanced Tat1-72 uptake by undetermined mechanisms.

Evidence indicates that astrocytes may contribute to the pathogenesis of HIV encephalopathy by regulating the production of neurotoxic factors by infected cells. Tat has been shown to induce functional changes in astrocytes. To understand the mechanism by which Tat initiates the effects on astrocyte, we characterized the Tat binding properties on

the astrocyte cell membrane. By immunocytochemical staining, we have demonstrated that Tat specifically bound to the astrocyte surface. This result was confirmed by ^{125}I-Tat binding assay on astrocytes in the presence of excess unlabelled Tat or anti-Tat antibody. This binding of Tat on astrocytes does not depend upon integrins αvβ3, α5β1 or αvβ5, which has been reported to be Tat binding proteins on other cell lines. In an attempt to determine the epitope of Tat involved in binding to astrocytes, we used 15 mer peptides overlapping by 10 amino acids each and spanning the entire sequence of Tat1-86 in an attempt to block the binding of Tat. None of the peptides were able to inhibit Tat binding. In contrast, Tat binding on astrocytes was enhanced by Tat28-42. By immunoprecipitation of ^{125}I-astrocytes with Tat, 35 and 43 kDa proteins were detected on the astrocyte surface. these may represent novel Tat binding proteins on astrocytes. These findings may be important for understanding the neuro-pathogenesis of HIV-1 infection.

II. INTRODUCTION

1. Structure of HIV-1

HIV-1 is a member of the lentiviridae subfamily of retroviruses. It has a cone-shaped core, and is composed of four nucleocapsid proteins p24, p17, p9, and p7 with an envelope outside. The p24 protein forms the main component of the inner shell of the nucleocapsid (NC). The NC proteins (p9 and p7) are tightly associated with the viral genome. The envelope is composed of two glycosylated proteins: gp120 and gp41. The inner portion of the envelope is surrounded by myristylated p17 protein that provides the matrix (MA) for the viral structure and is important for the integrity of the virion (Yu *et al.* 1992). Inside the capsid is the HIV-1 genome which has two identical RNA strands with the polarity of mRNA. The RNA genome (~9.8 kb) contains *gag, pol*, and *env* genes for viral structural proteins, *tat*, *rev*, and *nef* for regulatory proteins, *vif*, *vpr*, *vpu*, *vpt*, and *tev/tnv* for accessory proteins, and long terminal repeats at 5' and 3' ends (fig. 1).

2. Gp120 and Tat proteins of HIV-1

Gp120 and Tat, two of the viral proteins described above have been used in this study. Gp120 is a structural glycoprotein encoded by the *env* gene and initially synthesized as an 88 kDa precursor. This precursor is then inserted into the rough endoplasmic reticulum where the addition of high-mannose N-linked carbohydrate chains as well as folding into an appropriate tertiary structure takes place (Fennie and Lasky, 1989). The carbohydrate chains are terminally modified in the Golgi complex to form gp160. The gp160 precursor is then cleaved by a cellular protease into mature envelope

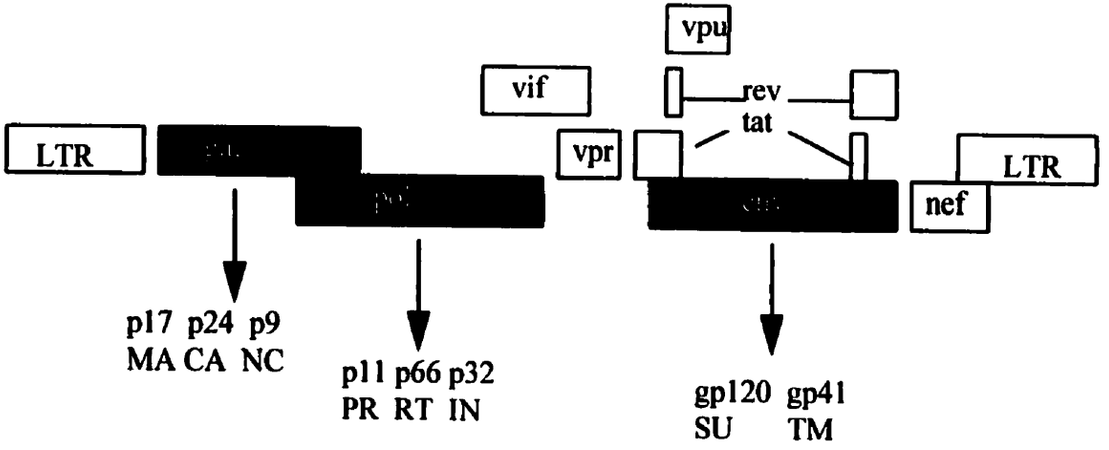


Figure 1 Structure of HIV-1 genome

gp120 and gp41 which are transported to the cell surface (Willey *et al.* 1988). The gp41 is a transmembrane protein which exists as a non-covalent complex with extracellular gp120 on the viral envelope. Despite the considerable variability of the HIV-1 envelope, there are regions or structural features that are highly conserved. For example, all 18 cysteine residues located within gp120, as well as most cysteine residues in gp41, are conserved (Modrow *et al.* 1987), suggesting that cysteine residues may be necessary to maintain a proper three-dimensional structure of the envelope protein. There are also several highly conserved regions C1- C4 that are interspersed with regions of high variability (V1-V5) within gp120. This protein plays an important role in virus-host interaction. Glycosylation of gp120 is crucial for its binding to the primary receptor CD4 since it helps fold gp120 into the proper conformation (Li *et al.* 1993). However, once gp120 has acquired the proper confirmation carbohydrate chains on gp120 are not required for the interaction between gp120 and CD4 since enzymatic removal of carbohydrate chains by endoglycosidase does not affect gp120 binding activity (Li *et al.* 1993). Interaction between gp120 and CD4 is discussed further in related sections.

Tat is one of the regulatory proteins. The *tat* gene is encoded by two exons, one exon preceding the *env* gene coding for 72 amino acids and the other exon within the *env* gene coding for 14 to 36 amino acids (Arya *et al.* 1985) (fig. 1, 2). A predominant form of Tat protein in the infected cells is 86 amino acids long (15 kDa) derived from two-exon mRNAs (Rice *et al.* 1993; Goh *et al.* 1986). Because of a stop codon immediately following the splice donor site of the first *tat*-coding exon, a minor form of 72 amino acids (14 kDa) Tat is expressed from additional unspliced one-exon mRNA. The Tat

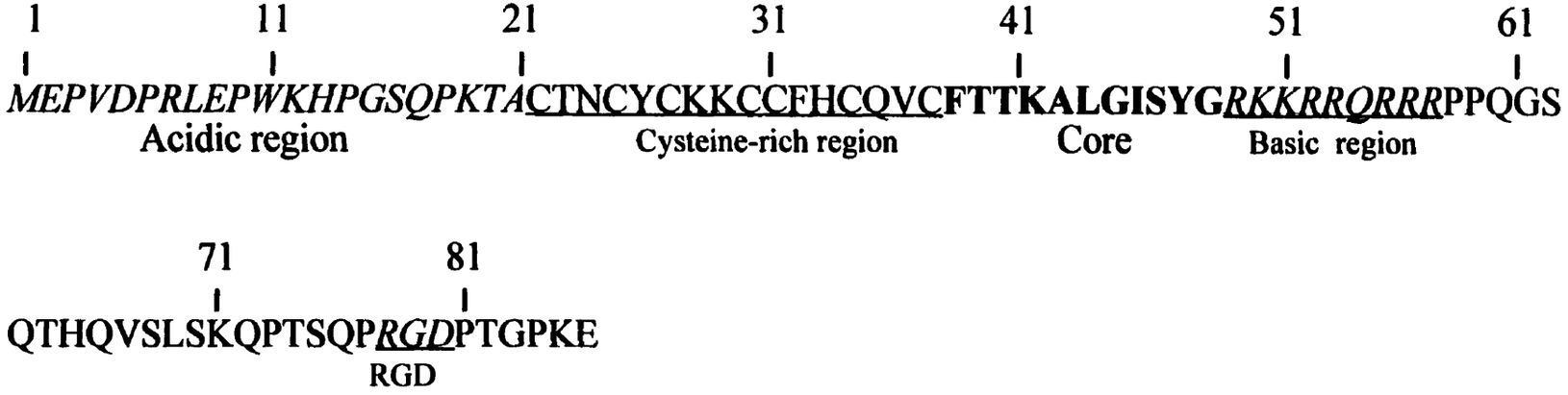


Figure 2 Tat sequence and its functional domains of HIV-$1_{BRU.}$

Acidic region (1-21):	likely form α-helix as activation domain
Cysteine-rich region (22-37):	metal binding
Core region (38-48):	most conserved
Basic region (49-57):	nuclear localization, binding to αvβ5
RGD (78-80):	adhesion to α5β1 and αvβ3
Core and basic regions (38-57):	neurotoxicity

protein is a transactivator of LTR-directed gene expression (Sodroski *et al.* 1985). The N-terminal 72 amino acids of Tat protein, encoded by the first exon, appears sufficient for full transactivation of HIV-1 LTR-specific gene expression (Sodroski *et al.* 1985). Three important functional domains have been identified in Tat protein for its transactivation function (fig. 2). The acidic region in N-terminus has been proposed to have a periodic arrangement of acidic, polar and hydrophobic residues consistent with an amphipathic α-helix (Rappaport *et al.* 1989), a feature reminiscent of activation domains of many transcription factors. Whether the acidic region of Tat folds into an α-helix and serves as an activation domain requires further study. A cluster of seven cysteine residues, highly conserved among divergent isolates of HIV-1 Tat proteins, constitutes the second domain. Mutation of these cysteine residues destroys Tat activity (Garcia *et al.* 1989; Sadaie *et al.* 1990; Sadaie *et al.* 1989; Sadaie *et al.* 1988). It has been proposed that Tat forms a metal-linked dimer with metal ions bridging cysteine-rich regions from each monomer (Frankel *et al.* 1988). However, the existence of Tat dimers *in vivo* and their potential role in Tat activity are yet to be established. In fact, Tat1-47 including the acidic region and the second domain was determined to be as active as native Tat in cell-free transcription system (Jeyapaul *et al.* 1991; Jeyapaul *et al.* 1990), suggesting these two domains are essential for the transactivating activity of Tat. However, a stretch of basic amino acids, from 49 to 57 containing two lysines and six arginines, constitutes the third domain and is required for nuclear localization (Mann *et al.* 1991; Hauber *et al.* 1989). Mutations within this region yield a cytoplasmic Tat protein which is nonfunctional. Moreover, peptide 49 to 57 alone does not have any transactivation function, suggesting

other regions of Tat are required. The second exon of Tat is of variable length encoding 14 to 32 amino acids. The biological function of the C-terminal region encoded by the second exon is still unclear.

Tat plays an extremely important roles in transcriptional and posttranscriptional regulation of HIV-1 gene expression. Tat dramatically induces the expression of viral protein directed by HIV-1 LTR and consequently for viral replication. The LTR (HIV-1 long terminal repeats) consists of a 453 bp U3 region, 98 bp R region, and an 83 bp U5 region. Within the U3 region and R regions are multiple cis-acting elements involved in HIV gene expression (fig. 3). It contains regulatory sequences recognized by various host transcriptional factors and viral regulatory proteins. The *cis*-acting TAR (tat responsive element) is localized to nucleotides +1 to +80 within the viral LTR (Rosen *et al.* 1985). Because of its location, TAR is present at 5' end of all HIV RNAs. The TAR RNA assumes a stable stem-loop structure *in vitro*, as determined by nuclease mapping (Muesing *et al.* 1987). Structural features of TAR that are important for Tat-mediated transactivation include the primary sequence in the loop (nucleotides 31 to 34), the 3-nucleotide bulge (nucleotides 23 to 25), and an intact stem (Roy *et al.* 1990a; Garcia *et al.* 1989a; Hauber *et al.* 1988a). Tat has been shown to interact with TAR RNA (Roy *et al.* 1990a; Dingwall *et al.* 1990a). Use of mutant TAR demonstrated that the bulge was important for both interaction of Tat with TAR as well as Tat-mediated transactivation. In contrast, mutations in the loop and the stem, which reduced Tat-mediated transactivation, had no effect on Tat binding (Roy *et al.* 1990a). Therefore, direct binding of Tat to TAR appears to be important but not sufficient for transactivation. Cellular factors have been

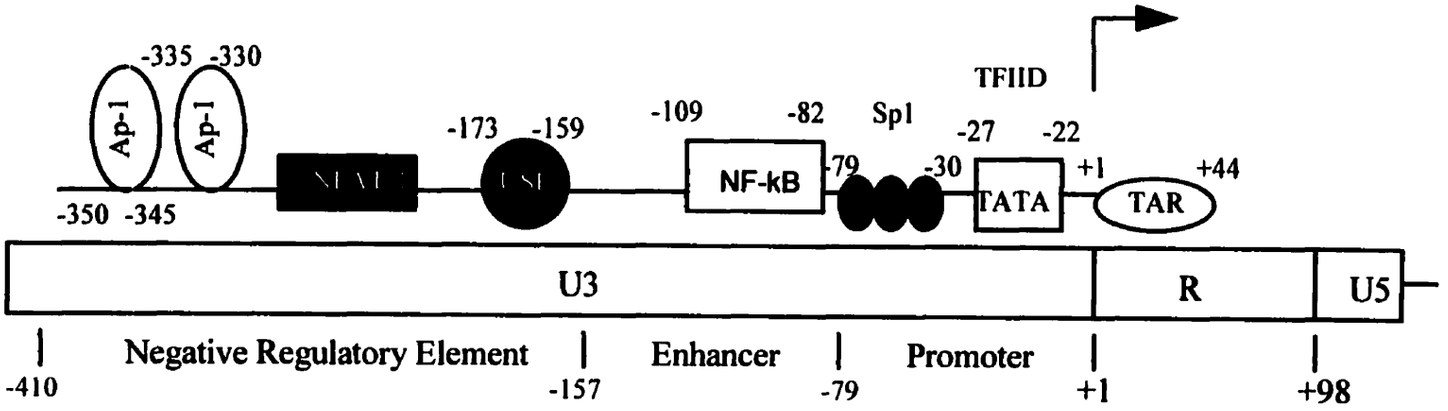


Figure 4 Organization of HIV-1 LTR. Binding sites of various factors are shown along with the coordinates of the recognition sequences.

postulated to play a role in Tat-mediated transactivation. It was reported that a number of human proteins are bound to TAR RNA (Gatignol *et al.* 1989; Gaynor *et al.* 1989). Recently, some of these factors have been characterized further, among them, eight proteins associated with either bulge, loop, or stem of TAR. TAR loop binding proteins include p68 that was identified by UV-cross-linking to TAR RNA (Marciniak *et al.* 1990), and TRPi/TRP185 (185 kDa) (Wu *et al.* 1991; Sheline *et al.* 1991). However, TRP2 (TAR RNA loop-binding protein, 70-110 kDa protein) and BBP (Bulge binding protein, 38 kDa) have been shown to bind to TAR-bulge (Baker *et al.* 1994; Sheline *et al.* 1991), which might inhibit Tat action. Proteins that complex with the double-stranded stem of TAR RNA consist of PKR (interferon-induced 68 kDa protein kinase) (Roy *et al.* 1991; McCormack and Samuel, 1995), SBP (140 kDa) (Rounseville *et al.* 1996) and TRBP (TAR RNA binding protein) (Gatignol *et al.* 1991). Similarly, many proteins that interact with Tat have been described to affect Tat transactivation. Besides binding to LTR, SP1 and TFIID, factors for eucaryotic polII transcriptional machinery, also bind to Tat. A direct interaction between Tat and SP1 has been well documented to play a critical role in Tat transactivation (Jeang *et al.* 1993; Berkhout and Jeang, 1992). In addition, there is evidence that Tat also contacts TFIID (Kashanchi *et al.* 1994). Recently, Tat has been demonstrated to induce NF-kB activation by either a pathway in the cytoplasm (Demarchi *et al.* 1996) or membrane binding (Conant *et al.* 1996). A large family of proteins related to the 26S protease are also reported to be Tat binding proteins. Among them are TBP1 (Tat binding protein 1), a 45 kDa protein (Ohana *et al.* 1993; Nelbock *et al.* 1990), TBP7 (Tat binding protein 7) (Chang and Sharp, 1990; Ohana *et al.* 1993),

SUG1 (suppressor of GAL4) (Swaffield *et al.* 1992), and MSS1 (mammalian suppressor of sgv1) (Shibuya *et al.* 1992). The cellular functions of these proteins are not clear. The 26S protease appears to modulate the stability of oncoproteins such as p53 (Dubiel *et al.* 1994; Dubiel *et al.* 1993). However, the potential role of most of the Tat- or TAR-binding proteins in Tat-mediated transactivation needs further investigation.

3. Replication cycle of HIV-1: A brief overview

A. Attachment of HIV-1 to host cells

The first step in the initiation of HIV-1 infection is the interaction between the virus particle and a specific receptor on the host cell. Like other envelope viruses, the interaction with the receptor is mediated by the envelope glycoprotein. The primary receptor first discovered for HIV-1 is the CD4 molecule, also known as T4 or Leu3, which is a 62 kDa glycoprotein that is predominantly expressed on T lymphocytes and monocytes, and that mediates efficient T lymphocyte-target cell interaction in the immune response. The major targets of HIV-1 are CD4+ T cells and monocytes that serve as both essential regulators and effectors of the normal immune response (Dalgleish *et al.* 1984; Sattentau and Weiss, 1988; Sattentau and Moore, 1993). The envelope glycoprotein, gp120 of HIV-1 binds to CD4 on the target cell with strikingly high affinity (Kd: 2-5 x 10^{-9} M) (McDougal *et al.* 1986; Dalgleish *et al.* 1984; Sattentau and Weiss, 1988; Deen *et al.* 1988). Further studies have indicated that the CDR2 domain in V1

region of the CD4 molecule interacts with a CD4-binding region in the fourth conserved portion (C2, C3, C4) near the C-terminal region of the HIV envelope gp120 (Lasky *et al.* 1987; Broliden *et al.* 1992; Broliden *et al.* 1990; Bowman *et al.* 1990). The initial attachment of HIV-1 to CD4 molecules most probably leads to exposure of epitopes in both gp120 (V3 loop) and perhaps CD4 (immunodominant epitope). Such a conformation would induce proteolytical cleavage of the third variable loop of gp120, the V3 loop. The V3 loop is susceptible to cleavage by several proteases, including the T-cell activation antigen CD26 (Callebaut-C 1993), thrombin, and trypsin (Kido *et al.* 1993). Subsequent displacement of gp120 or cleavage of the envelope protein by cellular enzymes such as CD26 (Oravecz *et al.* 1995) causes another change in the viral envelope, permitting viral entry (Sattentau and Moore, 1993; Sattentau and Moore, 1991). Other sites on CD4 could also be involved in gp120 binding and or fusion, such as the CDR3 domain of the V1 region (Corbeau *et al.* 1993; Autiero *et al.* 1991). Observations suggesting a role for this region include the blocking of CD4-gp120 interaction by CDR3 related peptides (Lifson *et al.* 1988) and decreased fusogenic activity of a virus with a mutation in this region (Camerini and Seed, 1990). However, further investigations are required to confirm these observations since a point mutation in this region did not affect gp120 binding (Ashkenazi *et al.* 1991) and virus fusion (Nussbaum *et al.* 1994; Broder *et al.* 1993; Broder and Berger, 1995).

A number of studies examining the infection of many types of non-CD4 cells with HIV-1 have indicated that receptors other than CD4 molecules on these cells are involved in gp120 binding. These cells include human skin fibroblasts (Tateno *et al.* 1989), human

trophoblast cells (Zack *et al.* 1990) follicular dendritic cells (Stahmer *et al.* 1991; Patterson and Knight, 1987; Patterson *et al.* 1995), human liver carcinoma cell lines (Cao *et al.* 1990) bowel epithelium (Nelson *et al.* 1988b; Ullrich *et al.* 1992b) and renal epithelium (Cohen *et al.* 1989; Detwiler *et al.* 1994). Many brain-derived cells such as astrocytes and oligodentrocytes which are non-CD4 are also reported to be infected with HIV-1. Interaction of those non-CD4 cells with HIV-1 is discussed later (II, 5B).

B. Internalization of the virus

Receptor-bound HIV-1 virions are brought inside the cell by either classic receptor-mediated endocytosis (Maddon *et al.* 1988) or virus-mediated membrane fusion (Stein and Engleman, 1991; Stein *et al.* 1987). Accumulated evidence supports membrane fusion as the dominant mechanism for virus entry. HIV-1-induced fusion, like that induced by most retroviruses, is pH-independent. The cascade of fusion events is initiated by the binding of gp120 to CD4 molecule and finally results in the insertion of the hydrophobic N-terminal region of gp41 into the plasma membrane of the target cell. As discussed above, in this event, the gp120 is displaced or cleaved after attachment to the CD4 molecule, leading to uncovering of the domains on the gp41 that are required for insertion (Bedinger *et al.* 1988, Sattentau and Moore, 199i; Veronese *et al.* 1985). previous results suggested that gp120 binds to putative second receptors in order to carry out the viral fusion (Qureshi *et al.* 1990; Henderson and Qureshi, 1993; Ebenbichler *et al.* 1993; Chen *et al.* 1992) including 44, 98, and 106 kDa proteins on cell surface.

The proteins involved in gp41 fusion are still unknown. However, they are believed to play an important role in viral internalization since murine cells which lack a 44 kDa surface protein can not be infected by HIV-1 after transfection of human CD4. Moreover, HIV-1 induced fusion requires calcium ions (Dedera *et al.* 1992). This may be due to calcium binding activity of gp41 which interacts with the gp41 putative receptors in a calcium-dependent manner (Stoiber *et al.* 1995). Thus, as noted above, HIV-1 enters cells by fusion with the cell membrane. This fusion is most likely mediated by gp41 binding to its receptor on the cell membrane following a conformational change in CD4 as well as dissociation of gp120 or exposure of its V3 loop to cleavage.

Recently, chemokine receptors have been shown to be coreceptors for HIV-1. gp120 interacts with these coreceptors to facilitate HIV-1 entry (Alkhatib *et al.* 1996, Feng *et al.* 1996). The family of chemokine receptors are variably expressed on lymphocytes and macrophages and in part select for viral strains specific for each of these cell types (Feng *et al.* 1996, Cocchi *et al.* 1996). Macrophages express CC-CKR5, while lymphocytes express CXCR4, also called fusin or lester. Additionally, CKR3 and CKR2b have also been shown to be fusion coreceptors for HIV entry in monocytes (Doranz *et al.* 1996). Interaction of HIV gp120 with those coreceptors has been further mapped to the V3 loop of the gp120 molecule. These chemokine receptors are seven-transmembrane domain proteins, which are coupled with G-protein (Horuk 1994). Bound with high affinity by related chemokines, the chemokine receptors play a major role in the mobilization and activation of the cells in the immune system. Out of over 20 different members of the growing chemokine family, RANTES (regulated on activation normal T-

cell expressed and secreted), MCP-1 (monocyte chemotactic protein -1) and MIP-1α and -1β (macrophage inflammatory protein-1α and -1β) have been shown to inhibit HIV entry by blocking interaction of V3 loop of HIV gp120 with the chemokine receptors (Cocchi *et al.* 1996, Dragic *et al.* 1996, Doranz *et al.* 1996, Oravecz *et al.* 1996). However, it remains to be explored how the coreceptors function on the biochemical level to allow HIV-1 to enter cells. Expression of chemokine receptors on cells within the nervous system has yet to be determined.

C. Reverse Transcription, Integration

After internalization of viral core into the cytoplasm of a susceptible cell, viral RNA is transcribed into double-stranded proviral DNA by the RNA/DNA-dependent DNA polymerase and ribonuclease H activities of RT (reverse transcriptase). The process of synthesis of viral DNA is complex. *In vitro* studies have shown that synthesis of the negative-strand DNA initiates by making a short DNA attached to 5' end of viral genome. In this process, tRNA primer at the 5' end of genome is crucial. The newly made DNA as a primer consists of R and U5 sequence. It must be transferred to the 3' end of the genome to synthesize a full negative-strand DNA. Once synthesis of the negative-strand DNA is finished, the RNase H activity of RT removes the viral RNA from the RNA:DNA hybrid, leaving the DNA free to make positive-strand DNA by RT.

Once formed, the double-strand proviral DNA, presumably in the form of a nucleoprotein complex, migrates to the nucleus where it is integrated into the host cellular DNA by the endonuclease activities of the viral integrase (Coffin, 1990). This

integration of the proviral DNA appears to be random and is essential for the cell to produce progeny viruses.

D. Assembly and release of mature Virus

HIV-1 assembly is an unique process in which the products of *gag* and *pol* are incorporated into virions in the form of their polyprotein processors during assembly and are proteolytically cleaved during or after budding. As noted above, the Gag and Pol proteins are synthesized in the form of their respective processors $Pr55^{gag}$, NH2-p17-p24-p9-p7-COOH, and $Pr160^{gag\text{-}pol}$, NH2-p17-p24-p9-p7-p10-p66-p32-COOH. The p17, p24, and p9 Gag domains as well as p10 protease domain of the Gag and Gag-Pol processors play important roles in the assembly process. The interactions between Gag proteins, plasma membrane, and viral RNA control the process. Under control of $p17^{gag}$, which is posttranalationally modified by the addition of myritic acids (Veronese *et al.* 1985; Bathurst *et al.* 1989), the $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ are first aggregated inside the plasma membrane. The viral genomic RNA is then packaged into viral particles by the p9 domain of Gag or Gag-Pol precursor. A signal of viral genomic RNA is required for the packaging. Studies have shown that the major packaging signal resides in the leader region between U5 and the *gag* gene initiation codon (Bender *et al.* 1987; Adam and Miller, 1988). The interaction of p24 domains of the Gag or Gag-Pol precursors is required for the formation of a protein shell surrounding the nucleocapsid (Peng *et al.* 1991). However, the function of p7, a proline-rich carboxy-terminal product of Gag precursor, is unclear.

During the viral budding, the Gag and Gag-Pol precursors are cleaved by the viral protease p10 to produce individual proteins as noted above (Kohl *et al.* 1988; Peng *et al.* 1991). This process is crucial for maturation of the virus. With mutations in the p10 gene, the assembly and budding steps of virus were not affected, but virions produced resembled immature core particles. Moreover, the mutant particles were not infectious.

The envelope glycoproteins are synthesized initially as a precursor gp160 and then incorporated in virions by a different pathway. The gp160 is cleaved intracellularly into gp120 and gp41, which are inserted into the plasma membrane. The incorporation of the envelope glycoproteins into virus is probably mediated by the interaction between the p17 domain of Gag precursor and Env protein complex.

4. HIV-1-induced immune deficiency

HIV-1 has been clearly identified as the primary cause of the acquired immunodeficiency syndrome (AIDS) (Barre Sinoussi *et al.* 1983d) and is spread by sexual contact, exposure to infected blood or blood products, and prenatal transmission from mother to child (Curran *et al.* 1988). The mechanism by which HIV causes immunodeficiency is one of the major mysteries of AIDS. Many studies suggest that immune abnormalities can be observed in T cells, B cells, and macrophages early in infection even before the loss of CD4 cells begins (Shearer and Clerici, 1992; Shearer and Clerici, 1993; Miedema *et al.* 1988; Clerici *et al.* 1992). The potential mechanisms of HIV-1-induced immune deficiency are discussed below.

A. Direct cytopathic effects on CD4 cells

In patients with HIV-1 infection, the first immunosuppresion recognized was loss of CD4 cells. *In vitro* syncytia formation occurs through direct HIV-mediated cytopathic effects (Lifson *et al.* 1986b; Garry, 1989b). The formation of syncytia involves fusion of cell membrane of infected cells with cell membranes of uninfected CD4 cells, which results in giant multinucleated cells. However, syncytia have been rarely been seen *in vivo*. Several observations have associated CD4 cell death with direct toxicity from virus or viral proteins. The use of interviral recombinants has shown that cytopathogenesis, including cell fusion, may be linked to a region of gp120 (Cheng Mayer *et al.* 1990). Moreover, the cell fusion that often leads to cell death has been associated with gp120 and Tat (Lifson *et al.* 1988; Lifson *et al.* 1986; Cheng Mayer *et al.* 1991). Further, addition of gp120 to PBMC caused cell killing in a dose-dependent manner. The gp41 can also be toxic to cells (Miller *et al.* 1991); a change in the viral gp41 has modified or produced cytopathic variants (Kowalski *et al.* 1991). The mechanism for this induction of cell death by the viral proteins are not clear. Disturbances in membrane permeability could be involved, as reflected by the balloon degeneration in cells observed *in vitro*. The death of CD4 cells by apoptosis has been suggested as cause for CD4 cell loss in HIV infection (Laurent Crawford *et al.* 1991; Laurent Crawford *et al.* 1995; Groux *et al.* 1992). Whether apoptosis results from direct effects of HIV-1 itself or its viral proteins remains to be determined. Recent results suggested that gp120, or Tat can elicit apoptosis of CD4 cells (Laurent Crawford *et al.* 1993; Li *et al.* 1995; Terai *et al.* 1991). Additionally, the virus infection could suppress the production of the early processors of

CD4 cells (stem cells) and reduce the quantities of the fresh lymphocytes added regularly from bone marrow to the peripheral blood (Folks *et al.* 1989).

B. Effect on signal transduction of CD4 cells

A protein kinase cascade and protein phosphorylation occur when natural ligands bind to the CD4 antigen or interact with other membrane surface proteins to bring about T-cell activation and effective immune response. The gp120 has been found to form an intracellular complex with CD4 and $p56^{lck}$ in the endoplasmic reticulum (Crise and Rose, 1992). The retention of this tyrosine kinase in the cytoplasm could be toxic to the cell or affect its function.

C. Cytotoxic CD8 cell

CD8+ cells are also believed to play a critical role in controlling HIV-1 infection, particularly during the phase of clinical latency and long-term nonprogressors (Paul, 1995). To do so, CD8+ cells have been shown to secrete some HIV-SF (HIV-suppression factors) that have recently been identified as chemokines RANTES, MIP-1α and MIP-1β (Nelbock *et al.* 1990; Mackewicz *et al.* 1995; Cocchi *et al.* 1995). Studies have also indicated that cytotoxic CD8+ cells might kill normal CD4 cells as well as those infected with HIV (Zarling *et al.* 1990; Pantaleo and Fauci, 1995). Two separate mechanisms are likely involved in the killing: direct lysis by releasing lytic granules or inducing apoptosis.

D. Autoimmune mechanism

In some of the early studies of HIV infection, antibodies to CD4 cells were detected (Dorsett *et al.* 1990; Ardman *et al.* 1990). Moreover, autoantibodies to the CD4 molecule itself have also been detected in HIV-infected individuals (Favre *et al.* 1986; Chams *et al.* 1988). Those autoantibodies might be responsible for CD4 cell death. The reason for this origin is not known.

E. Effects of cytokines

Several cytokines are produced by a variety of immune cells after the activation by infection and inflammation. For example, IL-1 (interleukin-1) and TNF-α are induced by macrophages infected by HIV or exposed to the viral gp120 or Tat, where IL-1 normally stimulates CD4 cell maturation and TNF-α can be toxic to CD4 cells and can induce apoptosis (Merrill *et al.* 1989). Viral gp120 was reported to potentiate TNF-α-induced NF-kB activation by stimulating a signal pathway that involves p^{56lck} and increased formation of reactive oxygen intermediates such as H_2O_2 in Jurkat cells (Shatrov *et al.* 1996). Moreover, induction by HIV of IL-6 production by human B lymphocytes has also been described (Boue *et al.* 1992), which might cause the polyclonal activation of B cells. IL-2, as a T cell growth factor, is also disregulated by HIV-1 Tat. However, the relative extent of cytokine expression during HIV infection is not clear, and whether these cellular products act as cofactors to influence the CD4 cells destruction or compromise their function needs further evaluation.

5. HIV-1-induced encephalopathy

A. Neuropathological findings in AIDS

It has been estimated that ~17 million people worldwide are infected with HIV-1 at the end of 1996 and one-third of these individuals will develop a dementing illness. HIV-1 infection is now the leading cause of dementia in the people less than 60 years of age (McArthur *et al.* 1993; Janssen *et al.* 1992). The clinical manifestations encompass impairment of motor control, cognition and behavior (Price *et al.* 1988) so called AIDS dementia complex. The gross pathology of the brain in AIDS dementia complex is variable. As expected from the nature of clinical findings in AIDS dementia complex, subcortical lesions are most prominent, particularly in the lobar white matter and deep gray nuclei (e.g., the thalamus). Subcortical atrophy ranges from slight to marked. In the latter event, there is usually significant enlargement of lateral ventricles (Dal Canto, 1989). Microscopic examination of the brain with AIDS dementia complex may show different degrees of involvement in different structures. Accumulation of mononuclear cells accompanied by multinucleated giant cells is evident. The giant cells are monocytic. HIV has been demonstrated in these cells in white matter infiltrates (Dal Canto, 1989). Abnormalities of cerebral myelin are also seen in association with AIDS dementia complex, most prominently progressive diffuse leukoencephalopathy, also termed defuse myelin pallor (Glass *et al.* 1993). Another neuropathological finding is astrocytosis that is defined by increases in density (hyperplasia) and size (hypertrophy) of astrocytes (Vitkovic and da Cunha, 1995). Astrocytosis is the most frequent pathological change occurring in adult and developing brains of HIV-1-infected individuals during the early

stage of infection (Everall *et al.* 1993). Furthermore, astrocytes can be productively infected with HIV-1 and produce all of the structural proteins and regulatory proteins of the virus (Tornatore *et al.* 1994, Saito *et al.* 1994, Ranki *et al.* 1995, Nath *et al.* 1996b). The infected astrocytes then can release infectious virus. However, astrocytes can establish a latent infection. HIV may alter the function of astrocytes when it integrates at a site within the chromosomal DNA. More importantly, neuronal loss occurs in cortexes of brains in the patients with AIDS dementia. Pathology in HIV-1 associated motor cognitive complex is characterized by a loss of large neurons in the orbitofrontal region of the cortex (Gray *et al.* 1991; Ketzler *et al.* 1990). Neuronal losses of 50% to 90% were observed in the interneurons of the hippocampus (Masliah *et al.* 1992a). Golgi analysis of the frontal region showed a 40% loss of dendrites and 40% to 60% loss of spine density along apical dendrites of the large pyramidal neurons; the affected dendrites are dilated, tortuous, and vacuolated with decreased lengths and branching points (Masliah *et al.* 1992c). In addition to neuronal loss, more sensitive techniques have shown that a loss of synaptic contacts and vacuolar changes in the neurons in both the deep gray matter and cortex in patients dying with AIDS (Masliah *et al.* 1992b; Wiley *et al.* 1991b). Recently, using in situ technique, apoptosis of neurons has been demonstrated in cerebral and basal ganglia of brains that had HIV-1 encephalitis with progressive encephalopathy (Gelbard *et al.* 1995). However, the mechanisms underlying these pathological changes and relationships between these pathological changes and AIDS dementia complex are not fully understood. For example, neuronal loss is most unlikely due to direct infection since neurons are rarely infected with HIV-1. Moreover, multinucleated giant cells and diffuse

myelin pallor were found in 50% of specimens from patients with dementia while half of the tissue samples from the patients with dementia exhibited no such abnormalities. Thus, multiple factors are likely involved in reducing the complex of pathological changes. These factors may include: (i) infection of brain cells by HIV-1, (ii) the toxic effects of viral proteins (gp120, gp41, Tat, and Nef), (iii) autoimmune and other immune mediators, (iv) toxic effects of cytokines, (vi) viral cofactors.

B. HIV-1 infection of brain cells

Many studies have shown that the central nervous system is exposed to HIV early in the course of systemic infection. The exposure may continue throughout the course of infection. Although there is evidence of viral infection and of an immunologic reaction, it is not necessarily accompanied by clinical injury to the host. Therefore, some questions remain to be answered: how HIV-1 enters the CNS; how HIV infection of the brain relates to AIDS dementia complex and what types of cells are infected by HIV-1.

HIV-1 invades the CNS via means that are still not fully understood. On the basis of many studies, the monocyte or macrophage is believed to be the principal vector that carries HIV-1 across the blood-brain barrier into the brain (Koenig *et al.* 1986; Price *et al.* 1988). They may enter via endothelial cell spaces followed by infection of susceptible cells in the brain such as microglia and astrocytes (the Trojan horse hypothesis) (Power and Johnson, 1995). HIV entry by direct infection of capillary endothelial cells should also be considered (Harouse *et al.* 1989, Falangola *et al.* 1995). Virus also may enter the

brain via a disrupted blood-brain barrier since diffuse breakdown of the blood-brain barrier is an accompaniment of early infection by HIV-1 (Brightman *et al.* 1995).

In the brain, there are many types of cells in the brain susceptible to HIV-1 infection. The resident macrophages (CD4+) and microglia (CD4+) are the most frequently and productively infected with HIV-1 (Koenig et al 1985, (Wiley *et al.* 1986), Pumarola-Sune 1987, Budka et al 1987, Meyenhofer *et al.* 1987). HIV-1 has also been shown to infect primary human astrocytes (Wiley *et al.* 1986; Tornatore *et al.* 1994; Tornatore *et al.* 1991; Nath *et al.* 1995), brain-derived glia cells (Harouse *et al.* 1989; Clapham *et al.* 1989; Cheng Mayer *et al.* 1989) and brain capillary endothelial cells (Harouse *et al.* 1989). The extent of virus replication is generally low in those CD4- cells. For example, low copy numbers of the genomes are expressed in astrocytes after infection with HIV-1 (Tornatore *et al.* 1994b; Nath *et al.* 1995b). The limited amount of virus production may be due to inefficient viral entry because usually less than 1% of cells become infected (Kleinschmidt *et al.* 1994; Brack Werner *et al.* 1992) and once infection is established, subsequent virus replication can occur. This is supported by *in vitro* experiments in which HIV-1 expression can be induced by cytokines although HIV-1 infection of astrocytes is persistent or latent (Tornatore *et al.* 1991). The nature of the cell surface molecule(s) responsible for viral entry into non-CD4 cells such as primary human astrocyte is not known. But viral entry conceivably could involve fusion receptors. On brain-derived cells, a potential fusion receptor has been identified by using rabbit polyclonal antibodies to GalC (galactosyl ceramide) (Harouse *et al.* 1991). This

cell surface product appears to be the receptor for virus entry (Bhat *et al.* 1991). It binds to a portion of V3 loop on gp120 rather than the region for CD4 (Bhat *et al.* 1991). The same receptor has also been linked to infection of bowel epithelial cells (Yahi *et al.* 1995; Yahi *et al.* 1992). It is important to further confirm that the GalC acts as the HIV-1 receptor for non-CD4 cells since infection of GalC-rich neurons and oligodendricytes is still controversial. Furthermore, not all brain cells express this glycolipid. Human fetal astrocytes, for example, have been shown to be susceptible to HIV-1 infection without GalC on the cell surface (Nath *et al.* 1995; Ma *et al.* 1994), suggesting that other receptor(s) may be involved. A novel protein receptor has been demonstrated on non-CD4 glioma cells. This protein is approximately 180 kDa, distinct from CD4 and appears to induce tyrosine-specific protein kinase activity in the cells by binding to gp120 (Schneider Schaulies *et al.* 1992). Many other putative binding sites for virus entry have also been reported, such as the gp41 binding sites (Wild *et al.* 1992; Qureshi *et al.* 1990), mannose-binding lectin on the cell membrane for gp120 (Curtis *et al.* 1992) and lymphocyte-function associated antigen adhesion molecule for HIV-1 infection (Hansen *et al.* 1991). However, their role in virus entry and infection needs further evaluation.

Infection of microglia occurs via a CD4 mediated mechanism and leads to cytopathic effects and multinucleated giant cell formation (Watkins 1990, Jordan 1991). As described above, astrocytic infection does also occur, particularly in children (Blumberg *et al.* 1994; Blumberg *et al.* 1992; Nath *et al.* 1995; Tornatore *et al.* 1994, Takahashi *et al.* 1996). However, the fact of non-cytopathy of these infected cells raises the question of biological relevance. Recently, some *in vivo* studies have shown the links

between astrocyte infection and AIDS dementia complex. By examining the samples of adult human brain infected with HIV-1, Ranki et al. found that HIV Nef and Rev were abundantly expressed in astrocytes from six out of seven patients suffering from moderate to severe dementia (Ranki *et al.* 1995), suggesting that astrocytes are infected with HIV-1 and their infection is associated with AIDS dementia complex. Astrocytes are essential to neurons. Astrocyte dysfunction caused by either HIV-1 infection or viral proteins may be associated with AIDS dementia complex (see later section). Recently, *in vitro* experiments have shown that the endothelial cells of the central microvasculature and not the macrovasculature could be infected with HIV-1. The infection of these cells is independent of CD4 and cytopathic effects have not been reported (Howard and Griffith, 1993, Poland *et al.* 1995). The endothelial cells from the choroid plexus have also been infected with HIV-1 *in vitro* (Moses and Nelson, 1994; Harouse *et al.* 1989, Falangola *et al.* 1995). However, the relationship between AIDS dementia complex and infection of endothelial cells remains to be evaluated. Although some studies have shown that oligodendrocytes and neurons could be infected with HIV-1 *in vitro* (Albright *et al.* 1996), convincing evidence of infection of these cells *in vivo* is still lacking.

C. Toxic effects of viral proteins

Neurotoxic effects of viral proteins have been demonstrated in association with HIV-1 infection (Lipton, 1991). The viral proteins in the brain originate from microglia or macrophages infected with HIV-1. They may also enter the brain via disrupted blood brain barrier. Moreover, infection of astrocytes and endothelial cells may contribute some

viral proteins in the brain. Among the viral proteins are gp120, gp41, Tat, and Nef. The envelope protein gp120 is shed from the virus and has been shown to the neurotoxic *in vitro* and *in vivo* (Hill *et al.* 1993; Dreyer *et al.* 1990). *In vitro* experiments show that gp120 kills cultured rodent neuronal cells (Brenneman *et al.* 1994). Further studies have shown that the gp120 induces an early rise in intracellular Ca^{2+} concentration in mixed rodent neuronal cultures (Dreyer *et al.* 1990). Such increases in intracellular Ca^{2+} represent a common mechanism for neuronal death induced by a diverse group of acute and chronic neurological diseases (Lipton, 1991). The gp120-induced neurotoxicity could be prevented by either antagonists of the L-type voltage-dependent Ca^{2+} channels, such as nimodipine or antagonists of N-methyl-D-aspartate (NMDA) receptor-gated channel, such as MK-801, suggesting an invoivement of voltage-gated Ca^{2+} channels and NMDA receptor-gated channels (Dreyer *et al.* 1990; Lipton *et al.* 1991). In addition, certain regions of the gp120 (particularly a threonine-rich region called peptide T) appear to compete with neurotrophic factors, such as neuroleukin and vasoactive intestinal polypeptide (VIP) (Pert *et al.* 1988b; Pert *et al.* 1988b; Lee *et al.* 1987b). In this case, VIP can prevent the neuronal cell killing induced *in vitro* by gp120 (Dreyer *et al.* 1990). Thus gp120 might cause neurologic disorder by blocking those factors needed for the growth, communication, and maintenance of nerve cells. *In vivo* studies have also supported gp120 neurotoxicity. Brenneman et al. have found that intraventricular injections of gp120 into rat brain result in dystrophic neurites in hippocampal pyramidal cells as well as behavioral deficits (Brenneman *et al.* 1994). Based on the *in vivo* experiments, interaction between gp120 and NMDA receptor was suggested due to the

similarity of neuronal injury in both the brains of AIDS patients (Achim *et al.* 1991) and the brains of rats injected with gp120. However, it is as yet unknown if the effects of gp120 on the NMDA receptors act directly on neurons, or act via glia cells such as microglia and astrocytes, or by a combination of mechanisms (Lipton, 1991) since these experiments have been done in either the brain or mixed cultures. In fact, there are growing evidence to suggest that the relationship between gp120 and NMDA receptor is indirect. For example, in patch-clamp recordings, gp120 does not have glutamate-like effect on NMDA receptor, and degradation of the endogenous EAA (excitatory amino acid) glutamate protects the neurons from gp120-induced injury (Lipton *et al.* 1991). One possibility is that gp120 sensitizes neurons to the lethal effects of EAA on NMDA receptor. Interestingly, when L-leucine methyl ester was used to deplete mononuclear phagocytes from neuronal culture, gp120-mediated neurotoxicity was eliminated (Lipton, 1993). It is also believed that HIV-1 infection or gp120 stimulate microglia or astrocytes to release some toxins including arachidonic acid metabolites, TNF-α, IL-1β, quinolinic acid and etc. (Lipton and Gendelman, 1995; Gendelman *et al.* 1994; Pulliam *et al.* 1991; Giulian *et al.* 1990). Some of the toxins act directly or indirectly on NMDA receptors.

Tat has also be shown to be neurotoxic to neuroblastoma cell lines (Sabatier *et al.* 1991) and human fetal neurons (Magnuson *et al.* 1995, Nath, *et al.* 1996) *in vitro*. The basic region of Tat is important for its toxicity. However, 15 mer peptides containing the basic region of Tat could not induce neuronal death while Tat1-72 and Tat31-61 were neurotoxic, suggesting that the neurotoxic domain is confirmation dependent. Further studies determined that Tat can produce neuronal excitation and elevation of intracellular

Ca^{2+} concentration (Magnuson *et al.* 1995, Nath, *et al.* 1996). The Tat-induced effects can be prevented by antagonists of the non-NMDA receptor, such as CNQX (6-Cyano-7-nitroquinoxaline-2,3-dine), suggesting that Tat action is likely mediated, at least in part, by non-NMDA receptors. Tat also causes aggregation and adhesion of cerebellar neurons (Orsini *et al.* 1996). Tat is neurotoxic when inoculated intracerebrally into mice (Sabatier *et al.* 1991) and causes gliosis, macrophage infiltration, and apoptosis of neuronal cells (Jones *et al.* 1996). Tat has also numerous effects on cytokine production which are discussed in a later section (II, 6D).

By sequence analysis, Nef , and perhaps part of gp41, has been shown to be related to scorpion toxins (Werner *et al.* 1991; Garry and Koch, 1992). Nef can affect normal cellular transmembrane conduction. However, more evidence is needed for Nef neurotoxicity. Recently, one *in vivo* study has shown abundant expression of HIV Nef occurred in brain astrocytes and is associated with AIDS dementia complex (Ranki *et al.* 1995).

D. Neurotoxic effects of cellular factors

Several cytokines including TNF-α, IL-4, TGF-β and IL-1β are produced in the CNS after HIV infection or exposure to viral proteins. TNF-α in large quantities has been shown to damage neurons from rodent species, to be toxic for myelin and human glioma cell lines, and alter the permeability of the blood brain barrier (Power and Johnson, 1995; Selmaj *et al.* 1990; Rutka *et al.* 1988). In some studies, the most notable change associated with HIV-1 dementia was elevated mRNA expression for TNF-α with

decreased levels of IL-4 mRNA in the brain parenchyma (Wesselingh *et al.* 1993; Glass *et al.* 1993). Further studies have shown that TNF-α is likely produced by microglia in response to either HIV infection or viral proteins including gp120 and Tat (Merrill, 1992; Merrill *et al.* 1989; Matsuyama *et al.* 1991; Gallo *et al.* 1989). Induction of TGF-β (transforming growth factor beta) production by microglia and astrocytes has also been linked to CNS disorders (Wahl *et al.* 1991). TGF-β is a very potent chemotactic factor and can increase the production of other cytokines including TNF-α. Although TGF-β itself has not been shown to be directly neurotoxic, its expression was found in the brain. Furthermore, IFN-γ (interferon-γ) and IL-1β from microglia or astrocytes can also induce the production of arachidonic acid metabolites (Gendelman *et al.* 1994).

It has been postulated that arachidonic acid metabolites such as LTB4 (Leukotriene B4), LTD4 (Leukotriene D4) and PAF (platelet activating factor) may be involved in HIV-induced CNS disorders based on *in vitro* and *in vivo* studies. Arachidonic acid can inhibit high affinity uptake of glutamate into synaptosomes and astrocytes, and potentiates NMDA receptor-activated current by increasing open channel probability (Miller *et al.* 1992). Cultivation of HIV-1 infected monocytes with glia cells stimulated synthesis of LTB4, LTD4, and PAF, and led to injury of neurons present in the culture (Gelbard *et al.* 1994; Epstein and Gendelman, 1993; Genis *et al.* 1992). This process may be due to these arachidonic acid metabolites which may contribute to excessive NMDA receptor stimulation by increasing the release of glutamate, inhibiting its uptake, and ultimately enhancing its action at the NMDA receptor (Bito *et al.* 1992). Griffin et al. have shown that prostaglandin E2 levels in CSF (cerebrospinal fluid) of

HIV-infected individuals with dementia were increased. This increase was associated with severity of dementia.

Additional neurotoxic factors have been implicated in the pathogenesis of AIDS dementia complex including quinolinic acid and nitric oxide. Quinolinic acid is a tryptophan-derived NMDA receptor agonist which is toxic to neurons. High levels of quinolinic acid have been detected in the CSF of HIV-1 infected patients (Heyes *et al.* 1991), suggesting it may play a role in AIDS dementia complex. Nitric oxide has also been implicated in HIV-induced neurological diseases. Nitric oxide is a powerful endogenous mediator for numerous physiological responses including antimicrobial and antivrial activity, but may mediate brain injury as well (Dawson *et al.* 1993). NMDA receptor-mediated elevation of Ca^{2+} in neurons indirectly caused by HIV-1 infection or gp120 can stimulate the formation of nitric oxide, which contributes to the cascade of neurotoxic events (Dawson *et al.* 1991). Nitric oxide itself may not be directly neurotoxic. However, other free radicals, including superoxide anion, appear in response to the over stimulation of NMDA receptors (Lipton *et al.* 1993). Superoxide anion reacts with nitric oxide to yield a neurotoxic substance probably peroxynitrite or one of its breakdown products (Lipton *et al.* 1993). The relevance of nitric oxide to HIV-induced dementia has been supported by the following evidence. First, gp120 neurotoxicity in primary neuronal culture may be mediated in part by nitric oxide (Lipton *et al.* 1993; Dawson *et al.* 1993). Second, in one *in vivo* study, nitric oxide synthase has been reported high in brain tissue with AIDS dementia (Bukrinsky *et al.* 1995) although the links between AIDS dementia complex and nitric oxide needs further studies. Third, gp120 can

stimulate an inducible form of nitric oxide synthase activity in astrocytes which can be upregulated by IL-1β and IFN-γ (Mollace *et al.* 1993b). Fourth, the expression of inducible nitric oxide synthase in HIV-1-infected microglia correlates with their activation by endotoxin or cytokine (Bukrinsky *et al.* 1995). The low level of nitric oxide in HIV-1-infected or gp120 stimulated microglia or astrocytes suggest that this free radical is not the primary or sole factor in neuronal injury (Pietraforte *et al.* 1994; Bukrinsky *et al.* 1995; Lipton, 1994).

Additionally, Guilian et al. have identified a small molecule which has been shown to damage neurons *in vivo* and is secreted by HIV-infected macrophages and/or macrophages treated with gp120 (Guilian *et al.* 1990). The same group has also found that a neurotoxic amine, NTox, was produced by blood monocytes and by brain mononuclear phagocytes infected with HIV-1 (Giulian *et al.* 1996). The NTox was demonstrated to be a NMDA receptor-directed toxin associated with neuronal damage (Giulian *et al.* 1996).

E. Auto-immune mechanisms and coinfection with other viruses

Immunologic disorders and viral coinfection may be the two other mechanisms for HIV-1 pathogenesis in the brain. For example, antibodies to myelin basic protein have been detected in the CSF of patients with AIDS dementia complex (Liuzzi *et al.* 1992). Anti-gp41 antibodies cross-react with some proteins in astrocytes (Yamada *et al.* 1991). This cross-reaction is thought to compromise astrocyte functions. Moreover, antibodies to brain were also detected in the sera of infected patients with neurologic disease (Kumar *et*

al. 1990). In addition, antiviral or anticellular cytotoxic T lymphocytes could be generated to harm brain cells (Jassoy *et al.* 1992). Finally, coinfection with other infectious agents such as cytomegalovirus, herpes virus, and JC virus could worsen the neuropathologic changes caused by HIV-1 infection (Nelson *et al.* 1988a; Nelson *et al.* 1990a; Ho *et al.* 1991a). It is noteworthy that HIV Tat protein can enhance human cytomegalovirus gene expression and JC virus T-antigen expression (Chowdhury *et al.* 1990; Ho *et al.* 1991) and conversely, cytomegalovirus and JC virus have been shown to activate the HIV LTR-directed gene expression (Gendelman *et al.* 1986; Davis *et al.* 1987).

6. HIV-1-induced astrocyte alteration

A. Functions of astrocytes

The astrocyte is the most abundant brain cell, outnumbering neurons by about 10:1. Derived from the neuroectoderm of the neural tube, these cells are, in reality, a lineage representing a large family of cells that share certain biochemical and morphological specialization, while diverging in certain functional capabilities. Classically, there are two principal types of astrocytes which are classified on morphological basis. The two types are known as protoplasmic and fibrous astrocytes. Protoplasmic astrocytes are characterized by thick, branched processes with spiny projections and are localized primarily with the gray matter. The fibrous astrocytes, in contrast, consist of relatively long, thin processes with few branches and are the predominant type in the white matter. They play very important functions in maintaining

normal brain functions. For example, they support neuronal survival and activity by regulating the extracellular environment and the release of neuroactive compounds. The best understood function in adult brain is the metabolism of glutamate and the major inhibitory neurotransmitter, GABA (γ-amino butyric acid). The glutamate-GABA cycle is catalyzed by several enzymes compartmentalized between astrocytes and neurons. One of these enzymes is glutamate synthetase that is almost exclusively made in astrocytes. By consuming ammonia in the brain, it catabolizes glutamate to glutamine. This reaction detoxifies ammonia from the brain and removes excess glutamate which is excitoxic at high concentration in the brain. In addition, astrocytes take up synaptically released glutamate (Mennerick and Zorumski, 1994). Thus, astrocytes do not only protect neurons from excitoxicity but probably control synaptic currents. Astrocytes also have many other functions including scar formation after brain injury, influence of myelin turnover via gap junction and induction of the blood-brain barrier. Moreover, astrocytes participate in immune reaction by acting as an antigen-presenting cell and by releasing cytokines. Hence, alteration of astrocyte function could have disastrous consequences for normal brain function.

B. Astrocytosis and HIV dementia

Astrocytosis is consistently reported as the earliest neuropathological change in brains of HIV-1 infected individuals. It was detected as early as 15 days following infection with HIV-1 (Davis *et al.* 1992). A relationship between astrocytosis and the infection of the brain parenchyma with HIV-1 is unknown. HIV-1 antigens were not be

detected by immunocytochemistry in any of the 11 brains from HIV-1 seropositive asymptomatic individuals displaying astrocytosis (Gray *et al.* 1992). Furthermore, astrocytosis was present in all tissues from seropositive individuals whereas p24 antigen was only detected in some of the samples. These observations suggest that astrocytosis may occur independently of HIV-1 expression in the brain. Thus, astrocytosis may be indirectly associated with dementia.

Astrocytosis can be triggered by circulating cytokines before the entry of HIV-1 into the brain. Interleukin-1 (IL-1) and TNF-α concentrations in circulation increase upon systemic infection with HIV-1, secreted by HIV-1-infected monocytes (Merrill *et al.* 1989; Locksley *et al.* 1988). Circulating IL-1 stimulates directly and/or indirectly its own expression in the brain where IL-1 induces TGF-β1 (transforming growth factor-β1) in astrocytes and other glia cells of the frontal cortex (da Cunha and Vitkovic, 1992; da Cunha *et al.* 1993). These cytokines and probably others initiate and control astrocytosis. Astrocytosis may cause neuronal dysfunction underlying AIDS dementia complex. The functions of astrocytes could be diminished during astrocytosis by TGF-β1, which down regulates glutamine synthetase in cultured astrocytes (Toru Delbauffe *et al.* 1990). Thus, dysregulated astrocytosis may by itself disturb catabolization of glutamate and ultimately cause excitoxicity, one of the possible mechanisms for neuronal dysfunction in AIDS dementia complex (Lipton *et al.* 1995). Glutamate induces prompt and oscillatory elevation of cytoplasmic free Ca^{2+} in astrocytes (Jensen and Chiu, 1991; Cornell Bell *et al.* 1990). The Ca^{2+} signals can propagate from astrocytes to neurons (Nedergaard, 1994) suggesting that astrocytes can directly modulate neuronal intracellular Ca2+

concentration. The observation from Nedergaad et al. has also supported the potential relationship between astrocytosis and AIDS dementia complex. They have determined that astrocytosis in brains with AIDS dementia complex was at least two standard deviations of the mean higher than brains without AIDS dementia complex (Vitkovic and da Cunha, 1995). Together these findings strongly suggest that dysregulated astrocytosis can cause neuronal injury of AIDS dementia complex.

C. Effects of gp120 on astrocytes

As noted above, increasing evidence have suggested that astrocytes could be infected with HIV-1 and the infection is not mediated by CD4 molecules (Tornatore *et al.* 1994a; Blumberg *et al.* 1992a; Nath *et al.* 1995a). Indeed upon infection with HIV-1 or following exposure of glia cells to gp120, astrocytes have been found to undergo physiological, biochemical and morphological changes (Bubien *et al.* 1995; Benos *et al.* 1994; Ciardo and Meldolesi, 1993; Codazzi *et al.* 1995; Pulliam *et al.* 1993). First, human astrocyte cultures treated with gp120 showed decrease expression of GFAP (Glial fibrillary acidic protein), as well as the diminution of a major protein of 66 kDa (Pulliam *et al.* 1993). Second, some studies have indicated that gp120 can upregulate intracellular adhesion molecule-1 (ICAM-1) expression in primary rat and human astrocytes via a signal transduction pathway involving activation of protein kinase C and tyrosine kinase (Shrikant *et al.* 1996). Third, similar to the effects on the neurons, gp120 also increases Ca^{2+} concentration in astrocytes of rat cerebellum (Ciardo and Meldolesi, 1993; Codazzi *et al.* 1995). However, in contrast to neuronal response to gp120, astrocyte response to

gp120 most unlikely involved activation of either voltage-gated Ca^{2+} channels or NMDA receptors (Ciardo and Meldolesi, 1993). Fourth, gp120 has been shown to alter K+ and Na+ ion transport in astrocytes (Bubien *et al.* 1995; Benos *et al.* 1994). Fifth, as noted above, gp120 may enhance HIV-1-related neuronal damage. Mollace et al. demonstrated that gp120 stimulates an inducible form of nitric oxide synthase activity in cultured astrocytoma cells (Mollace *et al.* 1993a). Sixth, Levi et al. demonstrated that gp120 inhibited β-adrenergic regulation within astrocytes (Levi *et al.* 1993). Acute exposure to picomolar gp120 depressed β-adrenergic agonist-induced formation of cAMP altered cAMP-regulated functions in astrocytes. Finally, astrocytes can produce many types of cytokines including TNF-α, lymphotoxin, IL-1, IL-6, IFN-α, IFN-β and TGF-β. Most of those cytokines induce neurotoxicity via different mechanisms. HIV-1 infection or gp120 have been shown to stimulate astrocytes to produce IL-6 and TNF-α , which may injure neurons (Yeung *et al.* 1995). Induction of TGF-β production by astrocytes after HIV-1 infection, gp120 treatment or contact of infected macrophages has been linked to AIDS dementia complex (da Cunha *et al.* 1995; Wahl *et al.* 1991). Since TGF-β is a very potent chemotactic factor and can enhance the production of other cytokines including TNF-α, it may play an important role in AIDS dementia complex. Therefore, these findings may provide new insights into how gp120 can influence the involvement of astrocytes in AIDS dementia complex.

Although the mechanism by which gp120 interacts with an astrocyte cell membrane or by which HIV-1 enters astrocytes is unclear, HIV-1 infection of at least microglia in the brain appears to be mediated by the binding gp120 to the CD4 receptor

(Jordan *et al.* 1991). Furthermore, GalC has been determined to bind to gp120 in brain cell lines (Harouse *et al.* 1991; Bhat *et al.* 1991). A 180 kDa protein, gp120 binding protein, that is distinct from GalC and CD4 has also been described on a human glioma cell line (Schneider Schaulies *et al.* 1992). Since alteration of astrocytes caused by HIV-1 infection or gp120 is likely associated with AIDS dementia complex, characterization of molecules on astrocyte membrane for gp120 interaction will help in understanding the pathogenesis of AIDS dementia complex.

D. Effects of Tat on astrocytes

Tat effects a variety of cellular functions in addition to its transactivating activity of HIV-1 gene expression. Tat up-regulates tumor growth factor β-1 in lympocytic cells, glia cells, and bone marrow macrophages (Zauli *et al.* 1992; Cupp *et al.* 1993), interleukin-4 receptors in B lymphoblastoid cells (Puri and Aggarwal, 1992), (Westendorp *et al.* 1994) and TNF-β in T lymphocytic and B lymphoblastoid cells (Sastry *et al.* 1990; Buonaguro *et al.* 1994). Tat also suppresses host cell functions such as antigen-induced lymphocyte proliferation (Viscidi *et al.* 1989), major histocompatibility complex class I expression (Howcroft *et al.* 1993), protein kinase activity (Roy *et al.* 1990b) and manganese superoxide dismutase activity (Flores *et al.* 1993). Besides, Tat transforms keratinocytes (Kim *et al.* 1992) and acts as a growth factor for Kaposi's sarcoma cells (Barillari *et al.* 1993). Recent studies suggest that Tat may also be involved in cellular processes that control apoptosis (Zauli *et al.* 1993; Li *et al.* 1995).

Importantly, Tat has a number of effects on astrocytes. Extracellular Tat increases both NF-kB binding and protein kinase C activity promoters (Conant, *et al.* 1996, Taylor *et al.* 1995), implying that Tat probably affects many functions of astrocytes because NF-kB is a transcriptional factor that activates a number of cellular promoters. Tat also transactivates JC virus T antigen expression in astrocytes (Chowdhury *et al.* 1990), and enhances the expression of extracellular matrix protein in glia cells (Taylor *et al.* 1992). It can also alter normal organization of neuron and astrocytes in primary cell culture (Koken *et al.* 1994). Similar to the effects of gp120 on astrocytes, Tat has also been shown to stimulate TGF-β1 in human astrocytic glial cells (Cupp *et al.* 1993). In our laboratory, we have demonstrated that Tat could induce changes in intracellular calcium in astrocytes (Haughey *et al.* unpublished observation) and stimulate expression of IL-1β in astrocytoma cells (Chen *et al.* unpublished result).

One unique feature of Tat is that it is released from productively infected cells to extracellular medium (Ensoli *et al.* 1990, 1992) and subsequently the extracellular Tat can be taken up by many cell types and localized in the nucleus (Frankel *et al.* 1988; Green and Loewenstein, 1988). Interestingly, Tat has been shown to be predominantly localized to the nucleolus (Fawell *et al.* 1994; Miyazaki *et al.* 1992; Hauber *et al.* 1989; Frankel *et al.* 1988; Green and Loewenstein, 1988) while a nuclear localization signal in basic region is essential for Tat uptake. The ability of Tat to affect a number of cellular functions and be taken up, that there are suggests cellular receptors for the Tat. So far, besides the Tat binding proteins on the cell (discussed in II 2), Tat has been shown to bind to some proteins. For example, Tat binds to a 90 kDa surface protein on Molt cells

(lymphocytic cell line) which is important for cell attachment (Ohana *et al.* 1993; Nelbock *et al.* 1990; Weeks *et al.* 1993). Integrin α5β1 and αvβ3 are also the receptors for Tat protein. Tat binds to integrin α5β1 and αvβ3 via the RGD sequence located on the C-terminal region of Tat protein (Ensoli *et al.* 1994; Ensoli *et al.* 1990; Toyama *et al.* 1992). N-terminal region of Tat contains a basic region which mediates Tat binding to αvβ5 integrin (Vogel *et al.* 1993). Integrins are cell adhesion molecules, a family of transmembrane receptors (Vogel *et al.* 1993). The relevance of integrin binding of Tat to HIV-1 pathogenesis is not clear. It has been suggested that Tat can affect some cellular functions through this interaction between RGD sequence of Tat and integrin α5β1 and αvβ3 such as cell proliferation of Kaposi's sarcoma and organization of primary astrocytes. However, Tat binding to integrin αvβ5 is unlikely play a role in Tat uptake by cells since anti-integrin antibodies capable of blocking cell attachment to Tat were not able to block uptake of Tat into cells (Vogel *et al.* 1993). However, it is still unknown if Tat action on astrocytes is due to membrane binding or following internalization. Clearly, it is crucial for us to understand the mechanisms since astrocytes may play an important role in AIDS dementia complex.

7. Objectives

Infection with HIV-1 frequently causes a dementing illness, resulting from neuronal cell loss, astrocytosis, myelin pallor, and infiltration by blood-derived macrophages. The virus causes a productive infection in microglia and a latent infection in astrocytes. The mechanism of viral entry into microglia is likely similar to

macrophages and mediated via CD4 and chemokine receptors. However, mechanism of viral entry into astrocytes remains to be determined.

The infected cells release soluble substances that interact with uninfected glial cells and neurons resulting in cellular dysfunction. Two viral proteins, gp120 and Tat have been implicated in mediating these responses.

The objectives of the project are thus to:

1) establish pure cultures of astrocytes from human fetal brain.

2) synthesize and purify recombinant Tat protein.

3) identify the dynamics of interaction between the viral protein gp120 and Tat with astrocytes.

4) identify and characterize the molecules on the astrocyte cell membranes that bind to gp120 and Tat.

III. MATERIALS AND METHODS

Part I Collective Materials and Methods

1. Cell cultures

A. Astrocytes

Human fetal brain tissue was obtained from the Department of Obstetrics and Gynecology at the University of Manitoba with approval from both Hospital and University ethics committees. Tissue was obtained after written consent from women undergoing elective termination of pregnancy with no risk factors for HIV-1 infection. Brain tissue from human fetuses of 13 to 16 weeks gestational age was washed with 10 ml serum-free Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) in a sterile petrie dish, and then mechanically disrupted by aspiration through a 20-gauge needle twice. The tissue was washed in DMEM and spun at 400g for 10 min and plated into 75 cm^2 tissue culture flasks (GIBCO). Each brain specimen was plated separately without pooling of tissues from similar or different gestational ages. Cells were cultured in 20 ml of DMEM with 10% (v/v) heat inactivated fetal bovine serum (FBS) (GIBCO), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 0.25 µg/ml amphotericin (Sigma) at 37°C with 5% CO_2 for four to six weeks with medium change once a week. To prepare pure cultures of astrocyte cells, the cultures were shaken in an orbital shaker (300 rpm) at room temperature for two to three hours to release the neurons that are loosely attached to astrocytes. The cells released from the cultures were discarded. The adherent cells were then harvested with 0.05% (w/v) trypsin and 0.53 mM EDTA in Hanks buffered saline solution, added to new 75 cm^2 flasks

with fresh culture medium and incubated at 37°C for 30 to 40 min to allow for microglial cells to adhere. The non-adherent cells in the supernatant, mainly astrocytes, were plated into new flasks. Aliquots of cells were routinely seeded onto glass coverslips and stained with antibody to glial fibrillary acidic protein (GFAP). Only cultures with >99% cells that were GFAP positive were used in the assays.

B. HeLa-CD4 cells

HeLa-CD4 cells, a cell line which can express CD4 molecules on cell surface, (provided by Dr. Michael Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were cultured in DMEM with 10% (v/v) FBS, 0.2 mg/ml G418 (Sigma) and 0.1 mg/ml hygromycin B (Sigma) at 37°C with 5% CO_2. To propagate the cells, the medium from an established cell culture flask was discarded. The cells were rinsed with 5 ml serum-free DMEM and incubated with 1.5 ml 0.05% (w/v) trypsin with 0.53 mM EDTA solution at 37°C until the cell layer started to detach. 10 ml DMEM with 10% (v/v) FBS was then added to disperse the cells. The cells were used for assays or for maintenance, 1/3 of cell suspension was subcultured in the above medium.

C. SK-N-MC cells

A neuroblastoma cell line, human SK-N-MC, (American Type Culture Collection, Rockville, MD), was cultured in Eagle's minimum essential medium (MEM, GIBCO) with sodium pyruvate (GIBCO) and 10% (v/v) FBS.

D. Human fetal neurons

Preparation of human fetal neurons has been done as described for preparation of human fetal astrocytes except culture medium. Briefly, fetal brain tissues were washed with serum-free OptiMEM (GIBCO) in a sterile petrie dishes and dissociated through a 20 gauge needle with 10 cc syringe. The dissociated tissue was then plated into a 75 cm^2 flask and cultured in Optimem containing 5% FBS (v/v), 100 U penicillin/ml, 100 μg streptomycin/ml and 0.25 μg amphotericin/ml. and 1% (v/v) N2 supplement (GIBCO) for more than four weeks prior to use. Purity of neurons was determined by immuno-staining for microtubule associated protein-2 (MAP-2), Cultures were used only if >70% cells were MAP-2 positive

E. NB41A3

NB41A3, a neuroblastoma cell line from mouse (American Type Culture Collection, Rockville, MD) was cultured in DMEM with 10% (v/v) FBS, 100 U penicillin/ml, 100 μg streptomycin/ml and 0.25 μg amphotericin/ml.

F. U373 cells

U373 cells (astocytoma cell line from human) were purchased from American Type Culture Collection, Rockville, MD and cultured in DMEM with 10% (v/v) FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U penicillin/ml, 100 μg streptomycin/ml and 0.25 μg amphotericin/ml. U373 cells were >99% pure as determined by positive staining for GFAP.

G. SupT cells

SupT cells (T lymphocyte cell line) were purchased from American Type Culture Collection, Rockville, MD and cultured in RPMI with 10% (v/v) FBS, 100 U penicillin/ml, 100 μg streptomycin/ml and 0.25 μg amphotericin/ml. The cells were grown in suspension at 37°C. For propagation, 1 ml of the cells was diluted with 20 ml fresh medium once a week.

2. Antibodies

Table 1

Name	Species	Dilution range/working concentration	Producer
Dako-T4-FITC	mouse	1 μg/ml	akopactt
OKT4a	mouse	1-4 μg/ml	Ortho Diagnostics
Anti-GalC	rabbit	1:10-1:200	Chemicon
Anti-Tat sera	rabbit	1:1000	NIH, AIDS Repository
Anti-rabbit IgG	goat	1:1000	Chemicon
Anti-αvβ3	rabbit	1:200	Dr. J. Wilkins (U of Manitoba)
Anti-αvβ5	rabbit	1:200	TELIOS
Anti-α5β1	mouse	1:100	Chemicon

3. Protein concentration determination

Two different methods were used to estimate protein content of an unknown sample. One of these was based upon an assay designed by Bradford (1976). The 5 to 50 μl sample was diluted to 800 μl with ddH_2O and then added into 200 μl dye Reagent Concentrate (Bio-Rad). The reaction solution was vortexed, allowed to stand for at least

five minutes, and read at 595 nm against a reagent blank without protein. A standard curve that was used for calculation of protein concentration was made in the same way using bovine serum albumin (BSA). To further confirm the protein concentration, a second method was used based on the absorbence of the peptide bond. The protein sample was diluted in 0.01% (w/v) of Brij 30 solution (Sigma) and read with a one cm cuvette at 205 nm while 0.01% (v/v) Briji solution alone was used for a control. The concentration of the sample protein was then estimated using the following equation: concentration (μg/ml)= 31 x ABS 205. This method of determining protein concentration can only be used for pure protein samples since many other reagents such as high salt and SDS can affect the reaction. Thus, the Brij reaction was used only for Tat protein determination in this study.

4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were used to separate proteins according to their molecular mass. The discontinuous buffer system of Laemmli (1970) was used. The unit used was a Mini Protein II (Bio-Rad). The protein was dissolved in a sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) ß-mercaptoethanol, and 0.01% (w/v) bromophenol blue and heated in boiling water for about 4 minutes. After loading the samples to the gel they were electrophoresed at 150 volts in a running buffer (0.025 M Tris-HCl, pH 6.6, 0.192 M glycine and 0.1% SDS) until the tracking dye reached the bottom of the gel. The following molecular mass markers (GIBCO) were used to estimate the molecular mass: insulin (2.9 kDa), bovine trypsin

inhibitor (6 kDa), lysozyme (14 kDa), β-lactoglobulin (18 kDa), carbonic anhydrase (28 kDa), ovalbumin (43 kDa), BSA (68 kDa), phosphorylase B (97 kDa) and Myosin (H-chain, 200 kDa). The gels were stained with Coomassie blue by placing it in the staining buffer [20% (v/v) methanol, 7.5% (v/v) acetic acid, 0.04% (w/v) Coomassie brilliant blue] for 1-2 hours with gentle shaking. The gel was then transferred into destaining buffer [20% (v/v) methanol and 7.5% (v/v) acetic acid] and destained at room temperature until the protein band(s) were clearly presented. Alternatively, the gels were electro-blotted (see section III. 5. Western blot) to transfer the protein band to a nitrocellulose membrane for Western blot analysis or fixed in a solution containing 10% (v/v) glacial acidic acid and 25% (v/v) methanol for at least 15 min followed by drying gel for autoradiography.

5. Western Blot

Following the SDS-PAGE, the gel was immersed in transfer buffer containing 25 mM Tris-HCl, pH 6.6, 192 mM glycine and 20% (v/v) methanol, pH 8.3 for 10 minutes. The nitrocellulose membrane (Bio-Rad, 0.45 μm) was cut to fit the gel and presoaked in the transfer buffer for 30 minutes. The membrane and gel were placed between two 3 mm chromatography papers presoaked in transfer buffer. The electroblotting proceeded in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 15 volts for 30 minutes. The membrane was then blocked by placing it in a solution of 5% (w/v) skimmed milk in PBS, pH 7.4 (0.01 M sodium phosphate, 150 mM NaCl, pH 7.4) (PBS-5% (w/v) skimmed milk) for 30 minutes at predetermined concentrations (see table 1).

The antibody was diluted in PBS-5% skimmed milk. The membrane was incubated with the diluted antibody at room temperature for one to two hours. It was then washed three times over 15 minutes in washing buffer (PBS with 0.05% (v/v) Tween 20) and then a species-specific secondary antibody was added (see table 1). For example, the secondary antibody might be goat IgG specific for rabbit immunoglobulins if the primary anti-sera was obtained from a rabbit. The secondary antibody conjugated with horse radish peroxidase (HRP) was diluted to 1:500 to 1:3000 in PBS-0.05% tween 20. This incubation step proceeded at room temperature for 90 minutes followed by three washes in washing buffer. The blot was developed with a developing solution [1xPBS, pH 7.4, 0.05% (w/v) 3,3'-Diaminobenzidine (DAB) (Sigma), 0.06% (v/v) H_2O_2 (Sigma) for 1 to 10 minutes. The reaction was then quenched by rinsing the membrane in dH_2O.

6. Immunocytochemical methods

The cells were plated on glass coverslips (Baxter) in 6-well plates and grown for 16 hours at 37°C. The cells were then washed once with serum-free medium and fixed in 2% (w/v) paraformaldehyde in PBS at room temperature for 30 minutes followed by three washes with PBS. After blocking for 30 minutes in PBS with 10% (v/v) horse serum (PBS-10% HS), the cells were incubated with the primary antibody diluted in PBS-10% HS at room temperature for 90 minutes. Unbound antibody was removed by three washes in PBS-1% BSA and the cells were incubated with a specie-specific secondary antibody conjugated with either HRP, FITC or Rhodamine (see table 1). The cells were then washed in PBS-1% BSA to remove the unbound antibody. If the second antibody conjugated with HRP was

used, the cells were developed with a developing solution [1xPBS, pH 7.4, 0.05% (v/v) DAB, 0.06% (v/v) H_2O_2] for 1 to 10 min. The reaction was then quenched by rinsing the cells in dH_2O. The cells stained with either HRP or immunofluorescence were dehydrated for 1 minutes in 50% (v/v), 75% (v/v), 95% (v/v), 100% (v/v) ethanol and then 100% (v/v) xylene, and mounted with Ghurr (BDH). The cells were viewed under a Olympus BH-2 microscope. Secondary antibodies alone without incubation in primary antibody were used as negative controls.

7. Cell Surface Radioiodination

Cells were surface radioiodinated by the lactoperoxidase technique (Ma *et al.* 1994) 5×10^7 cells were washed with 10 ml serum-free medium once and scraped off culture flasks with a rubber policeman. The cells were centrifuged at 400g for 5 min and resuspended in 1 ml PBS containing 1 mCi $Na^{125}I$ (DuPont) and 20 µg lactoperoxidase (Sigma) and following 0, 1, 5, and 10 min, 10 µl of 0.03% H_2O_2 (Sigma) were added. After the last addition of H_2O_2, the reaction was extended for an additional 10 min. The reactions were carried out at room temperature and were stopped by adding 5 ml of PBS containing 10 mM NaI. Free ^{125}I was removed by three washes with a total of 15 ml PBS. Labelled cells were lysed by adding 500 µl of detergent lysing buffer (LB) consisting of 0.5% (v/v) NP-40, 0.2% (w/v) sodium deoxycholate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.12 M NaCl, 50 µg PMSF/ml, and 20 mM Tris HCl, pH 7.4. Tubes were placed on ice for 15 min, and the nuclei were removed by centrifugation at 3000g for 20 minutes. The extracts were stored in -80°C until used for immunoprecipitation.

Part II Gp120 assay

1. Recombinant gp120

Recombinant gp120 of HIV-1_{SF2} (rgp120) was provided by Dr. Nancy Haigwood, Chiron Corporation through the AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH, was >90% pure as determined by Coomassie blue staining following SDS-PAGE. The activity of this protein was confirmed by Western blot analysis and by binding to CD4 on SupT cells (see IV, Part I 1). This binding activity was confirmed by cross-linking of radioiodinated gp120 to CD4 molecule on HeLa CD4 cells (see IV, Part I 2).

2. Radiolabelling of rgp120

The rgp120 was labelled with $Na^{125}I$ by lactoperoxidase with enzymobeads (Bio-Rad). The total reaction volume was 125 µl containing 50 µl phosphate buffer (0.2 M, pH 7.2), 10 µg gp120, 1.0 mCi Na ^{125}I, 25 µl β-D-glucose, and 50 µl Enzymobeads. The reaction proceeded at room temperature for 20 minutes and was quenched by applying the reaction solution to a PD-10 column (Pharmacia). The ^{125}I-labelled protein was separated from free ^{125}I using a PD-10 column preequilibrated with PBS-0.1% BSA. The column was eluted with 6 ml PBS-0.1% BSA and 0.5 ml fractions were collected. Radioactivity of labelled rgp120 was monitored by measuring 5 µl from each fraction. Two fractions containing the highest labelled rgp120 were pooled, dispensed into 25 µl aliquots and stored at -80°C until used. The specific activity of ^{125}I-rgp120 was 3.9×10^4 cpm/ng.

3. Flow Cytometry

The presence of CD4 molecules on the cell surface was determined by immunofluorescence flow cytometry. 5 x 10^5 of astrocytes were trypsinized briefly (30 seconds) at 37°C and removed from the monolayer primarily by mechanical force. The cells were washed once in 1 x PBS with 1% (w/v) BSA (PBS-1% BSA) followed by centrifugation at 400 g for 5 minutes. The cells were then resuspended and incubated in PBS-1% BSA with a monoclonal antibody (1 μg/ml) against CD4 molecule conjugated to fluorescent isothiocyanate (DAKO-T4-FITC) (Dakopatts) at room temperature for 2 hours. Background levels of fluorescence were determined by using goat anti-mouse IgG conjugated to FITC (Boehringer Mannheim). To ensure that trypsinization did not alter the epitope defined by DAKO-T4, 5 x 10^5 of HeLa-CD4 cells, which also form adherent monolayers, were used as a positive control and treated in an identical fashion. The cells were then washed with PBS-1% BSA three times for 30 minutes and fixed in 2% (w/v) paraformaldehyde in PBS for 30 minutes at room temperature. The cell-associated fluorescein were measured by a flow cytometer.

4. ^{125}I-rgp120 binding

Binding of ^{125}I-rgp120 to cells was performed essentially as previously described (To *et al.* 1992; Schnittman *et al.* 1988) with the following modifications. Prior to the binding assay, 2×10^4 astrocytes were plated into each well of 96 well plates and maintained at 37°C for 18 hours to allow the cells to attach to the bottom of the well. Final cell numbers

per well wwere approximately 4×10^4 at the time of assay. 2×10^4 HeLa-CD4 cells and SK-N-MC cells were plated into each well of 96 well plates and studied along with the astrocytes. The cells were washed twice with 200 µl ice-cold binding medium containing RPMI 1640, 0.5% (w/v) BSA and 50 µg bacitracin/ml. For kinetics, binding medium (50 µl) containing 1.0 nM ^{125}I-rgp120 was added into each well and incubated with the cells at 4°C for 1 to 5 hours with gentle shaking. For dose dependency, the cells were incubated with various concentrations of ^{125}I-rgp120 (0 to 120 nM) at 4°C for 5 hours. For the competition assay, the cells were incubated with 50 µl binding medium containing 1.0 nM ^{125}I-rgp120 in the presence of unlabelled gp120 (0 to 120 nM) at 4°C for 5 hours. Unbound rgp120 was removed by three washes in ice cold PBS-0.5% BSA. The cells were solublized in 100 µl 0.2 N NaOH and transferred into a scintillation vials to count in a LKB-Wallac gamma counter for cell associated radioactivity.

A monoclonal antibody against the gp120 binding site on CD4 (OKT4a) (Ortho Diagnostics) and rabbit anti-GalC (Chemicon) were used to determine if these two antibodies could block the binding of rgp120 to astrocytes. HeLa-CD4 cells were used as a positive control with OKT4a and SK-N-MC cells as a positive control with anti-GalC. The cells were preincubated with the respective antibodies at various dilution for 60 min at 37°C and then the cells were washed twice in ice-cold binding medium to remove the unbound antibodies. Subsequently, the cells were incubated with 1.0 nM ^{125}I-rgp120 for 5 hours at 4°C. The cells were then washed and counted as described above.

CD4 binding activity of ^{125}I-rgp120 was also tested on SupT cells. The cells (1 x 10^6/tube) were spun at 400 g for 5 min. Cell pellets were resuspended in 1 ml serum-free RPMI and centrifuged again. This washing cycle was repeated once more. Cells were then incubated with 2 μg/ml OKT4a in binding medium for 60 min at room temperature followed by centrifugation at 400 g for 5 min. Cell pellets were washed twice in 1 ml ice-cold binding medium to remove the unbound antibody. Cells were then incubated with 1.0 nM ^{125}I-rgp120 in 100 μl binding medium for 60 min at 4°C and centrifuged through an oil cushion [13 ml (556 cosmetic-grade silicone fluid) : 12 ml (550 cosmetic-grade silicone fluid) (Dow Corning)] to remove the free ^{125}I-rgp120. Pellets were transferred to scintillation vials followed by counting of cell-associated radioactivity.

5. Cross-linking of ^{125}I-rgp120 to CD4 molecule

Culture flasks (25 cm^2) containing confluent monolayers of HeLa CD4 cells were placed on ice and washed three times with 5 ml of ice-cold binding medium. Cells were then incubated with ^{125}I-rgp120 (500 ng) in 1 ml binding medium at 4°C for 5 hours. Unbound ^{125}I-rgp120 was removed by three washes with 5 ml of ice-cold PBS. Bound ^{125}I-rgp120 was then cross-linked to cell surface protein(s) using 0.2 mM DSS (Disuccinimidyl Suberate) (Pierce) (prepared as a 20 mM stock in DMSO); control cells lacking DSS received an equal amount of DMSO. Monolayers were incubated for 35 min at 4°C and then washed three times with 5 ml of ice-cold Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) followed by one wash using 5 ml of ice-cold PBS. Cells were then scraped off the flasks in 5 ml ice-cold PBS and pelleted by centrifugation at 400 g for 5

min. Cell pellets were lysed by treatment with 100 µl sample buffer followed by SDS-PAGE (6%). Radiolabelled cross-linked protein was visualized by autoradiography. Autoradiography was performed at -70°C for 2 days using Kodak X-Omat film (Kodak) and Du Pont Cronex Lightning plus intensifying screens.

6. Immunoprecipitation of gp120 binding protein

Human fetal astrocytes (5 x 10^7)were labelled (III, Part I, 8). The labelled cells were split into five microcentrifuge tubes and incubated with ^{125}I-rgp120 (0.1 µg) or rgp120 (1 µg) for 5 hours at 4°C with gentle shaking. The labelled cells without rgp120 were used as negative controls. Unbound rgp120 was removed by washing three times with ice-cold RPMI. The cell pellets were lysed by adding 400 µl of detergent lysing buffer consisting of 0.5% (v/v) NP-40, 0.2% (w/v) sodium deoxycholate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.12 M NaCl, 50 µg/ml phenylmethylsulfonyl fluoride, and 20 mM Tris HCl, pH 7.4. Tubes were placed on ice for 15 min, and the nuclei were removed by centrifugation at 3000 g for 10 min. Simultaneously, protein A agarose (20 µl) (Pharmacia) was washed twice with PBS and incubated with sera (150 µl) from a HIV-1 infected patient predetermined to have high titer antibody against gp120 or normal human sera for 60 min at room temperature. The beads were then washed three times with PBS-0.05% Tween 20. All lysates were added to the beads and incubated for 2 hours with constant rotation at room temperature. The protein A agarose absorbents were washed three times with lysing buffer. Absorbed beads were eluted at 100°C for 3 min with 30 µl of sample buffer and resolved by a 4 to 15% gradient SDS-PAGE. Dried gels were exposed to X-ray film (Kodak) for 5-7 days.

Part III Tat assays

1. Bacterial Expression and Purification of Tat1-72

A. PCR amplification of *tat1-72* gene

The *tat* DNA encoding 1 to 72 amino acids (first exon) was amplified from plasmid pSV2tat72 containing tat gene of HIV-1_{BRU} (obtained from Dr. Richard Gaynor through the AIDS repository, NIH) by using standard polymerase chain reaction (PCR) protocols and the following oligonucleotides *tat*1-72 initiation sequence primer 5'-CATGGAACCGGTCGACCCGCGT-3' and *tat*1-72 termination sequence primer 5'-CCGGGAGATCTTCACTGTTTAGACAGA-3'. The termination sequence primer contains a BglII site (underlined). These two primers were used to create a BglII site at 3' end of the amplified *tat*1-72 DNA and a blunt end at 5' site for NruI site insertion. 100 µl of PCR mixture containing 1 x PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM (each) deoxynucleoside triphosphates, 50 pM primer, 0.5 unit Taq polymerase, ddH_2O and 50 ng pSV2tat72 were placed in a 500 µl microfuge tube with cap (Bio-Rad); the mixtures were overlaid with 80 µl mineral oil to prevent evaporation. The PCR was carried out for 30 cycles; each cycle was for 1 min each at 95°C, 58°C, 72°C for denaturation, annealing, and elongation respectively. At the end of 30 cycles, the reaction mixtures were incubated at 72°C for an additional 7 min for the completion of the extension of the PCR products. A 200 bp *tat* DNA band was detected from the PCR products by agarose gel electrophoresis. The PCR products were transferred to a

microcentrifuge tube, adjusted to 0.15 M KOAc, pH 5.5 and precipitated with 2 volumes of 100% ethanol for 20 min at -70°C. Pelleted DNA was recovered by centrifugation at 12,000 g for 8 min and rinsed with 70% (v/v) ethanol, partially dryed and resuspended in 50 µl TE buffer (10 mM Tris, pH 8.0/0.1 mM EDTA, pH 8.0).

B. Subcloning of *tat* 1-72 DNA

Plasmid PinPoint Xa-2 (Promega) was cut with BglII and NruI. The *tat* DNA was also digested with BglII (GIBCO). Both plasmid DNA and *tat* DNA were extracted with an equal volume of phenol (GIBCO), followed by chloroform (GIBCO) to remove the proteins. DNA was precipitated as described (III, Part III, 1A). The *tat* DNA was then ligated into the BglII-NruI site of the plasmid. The ligation reaction contained 50 ng *tat* DNA, 200 µg plasmid DNA, and 0.5 unit of T4 ligase, and was carried out at room temperature for 5 hours. With this vector, the *tat* gene was expressed as a fusion protein that is naturally biotinylated at the N-terminus. The biotinylated protein is used as a purification tag.

C. Transformation of the plasmid

This plasmid containing the *tat* DNA were used to transfect competent *E. coli.* DH5αF'IQ (GIBCO). The 200 µl competent cells were mixed with 0.1 µg ligated plasmid and incubated on ice for 30 min followed by heat shocking the cells at 42°C for 2 min. The cells were then mixed with 1 ml SOC medium [2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.1 M NaCl, 0.025 M KCl, 20 mM Mg^{2+}, and 20 mM glucose] and grown

for 45 min at 37°C with shaking. Subsequently, 100 μl of the transfected cells were used to spread over the surface of LB agar plates with 100 μg ampicillin/ml. The plates were incubated at 37°C for 18 hours.

D. Plasmid "Mini-Prep"

The recombinant plasmids were isolated by a established "mini-prep" method. Several cell colonies were selected and grown 18 hours in 5 ml of LB-100 μg ampicillin/ml with shaking at 37°C. 1.5 ml of the cells was transferred into a microcentrifuge tube and centrifuged at 10,000 g for 1 min. The cell pellet was resuspended in 100 μl buffer I (0.025 M Tris, pH 8.0, 50 mM glucose, and 10 mM EDTA). Following incubation on ice for 5 min, 200 μl buffer II (0.2 M NaOH and 1% (w/v) SDS) was added. The cells were incubated on ice for 5 min followed by addition of 150 μl buffer III (3 M KAc, 11.5% (v/v) glacial acetic acid) to remove cellular DNA and proteins. The samples were pelleted by centrifugation at 10,000 g for 5 min and the supernatants were saved for plasmid extraction. The plasmid DNA was extracted with phenol/chloroform and precipitated with ethanol as discussed (III, Part III, 1A) . The DNA was resuspended in 20 μl TE buffer and digested with BglII and NruI followed by agarose gel electrophoresis. The clones in which the recombinant plasmid could produce a 200 bp DNA fragment were considered as positive clones, indicating the tat gene had been cloned. To confirm the *tat* reading frame to ensure that no base changes had occurred during PCR amplification, the recombinant plasmids were also subjected to dideoxynucleotide sequencing between restriction sites used for insertion.

E. Purification of Tat1-72

A positive clone was grown in 5 ml Terrific Broth (GIBCO) with 100 μg ampicillin/ml at 37°C for 18 hours. Bacteria were mixed with glycerol (1:5) and stored at -75°C. To express Tat proteins, the bacteria were grown in 200 ml LB (GIBCO) 18 hours and diluted into 2 L Terrific Broth the next morning followed by incubation at 37°C for one hour with shaking (150 rpm). Tat expression was then induced with 0.1 mM isopropylthio-β-galactoside (IPTG) (GIBCO). The bacteria were harvested 3-4 hours post-induction by centrifugation at 5000g for 15 min and the pellets were frozen at -20°C until used for Tat purification. The bacteria pellets from a 2 liter culture were resuspended on ice in 20 ml lysis buffer [50 mM Tris, pH 8.0, 50 mM NaCl, 4 mM dithiothreitol (DTT), 5% (v/v) glycerol, 0.1% (v/v) triton X-100 and 1 mM phenylmethylsulfonyl (PMSF)] and pressed twice by French Press. The resulting crude extract was clarified by centrifugation at 10,000 g for 20 min. The supernatant was saved for Tat1-72 purification. 5 ml of soft release avidin resin (Promega), equilibrated with the lysis buffer, was added to the supernatant and incubated for 2 hours on a rocker at 4°C. The resins were washed with 150 ml buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 4 mM DTT, 0.1% Triton X-100 (v/v) and 1 mM PMSF] and 100 ml factor Xa cleavage buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$ and 0.5 mM DTT) followed by incubation with 30 μg factor Xa in the cleavage buffer for 2 hours at room temperature. Subsequently, the cleaved Tat protein (Tat1-72) was eluted from the resins with cleavage buffer, desalted with a PD-10 column (Pharmacia) equilibrated with ddH_2O and

lyophilized. The Tat protein was > 95% pure as determined by (SDS-PAGE) followed by Coomassie blue staining. The purified product was further confirmed by Western blot analysis using polyclonal antisera to Tat (AIDS Repository, NIH), and the purified Tat1-72 also activated the HIV-LTR-CAT construct (AIDS repository, NIH).

Recombinant HIV-1_{BRU} Tat1-86 was obtained from Repligen. The Tat1-86 was >98% pure as determined by SDS-PAGE followed by Coomassie blue staining. Tat31-61, Tat31-71 and Tat48-85 were obtained as gifts from the AIDS Reagent Program of the Medical Research Council of U.K. Fifteen-mer Tat peptides, each overlapping by 10 amino acids and completely spanning the 86 amino acids sequence of Tat HIV_{BRU} were synthesized on a peptide synthesizer (Applied Biosystems) and purified by reverse-phase high-pressure liquid chromatography (done by Carol Martin, Department of Medical Microbiology). Stock solutions (1mM) of these peptides were prepared in 0.9% (w/v) NaCl (Table 2).

2. Immunocytochemistry staining of U373 cells with Tat1-72

10^5 U373 cells were plated on glass coverslips in 6-well plates and grown 18 hours at 37°C. The cells were then washed once with serum-free medium and incubated with 5 μg Tat1-72 in DMEM binding medium for 2 hours followed by three washes with the binding medium to remove unbound Tat1-72. The cells were then fixed in 2% (w/v) paraformaldehyde in PBS at room temperature for 20 minutes followed by three washes in PBS. The cells were blocked with 10% (v/v) horse sera in PBS for 30 min followed by incubation with anti-Tat sera (1:1000 diluted in 10% horse sera and 0.01% (v/v) tritonX-

100) for 1 hour. The cells were washed in PBS-1% BSA to remove the unbound antibody, goat anti-rabbit IgG conjugated with HRP was added, and reaction product was developed as described (III, PartI, 7). The cells treated with Tat1-72 followed by normal rabbit sera instead were used as controls.

3. Radiolabelling of Tat

Tat1-86 or Tat1-72 was labelled with $Na^{125}I$ by Iodo-beads (Pierce). An Iodo-bead was rinsed with PBS and added into 150 µl solution containing PBS pH 7.4, 1.0 mCi Na ^{125}I, and 10-50 µg Tat. The reaction proceeded at room temperature for 15 minutes and was quenched by removal of the bead. The labelled protein was separated from free ^{125}I using a PD-10 column (Pharmacia) equilibrated with PBS pH 7.4 containing 0.1% (w/v) BSA and 0.5 mM DTT. ^{125}I-Tat fractions were collected, dispensed into 25 µl aliquots and stored at -80°C until used. The specific activity of ^{125}I-Tat was 2.4×10^4 cpm/ng.

4. Cellular uptake assays of ^{125}I-Tat

Cellular uptake assays of ^{125}I-Tat were performed as previously described (Frankel *et al.* 1988, Mann and Frankel, 1991). Prior to the binding assay, 1×10^5 astrocytes were plated into each well of 24 well plates and maintained at 37°C until > 90% confluence. Final cell number per well was approximately 5×10^5 at the time of assay. Cells were plated were washed in 24-well plates with one ml of serum-free DMEM and incubated with varying concentrations of ^{125}I-Tat1-86 or ^{125}I-Tat1-72 (2 to 64 nM) in 0.2 ml binding

medium (DMEM and 0.1% (w/v) BSA) at room temperature for 30 min to two hours. Unbound ^{125}I-Tat was removed by three washes in ice cold DMEM. Cells were detached

Table 2

Name of Tat peptide	Amino acid sequence
Tat_{31-71}	FHCQVCFTTKALGISYGRKKRRQRRRPPQESQTHQVSLSKQ
Tat_{31-61}	FHCQVCFTTKALGISYGRKKRRQRRRPPQES
Tat_{48-85}	RKKRRQRRRPPQESQTHQVSLSKQPTSQSRGDPTEPKK
Tat_{1-15}	MEPVDPRLEPWKHPG
Tat_{3-17}	PVDPRLEPWKHPGSQ
Tat_{8-22}	LEPWKHPGSQPKTAC
Tat_{13-27}	HPGSQPKTACTNCYC
Tat_{18-32}	PKTACTNCYCKKCCF
Tat_{23-37}	TNCYCKKCCFHCQVC
Tat_{28-42}	KKCCFHCQVCFTTKA
Tat_{33-47}	HCQVCFTTKALGISY
Tat_{38-52}	FTTKALGISYGRKKR
Tat_{43-57}	LGISYGRKKRRQRRR
Tat_{48-62}	GRKKRRQRRRPPQGS
Tat_{53-67}	RQRRRPPQGSQTHQV
Tat_{58-72}	PPQGSQTHQVSLSKQ
Tat_{63-77}	QTHQVSLSKQPTSQP
Tat_{68-82}	SLSKQPTSQPRGDPT
Tat_{72-86}	QPTSQPRGDPTGPKE

by 0.05% (w/v) trypsin-0.53 mM EDTA-4Na (GIBCO BRL) for 10 min at 37°C. Cells were chilled to 4°C on ice, centrifuged at 400 g for 10 min, and supernatants were considered as the membrane-associated fraction of ^{125}Tat. Cytoplasmic and nuclear fractions were prepared as previously described (Ausubel *et al.* 1987). Cell pellets were washed twice with serum-free DMEM. Cells were lysed in the PBS buffer containing 0.5% (v/v) NP-40 at 4°C for 20 minutes and centrifuged at 3000 g for 10 minutes to

isolate the nuclei and supernatants were saved as the cytoplasmic fractions. The fractions were counted in a LKB-Wallac gamma counter for the associated radioactivity.

To further confirm the nuclear localization of Tat, astrocytes in 24-well plates were treated with 10 nM ^{125}I-Tat1-86 or ^{125}I-Tat1-72 for 30 min, 2 hours and 4 hours. Nuclear fractions were prepared and analyzed by 15% SDS-PAGE. The gels were then exposed to films for one day.

The competition of unlabelled Tat with a constant amount of ^{125}I-Tat was determined by incubating cells with 10 nM ^{125}I-Tat1-86 or ^{125}I-Tat1-72 in the presence of 20-100 fold molar excess of unlabelled Tat for 2 hours at room temperature. To define the regions of Tat which may be responsible for its uptake, we incubated cells with ^{125}I-Tat1-86 or ^{125}I-Tat1-72 in the presence of 500 to 1000 fold molar excess of various Tat peptides. To determine the effect of integrin αvβ3, αvβ5 and α5β1, cells were pretreated with ant-αvβ3 (kindly provided by Dr. J. Wilkins), αvβ5 (TELIOS) and α5β1 (Chemicon) at 1:100 to 1:200 for 90 minutes at room temperature followed by ^{125}I-Tat1-72 uptake assay. To determine the effect of polyanions, cells were treated with ^{125}I-Tat1-86 or ^{125}I-Tat1-72 in the presence of 0.2 - 3.2 μM concentrations of dextran sulfate. Cells were then harvested as above.

To further determine the specificity of Tat uptake, immunoabsorption of Tat was performed as described previously (Magnuson *et al.* 1995). 20 μl of protein A-sepharose (Pharmacia) was incubated with 1 ml PBS containing 10 μl rabbit anti-Tat antisera (1:100) (AIDS repository, NIH) or 10 μl normal rabbit serum for 90 min at room temperature. Bound sepharose was washed with 1 ml PBS-0.05% (v/v) Tween 20 three

times and incubated with 10 nM Tat for one hour at room temperature followed by centrifugation. Supernatants were added into astrocytes for measurements of Tat uptake and Cells were then harvested as above.

5. Tat1-72 transactivation assay

HL3T1 cells containing the HIV-1 LTR-chloramphenicol acetyltransferase (CAT) constructs were obtained from Drs. B. K. Felber and Dr. G.N. Pavlakis through the AIDS Repository, NIH and were propagated in DMEM with 10% (v/v) FBS. The Tat transactivation assay was performed as previously described (Felber *et al.* 1988; Frankel *et al.* 1988) with minor modifications and the CAT activity was measured by a simple phase extraction assay (Seed and Sheen, 1988). $1x10^6$ of HL3T1 cells were plated into 60 mm^2 culture dishes for 24 hours until 70% confluent. Cells were washed twice with 3 ml serum-free DMEM. 1 ml of DMEM with 10% (v/v) (with scrape-loading) to 0.5% (v/v) (without scrape-loading) FBS, Tat1-72 (0.1 to 0.5 μM), Chloroquine (100 μM) in the presence or absence of Tat peptides was added. Cells were scraped from the dishes and resuspended carefully and evenly by using a rubber policeman. Cells were cultured at 37°C for 24 hours and then washed twice with 5 ml PBS. Cells were then incubated with 1 ml TEN solution (40 mM Tris-HCL, pH 7.5, 1 mM EDTA, pH 8.0 and 150 mM NaCl) for 5 minutes on ice, scraped from the dishes by a rubber policeman, and transferred into a microcentrifuge tube on ice. The cells were then centrifuged at 400 g for 1 minute and the cell pellets were resuspended in 100 μl ice-cold 0.25 M Tris buffer, pH 7.5 and lysed by freezing in dry ice/ethanol slurry for 5 minutes and then thawing at 37°C for 5

minutes. This freeze-thaw cycle was repeated twice more, for a total of three freezes and thaws. The cell lysate was then centrifuged at 4°C for 5 minutes. Supernatants were saved for CAT assay. Following the protein concentration assay, 50 µl of cell extract were mixed with a 50 µl solution containing 0.004 µCi ^{3}H-chloramphenicol (DuPont), 25 µg butyryl-coA (Sigma) , 0.2 M Tris buffer, pH 8.0 and incubated 60 min at 37°C. 0.01 µCi ^{3}H-chloramphenicol was prepared as below (III, Part III, 6). The acylated ^{3}H-chloramphenicol was extracted by vigorously mixing the reaction solution with 200 µl tetramethylpentadecane/xylenes (2/1, v/v). The top organic phase was transferred into a scintillation vial containing 4 ml scintillation fluid and counted by a Beckman LS5000CE counter.

6. Chloramphenicol Acetyltransferase Assay

^{3}H-Chloramphenicol was purchased as 1 µCi/µl in ethanol. To prepare a 0.2 µCi/µl ^{3}H-chloramphenicol stock, 960 µl of 100% ethanol and 40 µl of 100 mg/ml of unlabelled chloramphenicol were added into 250 µl of 1 µCi/µl ^{3}H-chloramphenicol. This stock solution was stored in -20°C. To further create a working solution of 0.01 µCi ^{3}H-chloramphenicol, the 0.2 µCi/µl ^{3}H-chloramphenicol stock was diluted 20-fold in ddH_2O. The amount of the stock used depended upon the number of the reaction samples. The mixture was then extracted with an equal volume of xylenes by vigorous shaking. The phases were separated by centrifugation at 10,000 g for 1 min, and the top xylenes phase was discarded. The extraction was repeated one more time. This working solution of 0.01 µCi ^{3}H-chloramphenicol was ready for CAT assay.

7. ^{125}I-Tat1-72 binding assays

Binding of ^{125}I-Tat to human fetal astrocytes and U373 cells was performed as previously described (Mann and Frankel, 1991) with the following modifications. Cells were grown in 24-well plates until >90% confluence (approximately 5 x 10^5 cells/well). Cells were then washed with 1 ml of serum-free DMEM and incubated with varying concentrations of ^{125}I-Tat in 0.2 ml binding medium containing DMEM and 0.1% (v/v) BSA at room temperature for 30 min to five hours. Unbound Tat was removed by three washes in 1 ml of ice cold serum-free DMEM. The cells were treated with 0.05% (w/v) trypsin-0.53 mM EDTA-4Na (GIBCO) for 10 min. at 37 °C. The cells were chilled to 4°C on ice , centrifuged at 400 g for 10 min, and supernatants were counted in a LKB-Wallac gamma counter for cell associated radioactivity.

Competition of unlabelled Tat with a constant amount of ^{125}I-Tat was determined by incubating cells with 4 nM of ^{125}I-Tat and increasing concentrations of unlabelled Tat up to 400 nM for 2 hours at room temperature. To further determine the specificity of ^{125}I-Tat binding, cells were incubated with 4 nM ^{125}I-Tat in the presence of antisera to Tat (1:200) (provided by AIDS Repository, NIH). To define the region of the Tat which may be responsible for its binding, we incubated the cells with 4 nM ^{125}I-Tat in the presence of 500 to 1500 fold molar excess of various Tat peptides or 0.2 to 3.2 μM dextran sulfate. To determine the effect of integrin αvβ3, αvβ5 and α5β1, cells were pretreated with ant-αvβ3, αvβ5 and α5β1 at 1:100 to 1:200 for 90 min at room temperature. Following two

washes with serum-free DMEM to remove the unbound antibodies, cells were then incubated with 4 nM ^{125}I-Tat1-72 for the binding assay.

8. Immunoprecipitation of integrins

To detect αvβ3, α5β1 and αvβ5 on human fetal astrocytes and U373 cells, the cells were iodinated as described (III, Part I, 8). 20 μl of protein A Sepharose (Pierce) were washed twice with 0.5 ml PBS, pH 7.4 and incubated with 100 μl solution containing 10 μl of polyclonal antibodies to αvβ3, α5β1 and β5 or normal rabbit sera for 90 min at room temperature with gentle shaking. Unbound antibodies were then removed by three washes with PBS-0.05% tween 20. The 200 μl extracts of ^{125}I-human fetal astrocytes or ^{125}I-U373 cells were added onto antibody bound protein A sepharose for 2 hours at room temperature with gentle shaking. The protein A sepharose conjugated with antibody-ligand was centrifuged at 400 g for 3 min and washed four times with PBS, pH 7.4 containing 0.1% (w/v) BSA and 0.05% (v/v) tween 20. The beads were then boiled in electrophoresis sample buffer and run on 7.5% SDS-PAGE. The gels were dried and exposed to X-ray film (Kodak) O/N.

9. Affinity Chromatography

A. Preparation of Tat1-72 affinity column

A Tat affinity column was generated as described in the supplier's instruction for the Activated CH Sepharose 4B (Pharmcia Biotech). 1 gram of powdered Activated CH sepharose 4B was suspended in 5 ml of 1 mM HCl. The beads swell immediately and were washed with 300 ml 1 mM HCl on a scintered glass filter. 5 mg Tat1-72 was

dissolved in 6 ml coupling buffer (0.1 M $NaHCO_3$ pH 8, 0.5 M NaCl) and mixed with 3 ml of pre-swollen Activated CH sepharose 4B in a glass vial for 2 hours at room temperature with gentle stirring (no magnetic stirrer). The gel was then poured into 10 ml column (Bio-Rad) and washed with at least 15 ml of coupling buffer to remove unbound Tat1-72. The remaining active groups on the gel were blocked with 15 ml 0.1 M Tris-HCl pH 8.0 at room temperature for 1 hour. The gel was then washed thoroughly with at least 3 cycles of alternating pH. Each cycle consisted of a wash with 0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. The Tat column was then packed in PBS with 0.02% (w/v) sodium azide and stored at 4°C. For the control, 5 mg BSA was used to make a column as described for the Tat column.

B. Isolation of Tat1-72 binding proteins

$5x10^7$ U373 cells were washed with serum-free DMEM, scraped from culture dishes with a rubber policeman, and lysed in 2 ml of extraction buffer containing 137 mM NaCl, 1 mM CaCl, 1 mM MgCl, 0.75% (v/v) NP-40, 10% (v/v) glycerol, 50 μg (PMSF)/ml and 50 mM Tris-HCl pH 7.4 at 4°C for 30 min. The extracts were clarified at 3000g for 8 min and loaded into the Tat column which was preequilibrated with 8 bed volumes of the extraction buffer. The extracts ran through the Tat column with rate 0.1 ml/min. After the completely ran into the column, the extracts were incubated at room temperature for 2 hours. The column was then washed with 10 bed volumes of the extraction buffer. The bound materials were then eluted with 10 ml of 0.2 M glycine-HCl

pH 2.8 followed immediately by passing through a PD10 column that has been equilibrated with ddH_2O, for buffer exchange. The eluted materials were dispensed in 1 ml aliquots and lyophilized. The eluted proteins were either analyzed by SDS-PAGE followed by Coomassie blue staining or iodinated as described (III, Part III, 3) for immunoprecipitation below. The same amount of extracts was also passed through the BSA column as a control.

C. Immunoprecipitation of Tat1-72 binding protein

a) Immunoprecipitation from the proteins eluted from Tat1-72 affinity column

To confirm that the eluted proteins from the Tat column contains Tat binding protein(s), the eluted proteins were labelled with ^{125}I as described (III, Part III, 3) and were subjected to immunoprecipitation. 10 µl antisera to Tat (AIDS repository, NIH) was diluted into 90 µl PBS and mixed with 10 µl protein A-sepharose for 90 min at room temperature. Unbound antibodies were removed by three washes in 1.5 ml PBS-0.05% tween 20. Simultaeneously, 10 µg Tat was incubated with ^{125}I-eluted proteins for 2 hours at room temperature followed by mixture with a proteinA-antibody complex with constant rotation. The beads were washed three times with PBS-0.05% tween 20 and run on 9.5% SDS-PAGE. Protein A sepharose alone and normal rabbit sera which substituted anti-Tat sera were used as controls.

b) Immunoprecipitation from U373 cell membranes

To determine whether Tat binding protein(s) is located on U373 cell surface, $5x10^7$ U373 cells were surface-iodinated described (III, Part I, 8). The labelled cells were then extracted in the detergent lysing buffer containing 137 mM NaCl, 1 mM CaCl, 1 mM MgCl, 0.75% (v/v) NP-40, 10% (v/v) glycerol, 50 μg PMSF/ml and 50 mM Tris-HCl, pH 7.4. The extracts were then aliquoted (5 aliquots) for immunoprecipitation. 10 μl antisera to Tat (AIDS repository, NIH) was diluted into 90 μl PBS and mixed with 10 μl protein A-sepharose for 90 min at room temperature. Unbound antibodies were removed by three washes in 1.5 ml PBS-0.05% tween 20. Simultaneously, 10 μg Tat was incubated with ^{125}I-cell extract for 2 hours at room temperature followed by incubation with anti-Tat sera conjugated to protein A-sepharose for 90 min. The beads were washed three times with PBS-0.05% tween 20 and run on 9.5% SDS-PAGE. As controls, protein A sepharose alone or normal rabbit sera conjugated to protein A sepharose was used to incubated with ^{125}I-cell extract in the presence of Tat1-72. The anti-Tat sera conjugated to protein A sepharose was also used as a control to incubate with ^{125}I-cell extract in the absence of Tat1-72.

IV. RESULTS

Part I Characterization of gp120 binding on astrocytes

1. Binding of ^{125}I-rgp120 to CD4

CD4 is the primary receptor for gp120 on lymphocytes. To ensure that the rgp120 obtained was functional, CD4 binding activity of rgp120 was tested. SupT cells were incubated with ^{125}I-rgp120 with or without pre-treatment of the cells with OKT4a (a monoclonal antibody against the gp120 binding site on CD4 molecule). As shown in figure 4A, 70% of rgp120 binding on SupT cells was inhibited by OKT4a. To further confirm the CD4 binding activity of the rgp120, we used a homobifunctional noncleavable cross-linking reagent, DSS (disuccinimidyl suberate). This reagent has been successfully used in the characterization of several cell surface receptors including CD4 on H9 cells (McDougal et al., 1984). When ^{125}I-rgp120 was bound to HeLa CD4 cells, covalently cross-linked with DSS and analyzed by SDS-PAGE, in addition to the major rgp120 band, a higher molecular mass band (~180 kDa) was observed (fig. 4B) representing CD4 (58 kDa) bound to rgp120 (120 kDa). The 180 kDa band was not seen in the extracts from cultures without DSS (fig. 4B). Therefore, we considered that the function of rgp120 was well retained.

2. Binding kinetics of ^{125}I-rgp120 to astrocytes

To determine the properties of gp120 binding to astrocytes, human fetal astrocytes were cultured in 96 well plates and incubated with ^{125}I-rgp120 for various time intervals

Figure 4. **^{125}I-rgp120 binding to CD4 molecule.** (A) 1x10^6 SupT cells suspended in 0.2 ml binding medium were incubated with 0.5 nM ^{125}I-rgp120 in the presence or absence of 0.4 μg anti-CD4 antibody at 4°C for 2 hours. The bound ^{125}I-rgp120 was then separated from free ^{125}I-rgp120 and counted as described in Materials and Methods. 70% of ^{125}I-rgp120 binding on the cells was inhibited by anti-CD4 antibody compared to control (*$p < 0.005$, paired Student's *t* test). The values represent the mean ± standard error of two experiments done in triplicate. (B) Crosslinking of ^{125}I-rgp120 to CD4 molecules on HeLa-CD4 cells. ^{125}I-rgp120 was bound to HeLa CD4 cells and then crosslinked to CD4 molecules with DSS. The cells then lysed with sample buffer and analyzed by 7.5% SDS-PAGE followed by autoradiography. Lane 1 showed two bands: 180 kDa represented ^{125}I-rgp120 (120 kDa) crosslinked to CD4 (58 kDa) and ^{125}I-rgp120 (120 kDa). Lane 2 was a control without DSS and showed ^{125}I-rgp120 only. The experiments have been repeated for three times.

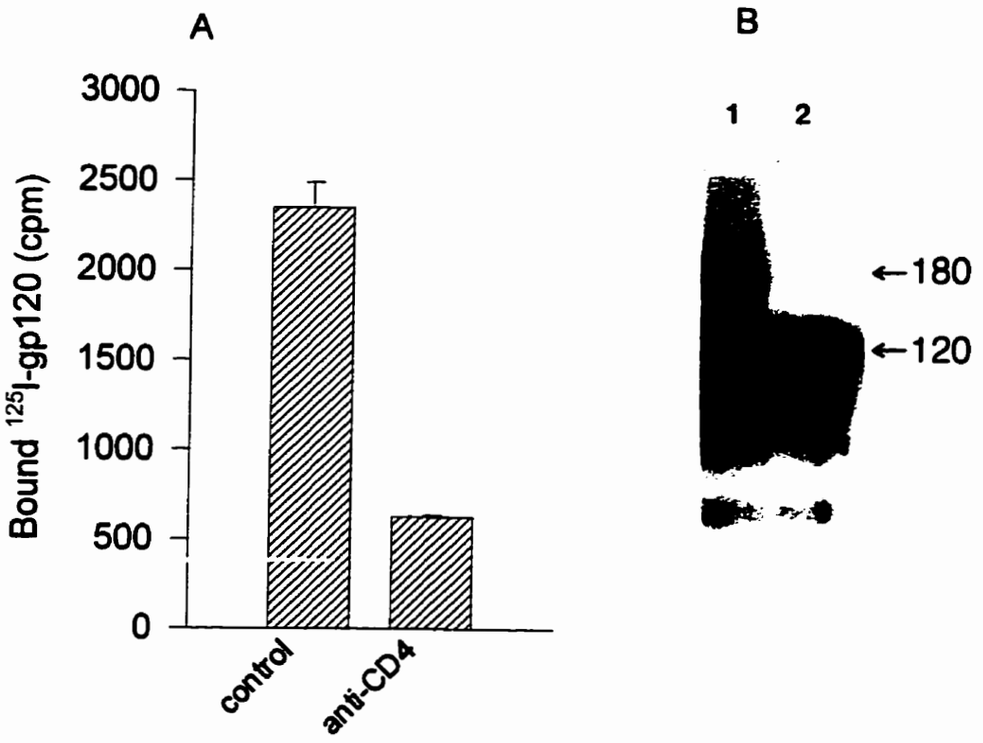

A
B
3000
2500
2000
1500
1000
500
0
Bound ^{125}I-gp120 (cpm)
control
anti-CD4
1
2
←180
←120

Figure 4

(10 min to 5 hours) at 4°C. Maximum binding of ^{125}I-rgp120 to astrocytes was observed at 5 hour incubation (fig. 5). The concentration-response of ^{125}I-rgp120 binding to astrocytes (5 x 10^5 cells/well) was performed in 24-well plates with increasing concentrations of ^{125}I-rgp120 ranging from 0.5 to 30 nM. The binding was saturated at 24 nM rgp120 (fig. 6). All subsequent experiments were performed in 96-well plates (4 x 10^4 cells/well).

Gp120 is the key molecule required for HIV-1 entry into cells. In order to do so, gp120 binds to specific cell surface receptor(s). To test directly for the presence of specific cell surface molecules that bind gp120, astrocytes were incubated with 1 nM ^{125}I-rgp120 in the presence of unlabelled rgp120 at 4°C for 5 hours. The binding of ^{125}I-rgp120 to astrocytes was competed with increasing concentrations (0 to 120 nM) of unlabelled rgp120 (Fig. 7). ^{125}I-rgp120 binding on astrocytes in the absence of unlabelled rgp120 was considered to be 100%. ^{125}I-rgp120 binding was also inhibited by unlabelled gp120 dose-dependently (fig. 7) and in the presence of 100 nM unlabelled rgp120, 50% of ^{125}I-rgp120 binding was inhibited. An increase in the concentration of unlabelled rgp120 to 120 nM did not inhibit further ^{125}I-rgp120 binding. Scatchard analysis of these data revealed a single class of binding molecules with an apparent dissociation constant (Kd) of 26 nM and an apparent maximal number of binding sites (Bmax) 29.9 fmoles/4×10^4 cells (4.5×10^5 binding molecules/cell) (fig. 7).

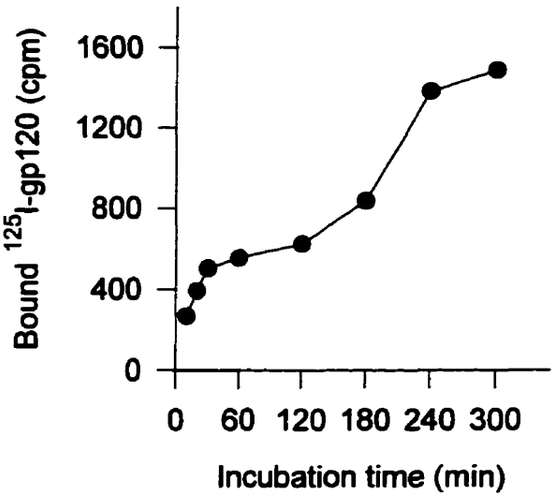


Figure 5. **Kinetics of ^{125}I-rgp120 binding to astrocytes.** Astrocytes (4×10^4 cells per well) were incubated with 1 nM ^{125}I-rgp120 for time periods ranging from 5 min to 5 hours at 4°C. The unbound ^{125}I-rgp120 was removed by three washes in ice-cold PBS-0.5% BSA. The bound ^{125}I-rgp120 was counted in a gamma counter. Maximum binding was apparently reached by 5 hours. Each experiment was done in triplicate and repeated three times. Data shown are the mean values from a single representative experiment.

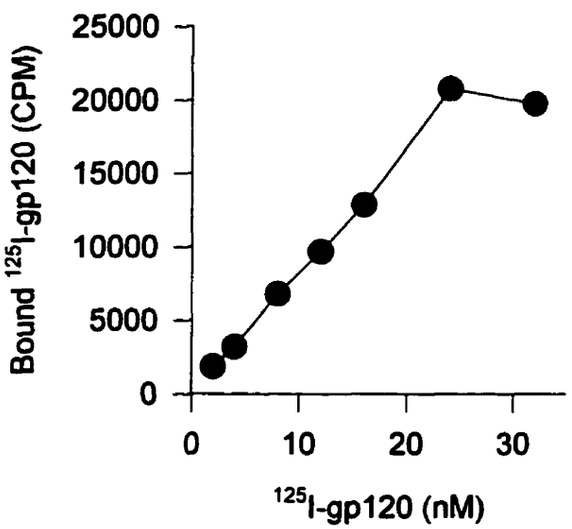


Figure 6. **Dose curve of ^{125}I-rgp120 binding to astrocytes.** Astrocytes were incubated with increasing concentrations of ^{125}I-rgp120 from 0.5 nM to 30 nM at 4°C. The unbound ^{125}I-rgp120 was removed by three washes in ice-cold PBS-0.5% BSA. The bound ^{125}I-rgp120 was counted in a gamma counter. Binding was saturated at 24 nM ^{125}I-rgp120. These values are from a single representative experiment done in 24 well plates.

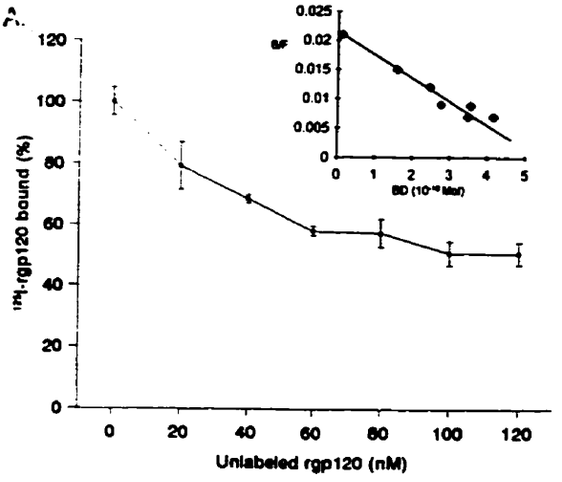


Figure 7. **Binding of ^{125}I-rgp120 to astrocytes.** Binding of ^{125}I-rgp120 to human fetal astrocytes was conducted by incubating 4×10^4 cells per well with 1 nM ^{125}I-rgp120 alone or with increasing concentrations of unlabelled rgp120 ranging from 0 to 120 nM for 5 hours at 4°C. The unbound radioactivity was removed by three washes in ice-cold PBS-0.5% BSA. The cell-associated radioactivity was measured in a gamma counter after solubilization of the cells in 0.2 N NaOH. The values represent the mean ± standard error of two experiments done in triplicate. Insert presents a saturation isotherm plot of the data from above. A single binding site was revealed with Kd of approximately 26 nM and maximal number of binding sites of 29 fMoles. B/F, bound/free ratio.

3. Rgp120 binding is CD4 independent

To determine if human fetal astrocytes express the CD4 molecule on the cell surface, we analyzed astrocytes by flow cytometry. Astrocytes were trypsinized briefly (30 seconds) and tapped to detach. Following inactivation of and removal of the trypsin, the cells were then treated with DAKO-T4-FITC. CD4 could not be detected on the cell surface of astrocytes while HeLa-CD4 cells, treated in an identical manner, exhibited strongly positive staining (Fig. 8). Considering the possibility that the CD4 epitope could be altered in part by trypsinization, we simultaneously stained the cells attached to glass coverslips with OKT4a and analyzed them by immunocytochemical staining. The CD4 molecule was present on HeLa-CD4 cells (Fig. 9A), however no specific staining on astrocytes was observed when compared to controls (Fig. 9B), indicating that CD4 was not expressed on human fetal astroyctes.

To further confirm that rgp120 binding was not mediated by CD4 on the surface of human fetal astrocytes, OKT4a was used to inhibit the binding of ^{125}I-rgp120 to astrocytes and to HeLa-CD4 cells as a positive control. 60% of the total binding on HeLa-CD4 cells was inhibited by 1 μg/ml OKT4a while OKT4a (up to 4 μg/ml) had no affect on the binding of ^{125}I-rgp120 to astrocytes (Fig. 10). Taken together, these results suggest that gp120 binding on human fetal astrocytes is CD4 independent.

4. Gp120 binding is GalC independent

GalC was reported to be an alternative receptor for gp120 and mediates HIV-1 infection in SK-N-MC cells (Harouse *et al.* 1991). To detect if the human fetal

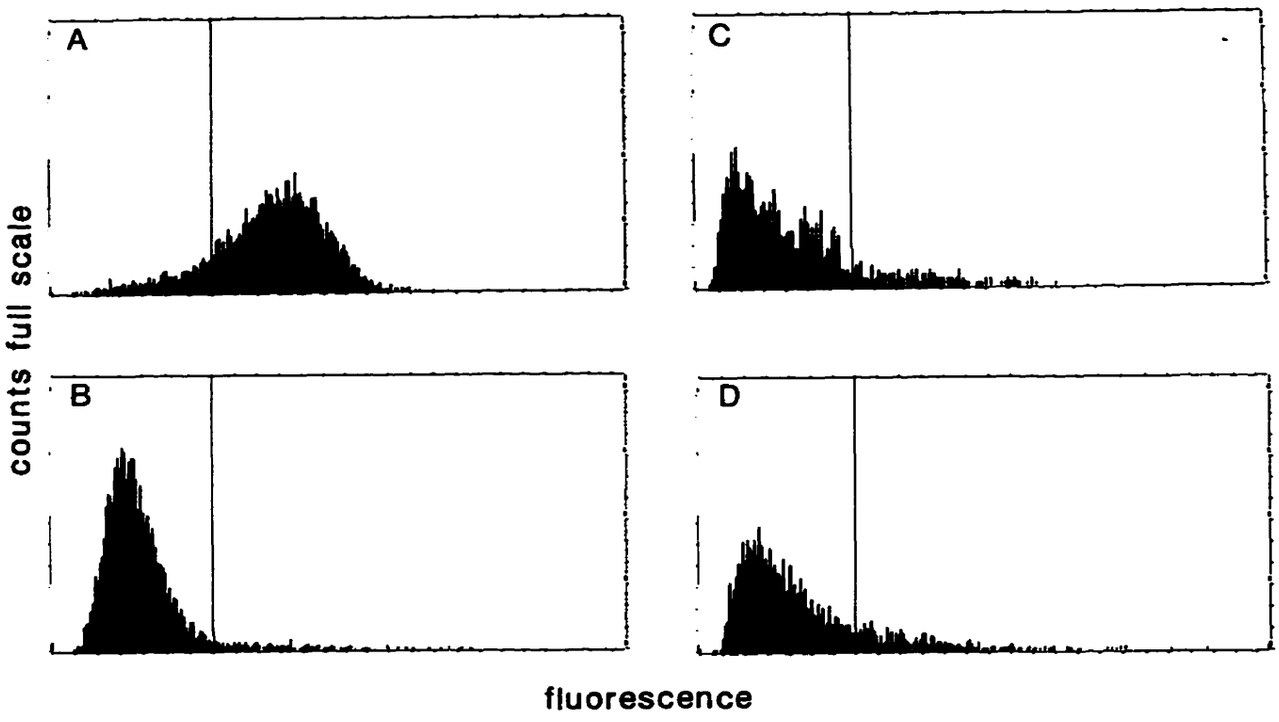
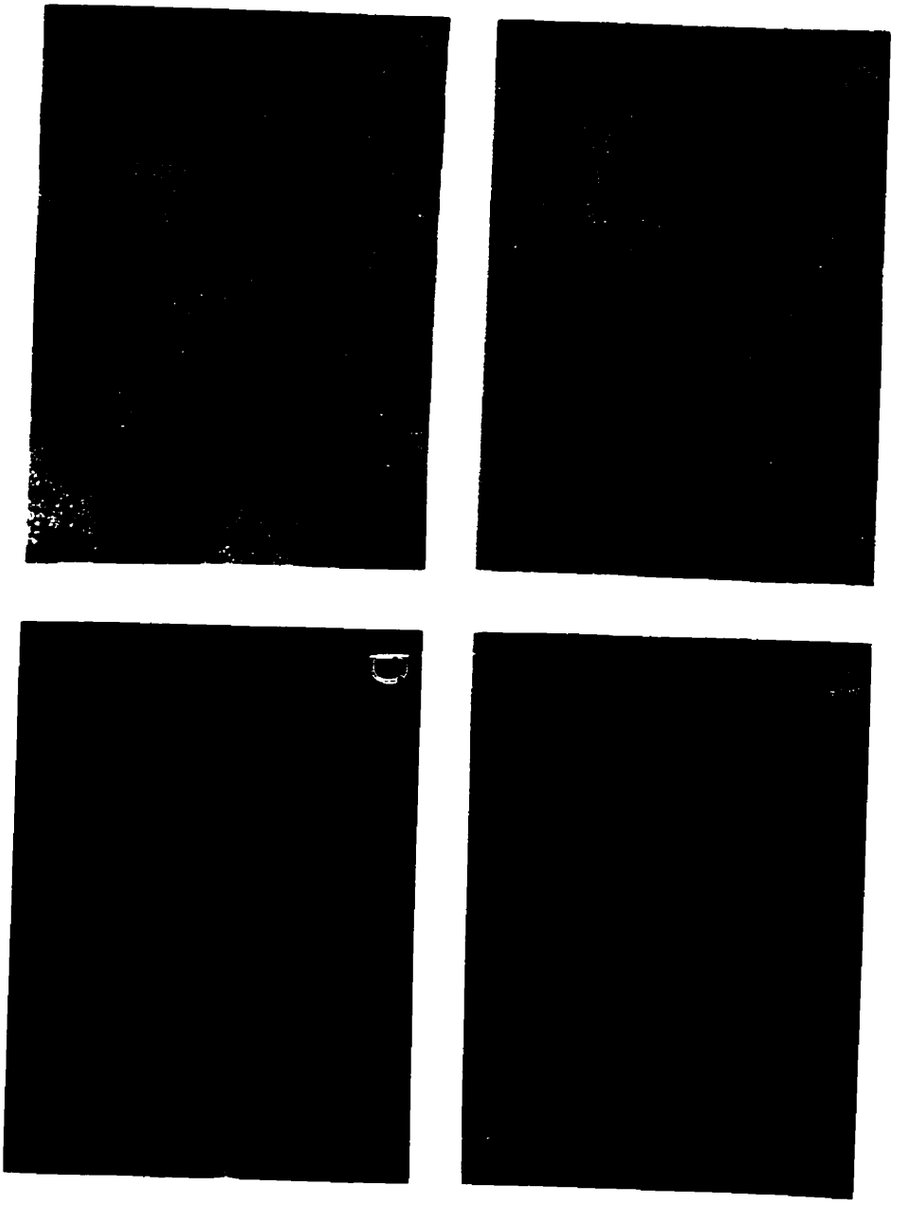


Figure 8. Expression of surface molecules determined by flow cytometry. (A) As positive controls, CD4 was detected on HeLa-T4 cells by flow cytometry with DAKO-T4-FITC. (B) Background level of fluorescence on HeLa-T4 cells with goat anti-mouse IgG conjugated with FITC alone. (C) CD4 could not be detected on astrocytes by flow cytometry with DAKO-T4-FITC. (D) Background level of fluorescence on astrocytes with goat anti-mouse IgG conjugated with FITC alone.

Figure 9. Immunocytochemical staining. Astrocytes or HeLa-CD4 cells on glass coverslips were fixed with 2% paraformaldehyde and incubated with OKT4a (1:100) for 90 min. After removal of unbound OKT4a by three washes in PBS, goat anti-mouse IgG conjugated with rodamine was added. The cells were viewed under fluroresence microscope with 400 x magnification. (A) HeLa-CD4 cells show staining for CD4 on cell membrane while (B) astrocytes were not be stained with OKT4a.

Astrocytes or SK-N-MC cells on glass coverslips were fixed with 2% paraformaldehyde and incubated with anti-GalC (1:200) for 90 min. After removal of unbound anti-GalC by three washes in PBS, goat anti-rabbit IgG conjugated with rodamine was added. The cells were viewed under fluroresence microscope with 400 x magnification. (C) SK-N-MC cells stained for GalC on cell membrane while (D) astrocytes were not stained with anti-GalC.



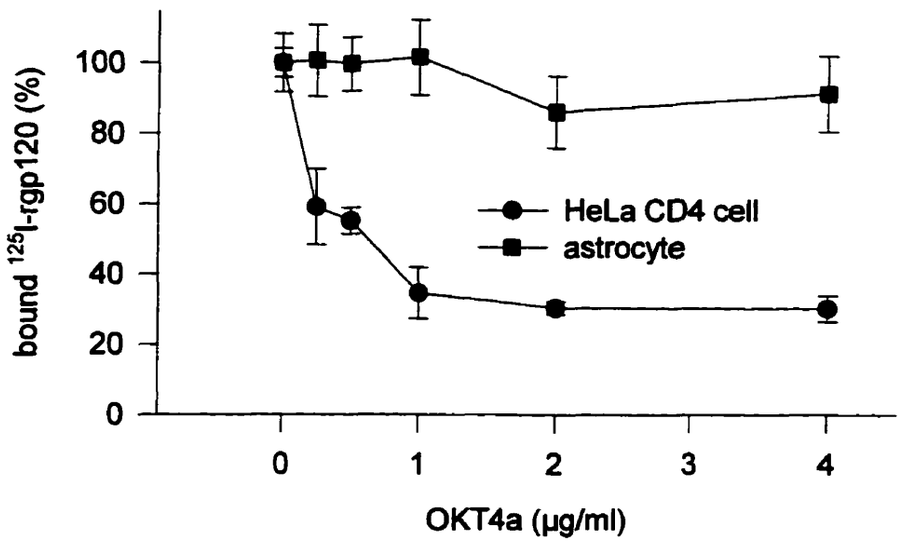


Figure 10. Effect of OKT4a on the binding of ^{125}I-rgp120 to astrocytes and HeLa-CD4 cells. Cells were preincubated with various concentrations of OKT4a for 60 min at 4°C. Following removal of unbound antibody by two washes, 1 nM of ^{125}I-rgp120 was added for 5 hours at 4°C. OKT4a was unable to block ^{125}I-rgp120 binding to astrocytes. The data points represent the mean ± standard error of two independent experiments performed in duplicate.

astrocytes express GalC on the cell surface, we performed immunocytochemical staining on human fetal astrocytes and SK-N-MC cells with rabbit sera against GalC. SK-N-MC cells which express GalC on their surface (Bhat *et al.* 1991) were used as a positive control. SK-N-MC showed membrane staining with anti-GalC (Fig. 9C), while astrocytes showed only background levels of fluorescence (Fig. 9D), indicating that GalC was not detectable on human fetal astrocytes.

To further determine if rgp120 binding on the surface of astrocytes was mediated by GalC, we pretreated the astrocytes with anti-GalC (1:320 to 1:10 dilution) followed by incubation with 1 nM ^{125}I-rgp120. ^{125}I-rgp120 binding without anti-GalC was considered to be 100% binding. SK-N-MC cells were used as a positive control. Inhibition of rgp120 binding to the SK-N-MC cells was inhibited by anti-GalC. A maximal inhibition of 70% ^{125}I-rgp120 binding on SK-N-MC cells was seen with anti-GalC at a dilution of 1:20 (Fig. 11). However, anti-GalC, even with the highest concentration, had no effect on ^{125}I-rgp120 binding to astrocytes. Thus, we conclude that rgp120 binding on astrocytes is GalC-independent.

5. Immunoprecipitation of rgp120 binding proteins

To identify the gp120 binding molecule on the astrocyte membrane, we radio-labelled surface proteins on astrocytes with ^{125}I. The labelled astrocytes were incubated with rgp120 (1 μg) or ^{125}I-rgp120 (0.1 μg) followed by lysing the cells. Protein A-agarose bound with either HIV-1 positive sera or normal sera (selected for high titer antibody to gp120) was added into the cell lysates to coimmunoprecipitate the rgp120 binding protein. A 260

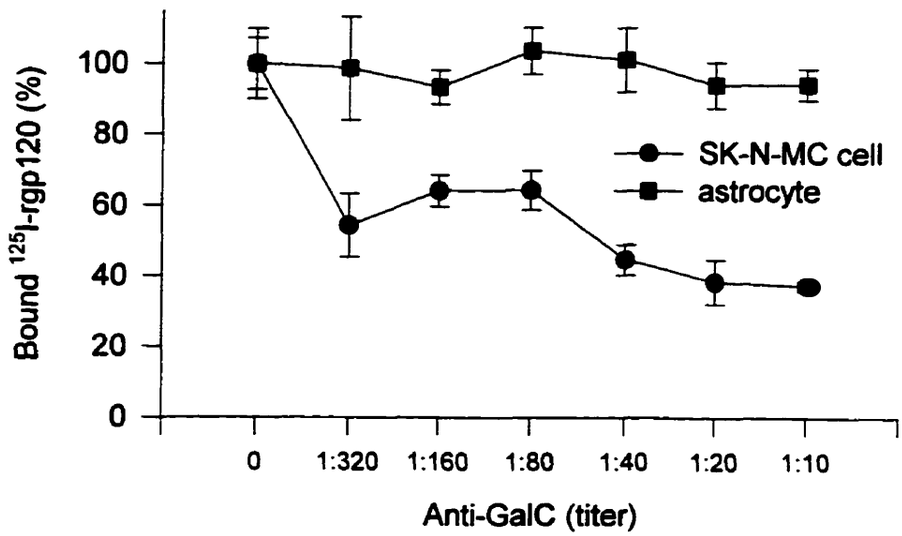
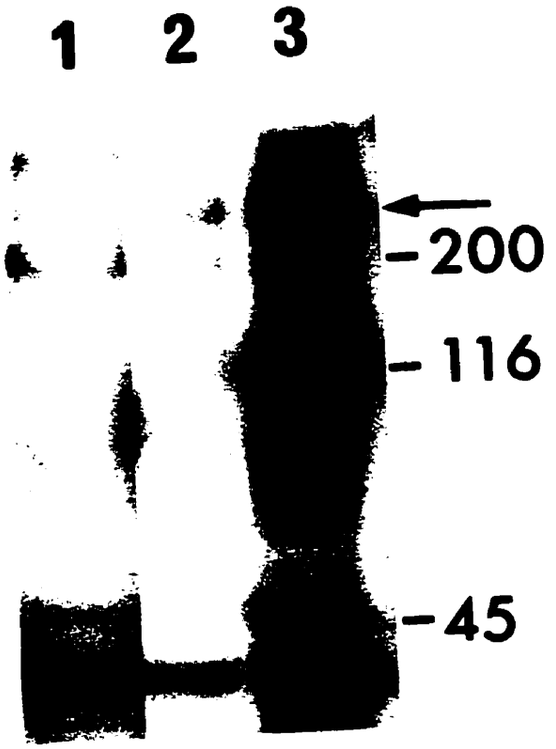


Figure 11. Effect of anti-GalC on the binding of ^{125}I-rgp120 to astrocytes and SK-N-MC cells. Cells were preincubated for 60 min at 4°C with various dilutions of anti-GalC. Following removal of unbound antibody by two washes, 1 nM of ^{125}I-rgp120 was added for 5 hours at 4°C. Anti-GalC antibody did not inhibit ^{125}I-rgp120 binding on astrocytes. The data points represent the mean ± standard error of two independent experiments done in duplicate.

kDa protein was identified as a putative gp120 binding protein (fig. 12). This protein could not be coimmunoprecipitated when anti-gp120 sera were substituted with normal sera. It was also absent in cells not treated with rgp120. Because the binding kinetics were determined with ^{125}I-rgp120, we treated ^{125}I-astrocytes with ^{125}I-rgp120 and coimmunoprecipitated them with anti-gp120 sera. The same 260-kDa protein was again observed, confirming that iodination of gp120 did not alter its binding properties.

Figure 12. Immunoprecipitation of gp120-binding protein. Astrocytes were surface-labelled with ^{125}I and then incubated with rgp120 or ^{125}I-rgp120 prior to detergent extraction and immunoprecipitation with anti-gp120-protein A agarose. Samples were analyzed by SDS-PAGE in 4% to 15% gradient gels followed by autoradiography. Lane 1, ^{125}I-labelled astrocytes only. Lane 2 and Lane 3 ^{125}I-labelled astrocytes incubated with rgp120 or with ^{125}I-rgp120 respectively. A 260 KDa band is seen in lanes 2 and 3 only (arrow). Five fold more protein was added to lane 3 to detect any minor bands in addition to the 260 kDa molecule.


1
2
3
200
116
45

Part II Molecular determinants for the cellular uptake of Tat

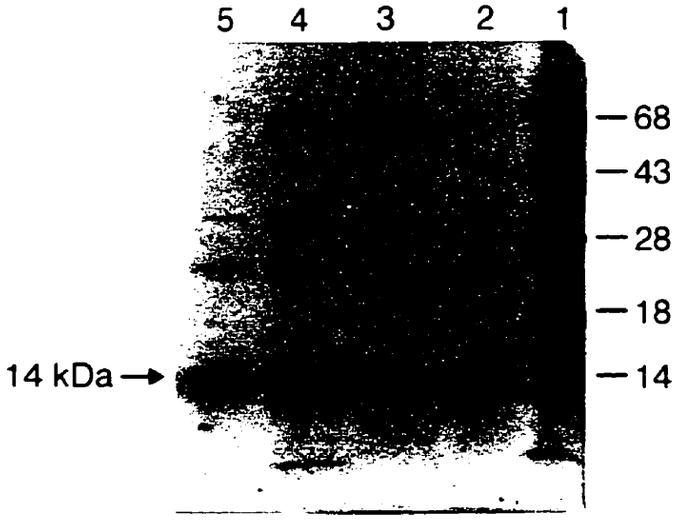
1. Tat1-72 expressed and purified from *E Coli*

Recombinant Tat1-72 was prepared as described as in Materials and Methods. Purified Tat1-72 was analyzed by SDS-PAGE followed by Coomassie blue staining (fig. 13A). A monomeric form of Tat1-72 (14 kDa) represented >90% of the total proteins. A dimeric form of Tat1-72 was present as a minor band (28 kDa). The enzyme Xa-factor which was used to cleave Tat1-72 from bound fusion protein was undetectable by Coomassie blue staining. Western blot analysis with rabbit anti-Tat1-72 serum confirmed the presence of monomeric and dimeric form of Tat1-72 (fig. 13B).

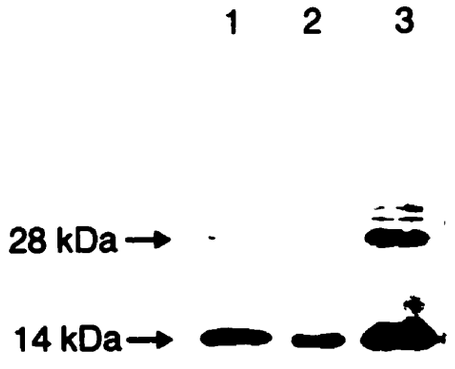
To confirm that purified Tat1-72 was transactivationally functional, we determined its ability to transactivate the HIV-LTR using LTR CAT assay. HL3T1 cells with the LTR-CAT construct were mixed with or without Tat1-72 followed by scrape-loading. For the specificity of Tat1-72 action, Tat1-72 was also pretreated with monoclonal antibody to Tat (kindly provided by Dr. Dawood) for 1 hour at room temperature followed by incubation with HL3T1 cells. After 24 hours incubation, HL3T1 cells were harvested for CAT assay. As shown in figure 14, Tat1-72 produced a 22-fold increase in LTR transactivation which was blocked by monoclonal antibody to Tat. This indicates that the function of recombinant Tat1-72 purified from E. Coli is well retained.

Figure 13. Purity of recombinant Tat1-72. (A). Purified recombinant Tat1-72 was analyzed by 15% SDS-PAGE followed by Coomassie blue staining. Lane 1 is molecular weight markers. Lane 2, 3, 4, and 5 were fractions eluted from the affinity column. The major bands at 14 kDa represent monomeric form of Tat1-72 and minor bands at 28 kDa were dimers of Tat1-72. (B). Western blot analysis of purified Tat1-72. Tat1-72 was transferred to a nylon membrane (Bio-Rad) following SDS-PAGE and incubated with rabbit anti-Tat1-72 serum (1:2000) for 90 min. The unbound antibodies were removed by three washed in PBS-0.05% tween 20. The goat anti-rabbit IgG conjugated with HRP (1:1000) was added to the membrane followed by development with DAB. Lane 1, 2 and 3 represented 0.5 μg, 0.1μg and 1 μg of Tat1-72 respectively. Major bands at 14 kDa and minor bands at 28 kDa were observed.

A



B



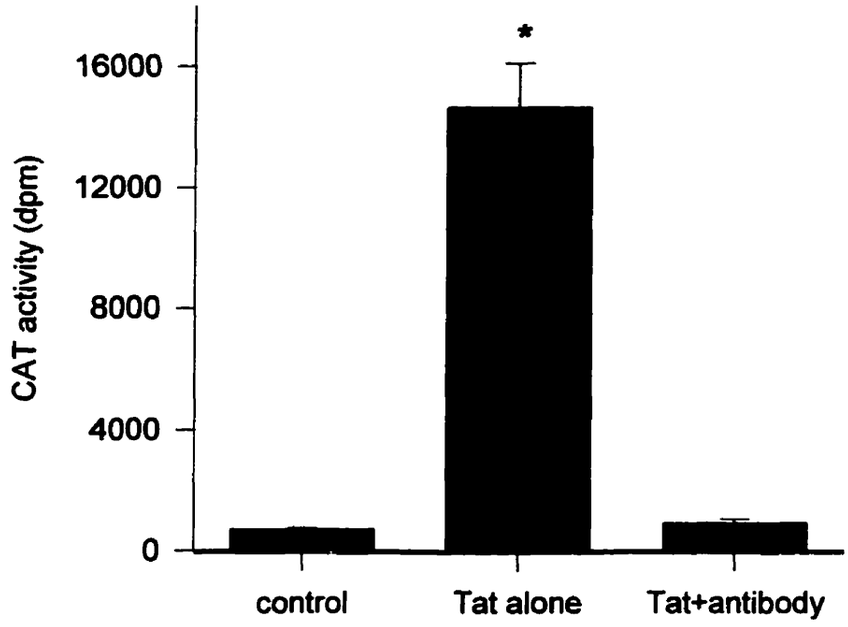


Figure 14. Tat1-72 transactivation activity. 1 μg Tat1-72 in 1 ml medium was scrape-loaded into 1 x 10^6 HL3T1 cells with the LTR-CAT construct and incubated for 24 hours at 37°C. The cells were then harvested for the CAT assay as described in Materials and Methods. Tat1-72 greatly transactivated the LTR-CAT activity (Tat alone) compared to the control group (without Tat1-72 treatment) (* $p < 0.005$, paired Student's *t* test). After Tat1-72 (1 μg) was preincubated with monoclonal antibody to Tat (1:10), transactivation activity of Tat1-72 was blocked completely. The values represent the mean ± standard error of two experiments done in triplicate.

2. Uptake of Tat1-86 and Tat1-72

To compare the cellular uptake of Tat1-86 and Tat1-72 by astrocytes, ^{125}I-Tat1-86 or ^{125}I-Tat1-72 was added to human fetal astrocyte cultures and incubated for 5 min to 4 hours at room temperature. Uptake of both ^{125}I-Tat1-86 and ^{125}I-Tat1-72 was time and dose dependent. However, the uptake of ^{125}I-Tat 1-72 was much lower than that of ^{125}I-Tat1-86 (0.02 ± 0.003 ng versus 0.12 ± 0.001 ng at 4 hours; $p<0.01$) (fig. 15). At maximal concentration (64 nM) of ^{125}I-Tat1-86 or ^{125}I-Tat1-72 tested, the uptake of the Tat could not be saturated (fig. 16). Further, > 90% of internalized ^{125}I-Tat1-86 or ^{125}I-Tat1-72 was localized in the nucleus while the cytoplasmic fraction represented only <10% in the astrocytes (fig. 17). To further confirm the nuclear localization and the fate of Tat within the nucleus, nuclear fractions were prepared from ^{125}I-Tat1-86 or ^{125}I-Tat1-72 treated astrocytes and analyzed by SDS-PAGE followed by autoradiography. ^{125}I-Tat1-86 or ^{125}I-Tat1-72 could be localized as a prominent 15 kDa or 14 kDa band respectively as shown in figure 18A and 18C. By 2 hours, a prominent band for both Tat1-86 and Tat1-72 were detected (fig. 18B and 18D). Bands of smaller molecular weights representing breakdown products of Tat were also noted (fig. 18). Similar results were observed in neurons, NB41 cells and SupT-1 cells (data not shown). All subsequent experiments were done with astrocytes only.

When the ^{125}I-Tat containing solution was pre-absorbed with polyclonal Tat antisera and conjugated with protein A sepharose, cellular uptake of ^{125}I-Tat1-86 and ^{125}I-Tat1-72 decreased by 79 ● 11% and 78 ± 3% respectively (fig. 19), demonstrating that the uptake of Tat was specific.

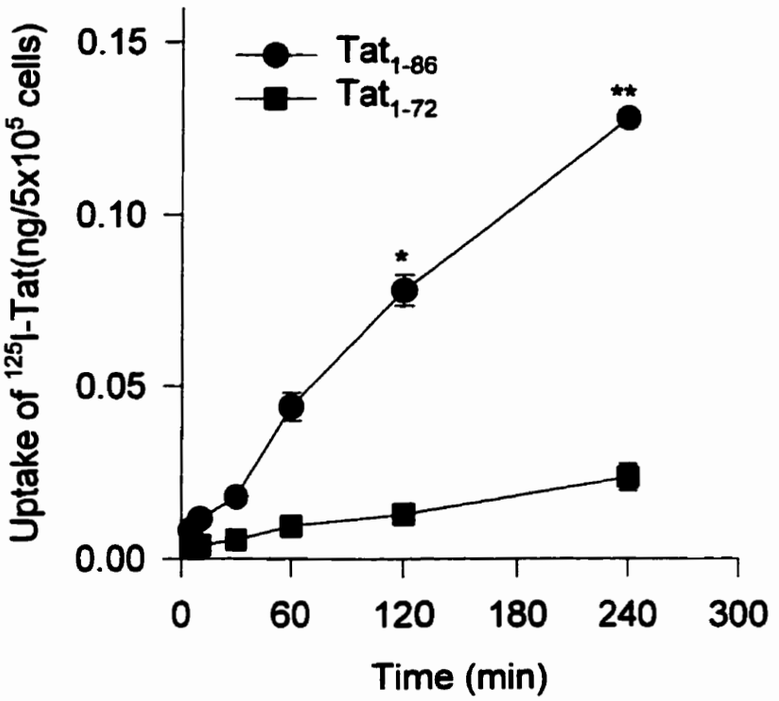


Figure 15. Kinetics of Tat1-86 and Tat1-72 uptake by astrocytes. 10 nM ^{125}I-Tat1-86 or ^{125}I-Tat1-72 in 0.2 ml binding medium was incubated with 5 x 10^5 astrocytes for 5 min to 4 hours. The cells were then washed to remove free ^{125}I-Tat1-86 or ^{125}I-Tat1-72. Nuclear fractions were isolated as described in Materials and Methods. Both ^{125}I-Tat1-86 and ^{125}I-Tat1-72 were internalized by astrocytes. Uptake of Tat1-86 was much more rapid and occurred in larger amounts as compared to Tat1-72 (*$p < 0.01$, unpaired Student's *t* test). Each value represents the mean ± standard error of two experiments, each done in triplicate.

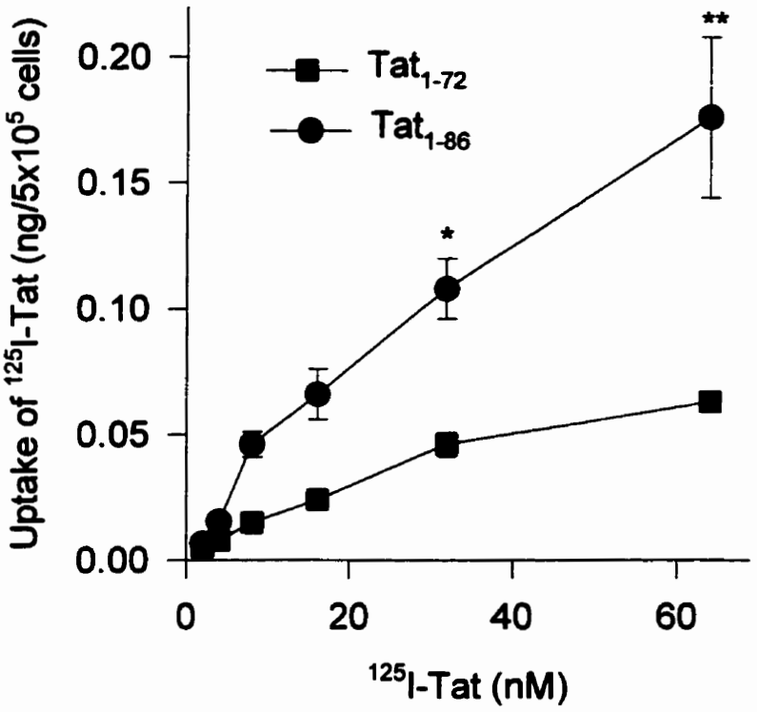


Figure 16. Dose dependency of Tat1-86 and Tat1-72 uptake into astrocytes. The cells were incubated with increasing concentrations of ^{125}I-Tat1-86 or ^{125}I-Tat1-72 for 2 hours. The cells were then washed to remove free ^{125}I-Tat1-86 or ^{125}I-Tat1-72. Nuclear fractions were isolated as described as Materials and Methods. ^{125}I-Tat1-86 and ^{125}I-Tat1-72 were internalized dose dependently. Uptake of Tat1-86 was more efficient (at 64 nM, *$p < 0.01$). Each value represents the mean ± standard error of two experiments, each done in triplicate.

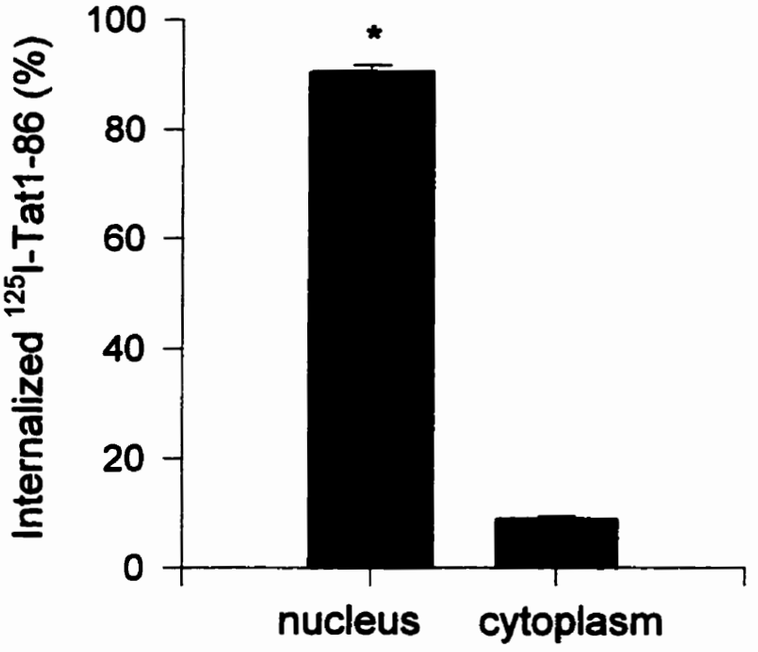
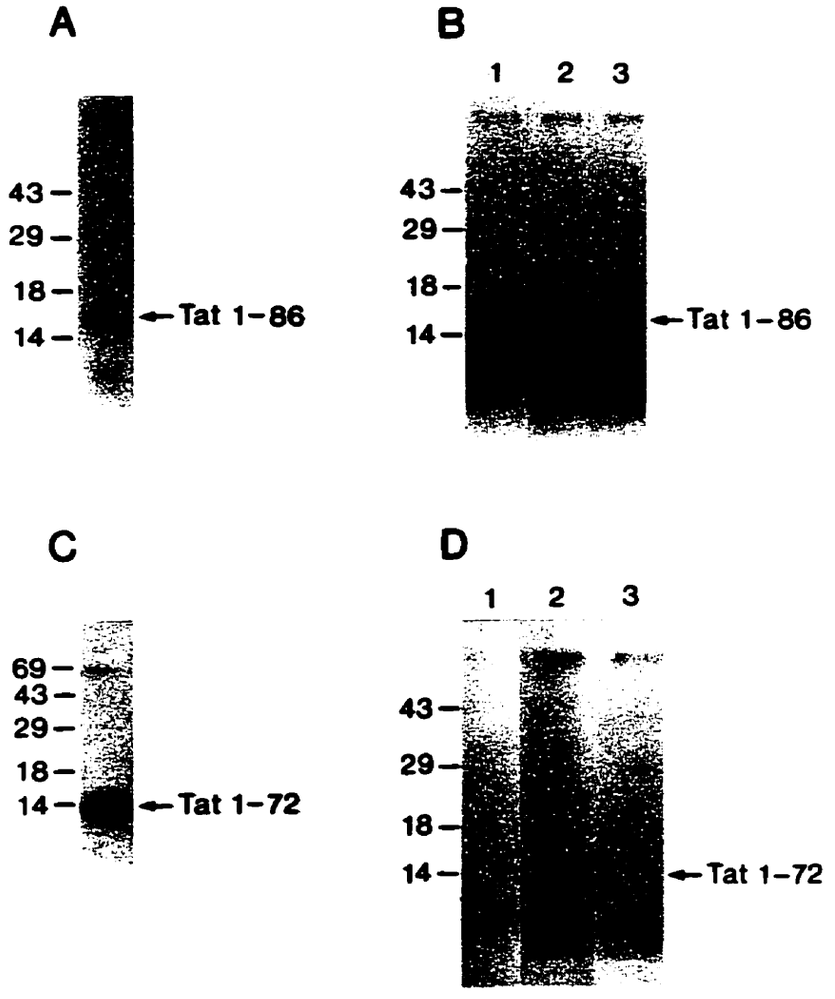


Figure 17. Nuclear localization of internalized Tat1-86. Cells were incubated with 10 nM ^{125}I-Tat1-86 for 2 hours. Cells were washed to remove free ^{125}I-Tat1-86. Nuclear and cytoplasmic fractions were isolated as described as Materials and Methods. Internalized ^{125}I-Tat1-86 was predominantly present in the nuclear fraction of astrocytes (*$p < 0.005$, unpaired Student's *t* test). Each value represents the mean ± standard error of two experiments, each done in triplicate.

Figure 18. Nuclear fraction following Tat1-86 or Tat1-72 uptake analyzed by SDS-PAGE. Astrocytes (5 x 10^5 cells) were incubated with 1 nM ^{125}I-Tat1-86 or ^{125}I-Tat1-72 for 2 hours at room temperature. Cells were washed to remove free ^{125}I-Tat1-86 or ^{125}I-Tat1-72. Nuclear fractions were isolated and resolved by 15% SDS-PAGE as described in Materials and Methods and analyzed by autoradiography. A and C represent ^{125}I-Tat1-86 and ^{125}I-labelled Tat1-72, respectively. B and D show internalized ^{125}I-Tat1-86 (15 kDa) and ^{125}I-Tat1-72 (14 kDa) in the nuclear fraction of astrocytes, respectively. Bands of breakdown products of ^{125}I-Tat1-86 or ^{125}I-Tat1-72 were also observed (B or D). Lane 1, 2, 3 indicate Tat uptake at 0.5 hours, 2 hours and 4 hours respectively.


A
43
29
18
14
Tat 1-86
B
1 2 3
43
29
18
14
Tat 1-86
C
69
43
29
18
14
Tat 1-72
D
1 2 3
43
29
18
14
Tat 1-72

3. Competition of Tat1-86 and Tat1-72 uptake by unlabelled Tat and dextran sulfate

Astrocytes were treated with 10 nM ^{125}I-Tat1-72 in the presence of increasing concentrations of unlabelled Tat1-72 ranging from 200 to 1000 nM. Uptake of ^{125}I-Tat1-72 was inhibited dose-dependently with maximal inhibition (60%) achieved in the presence of 800 nM unlabelled Tat1-72 (fig. 20). To determine if Tat uptake was charge dependent, human fetal astrocytes were treated with ^{125}I-Tat1-86 and ^{125}I-Tat1-72 in the presence of increasing concentrations of dextran sulfate (0.25 μM to 3.2 μM). Dextran sulfate inhibited ^{125}I-Tat1-86 or ^{125}I-Tat1-72 uptake dose-dependently (fig. 21). 80% of inhibition of ^{125}I-Tat1-86 and 75% of inhibition of ^{125}I-Tat1-72 uptake was noted in the presence of 0.4 μM and 1.5 μM dextran sulfate, respectively (fig. 21).

4. Role of integrins $\alpha_v\beta_5$ and $\alpha_5\beta_1$ or $\alpha_v\beta_3$ on uptake of Tat1-86

To determine if the basic region of the Tat49-57 or the RGD sequence in the Tat C-terminal region (72-86), previously shown to bind to integrins $\alpha_v\beta_5$ and $\alpha_5\beta_1$ or $\alpha_v\beta_3$, mediate cellular uptake of Tat, we treated astrocytes with 10 nM ^{125}I-Tat1-86 in the presence of 600-fold higher concentrations of either Tat48-86 containing the RGD sequence, or Tat31-72 containing the basic region. Both peptides failed to inhibit ^{125}I-Tat1-86 internalization in astrocytes (fig. 22).

Conversely, when astrocytes were treated with ^{125}I-Tat1-72 in the presence of Tat peptide31-72, a 5-fold enhancement of Tat uptake was observed ($p<0.01$) while Tat peptide48-86 had no effect on ^{125}I-Tat1-72 uptake by astrocytes (fig. 23).

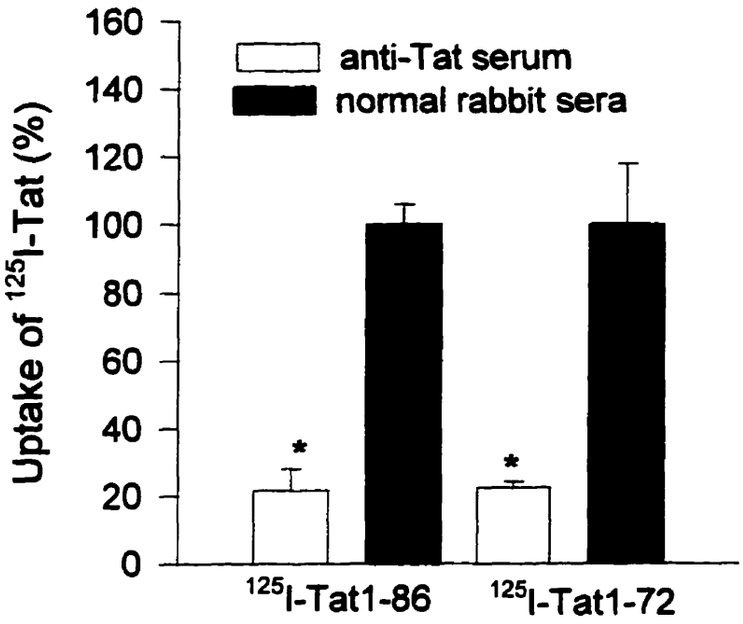


Figure 19. Specificity of Tat1-86 and Tat1-72 uptake by astrocytes. Rabbit anti-Tat sera (1:10) was conjugated to protein A sepharose. After removal of unbound materials, the conjugated beads were then incubated with 10 nM ^{125}I-Tat1-86 or ^{125}I-Tat1-72 in binding medium for 60 min. Control groups used normal sera instead. Supernatants were then used for Tat uptake assay as described in Materials and Methods. Uptake of both of ^{125}I-Tat1-86 and ^{125}I-Tat1-72 was abolished following preabsorption of Tat with polyclonal Tat antisera compared to control (*$p < 0.005$, unpaired Student's *t* test). Each value represents the mean ± standard error of mean of two experiments, each done in triplicate.

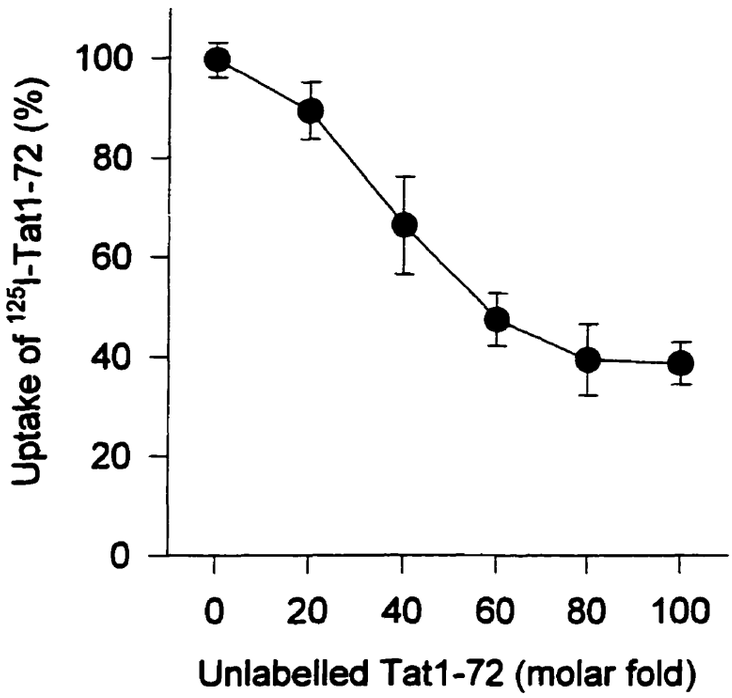


Figure 20. Competition of ^{125}I-Tat1-72 uptake by unlabelled Tat1-72 on astrocytes. Astrocytes (5 x 10^5) were incubated with 10 nM ^{125}I-Tat1-72 in the presence of increasing concentrations of unlabelled Tat1-72 (0 to 1000 nM) for 2 hours. Cells were washed to remove free ^{125}I-Tat1-72. Nuclear fractions were isolated as described in Materials and Methods. ^{125}I-Tat1-72 uptake was inhibited dose-dependently with maximal inhibition (60%) achieved in the presence of 800 nM unlabelled Tat1-72. Each value represents the mean ± standard error of two experiments, each done in triplicate.

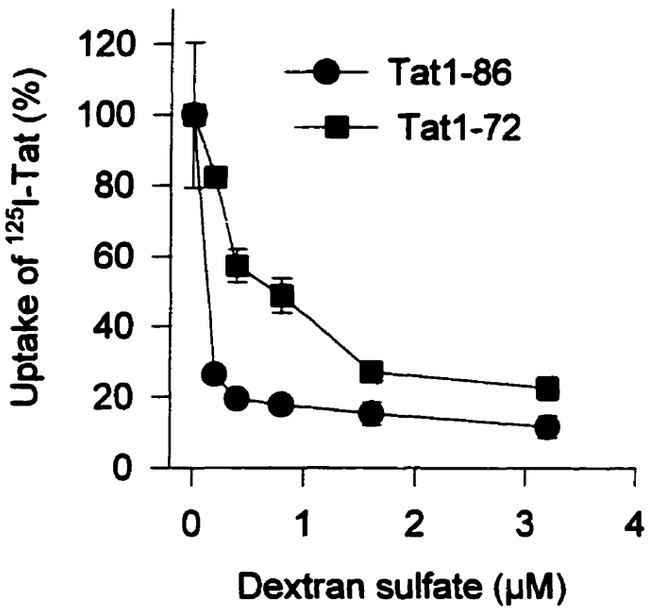


Figure 21. Effect of dextran sulfate on uptake of Tat1-86 and Tat1-72. Astrocytes (5 x 10^5 cells/well) were incubated with 10 nM ^{125}I-Tat1-72 in the presence of increasing concentrations of dextran sulfate (0.2 to 3.2 μM) for 2 hours at room temperature. The cells were then washed to remove free ^{125}I-Tat1-72. Nuclear fractions were isolated as described in Materials and Methods. Each value represents the mean ± standard error of two experiments, each done in triplicate. Dextran sulfate inhibited ^{125}I-Tat1-86 or ^{125}I-Tat1-72 uptake dose-dependently.

5. Effect of 15 mer Tat peptides on Tat1-72 uptake

To further determine the region responsible for the enhancement of Tat1-72 uptake, we incubated ^{125}I-Tat1-72 with astrocytes in the presence of 600-fold excess concentrations of various 15 mer Tat peptides. The peptides, completely spanning the 86 amino acid sequence of Tat were listed in Table 2. A 30-fold enhancement of Tat1-72 uptake was observed by Tat28-42 ($p < 0.005$). Tat23-37 and Tat18-32 also produced a 10-fold ($p < 0.005$) and a 2-fold ($p < 0.01$) enhancement of ^{125}I-Tat1-72 uptake, respectively while the remaining 15 mer peptides had no significant effect (fig. 24). Similarly, Tat72-86, which supplements the deleted region of the second exon, had no effect on ^{125}I-Tat1-72 uptake (fig. 24).

To determine if the internalized Tat1-72 retains its functional properties, transactivation of HIV-LTR was determined in HL3T1 cells containing the LTR-CAT construct treated with 0.5 μM Tat1-72 without scrape-loading in the presence or absence of 6 μM Tat1-15, Tat28-42 or Tat72-86. As shown in figure 25, compared to Tat1-72 alone, transactivation of Tat1-72 was enhanced by 9-fold in the presence of Tat28-42 ($p < 0.005$). Compared to the control (without Tat1-72), Tat1-72 significantly transactivated LTR ($p < 0.05$, paired Student's *t* test). Tat1-15 or Tat72-86, when co-incubated with Tat1-72, did not affect transactivation by Tat1-72. Tat28-42 did not have any transactivation activity.

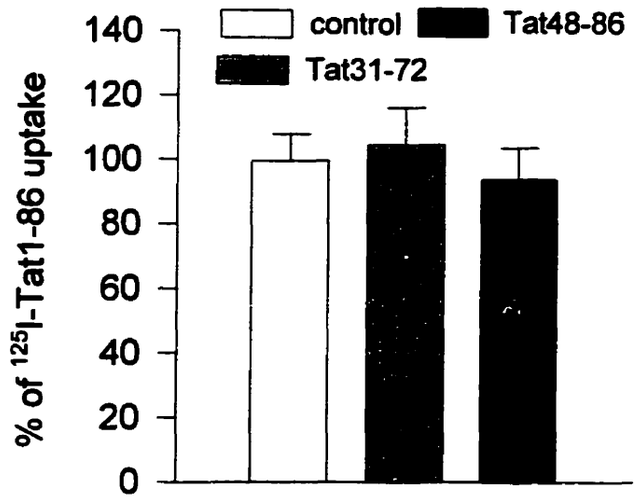


Figure 22. Effects of Tat31-72 and Tat48-86 on internalization of Tat1-86 into astrocytes. Astrocytes (5 x 10^5 cells/well) were incubated with 10 nM ^{125}I-Tat1-86 in the presence of 6 μM Tat31-72 or Tat48-86 for 2 hours. The cells were then washed to remove free ^{125}I-Tat1-72. Nuclear fractions were isolated as described in Materials and Methods. Uptake of ^{125}I-Tat1-86 was not affected by these peptides. Each value represents the mean ± standard error of two experiments, each done in triplicate.

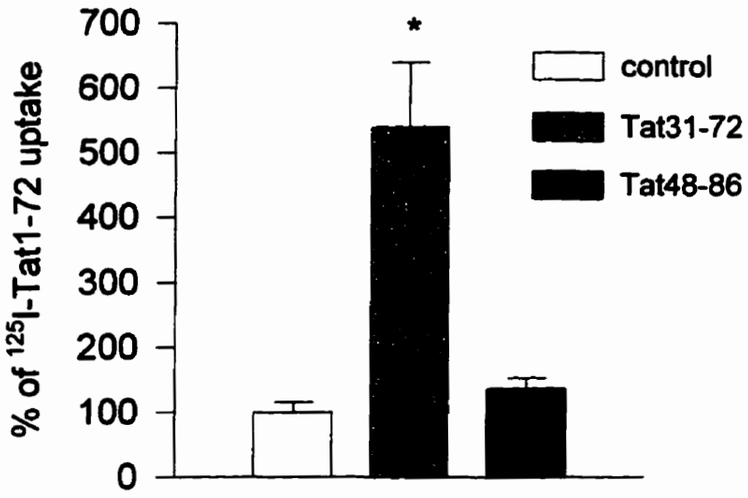


Figure 23. Effects of Tat31-72 and Tat48-86 on internalization of Tat1-72 into astrocytes. Astrocytes (5 x 10^5 cells/well) were incubated with 10 nM ^{125}I-Tat1-72 in the presence of 6 μM Tat31-72 and Tat48-86 for 2 hours. The cells were then washed to remove free ^{125}I-Tat1-72. Nuclear fractions were isolated as described in Materials and Methods. Uptake of ^{125}I-Tat1-72 (10 nM) in astrocytes was enhanced 5-fold (*$p<0.01$, unpaired Student's *t* test) by Tat31-72 (6 μM) while Tat49-86 had no effect. Each value represents the mean ± standard error of two experiments, each done in triplicate.

Figure 24. Effect of 15 mer Tat peptides on Tat1-72 uptake. Astrocytes were treated with ^{125}I-Tat1-72 (10 nM) in the presence of various 15 mer peptides (6 μM each). The unbound ^{125}I-Tat1-72 was removed by three washes. Nuclear fractions were isolated as described as Materials and Methods. ^{125}I-Tat1-72 uptake was enhanced by 30-fold (**$p < 0.005$), 10-fold (**$p < 0.005$) or 2-fold (*$p < 0.05$) in the presence of Tat peptides 28-42, 23-37 or 18-32, respectively while the other 15 mer peptides had no effect. Each value represents the mean ± standard error of two experiments, each done in triplicate.

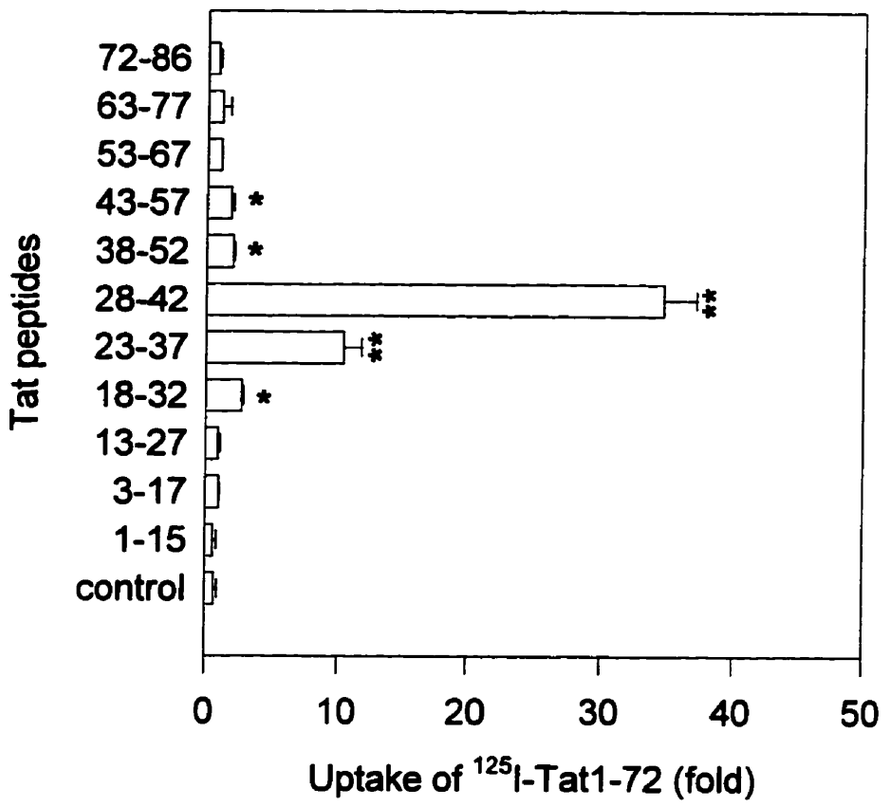

72-86
63-77
53-67
43-57
38-52
28-42
23-37
18-32
13-27
3-17
1-15
control
Tat peptides
*
*
**
**
*
0
10
20
30
40
50
Uptake of 125I-Tat1-72 (fold)

Figure 24

Figure 25. Effects of 15 mer Tat peptides on Tat1-72 transactivation. The HL3T1 cells were treated with 0.5 μM Tat1-72 and 100 μM chloroquine without scrape-loading in the presence or absence of 6 μM Tat1-15, Tat28-42 or Tat72-86. Compared to Tat1-72 alone transactivation of Tat1-72 was enhanced by 9-fold in the presence of Tat28-42 (**$p < 0.005$). Compared to the control (without Tat1-72), Tat1-72 also significantly transactivated LTR (*$p < 0.05$). Tat1-15 or Tat72-86, when co-incubated with Tat1-72, did not affect transactivation by Tat1-72. Tat28-42 did not have any transactivation activity by itself. Each value represents the mean ± standard error of mean from two experiments, each done in triplicate.

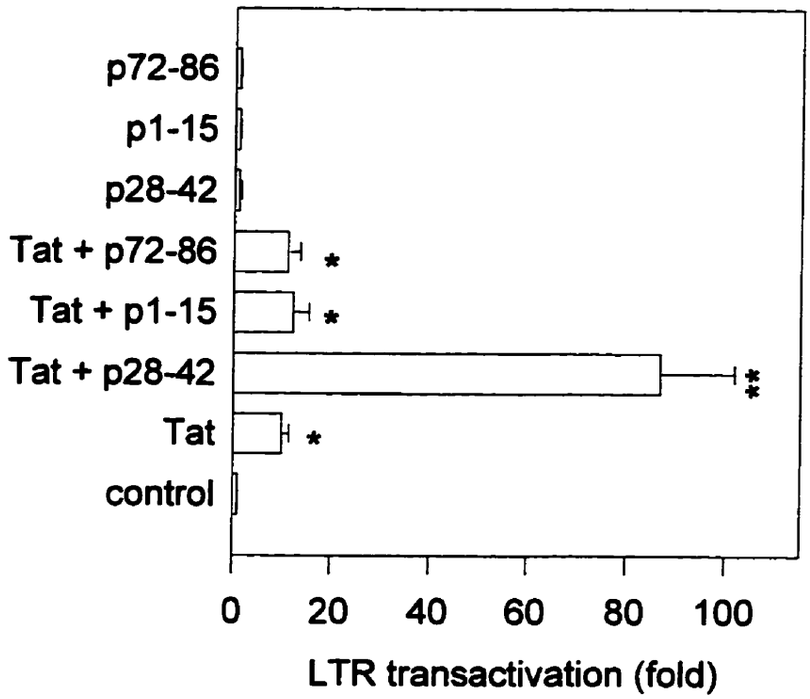

p72-86
p1-15
p28-42
Tat + p72-86
Tat + p1-15
Tat + p28-42
Tat
control
*
*
**
*
0
20
40
60
80
100
LTR transactivation (fold)

Figure 25

Part III Identification of Tat binding proteins

1. Membrane binding of Tat1-72 on U373 cells

To initially examine the membrane binding of Tat1-72, U373 cells on coverslips were treated with Tat1-72 for 2 hours. Unbound protein was removed by repeated washing. The bound Tat1-72 was detected by immunostaining with anti-Tat antibody. Tat1-72 treated U373 cells showed an intensive cell-surface staining (fig. 26A) which was completely absent in cells treated with normal serum instead (26B). The cells did not show any staining if treated with anti-Tat antibody followed by secondary antibody (fig. 26C) or secondary antibody alone (fig. 26D).

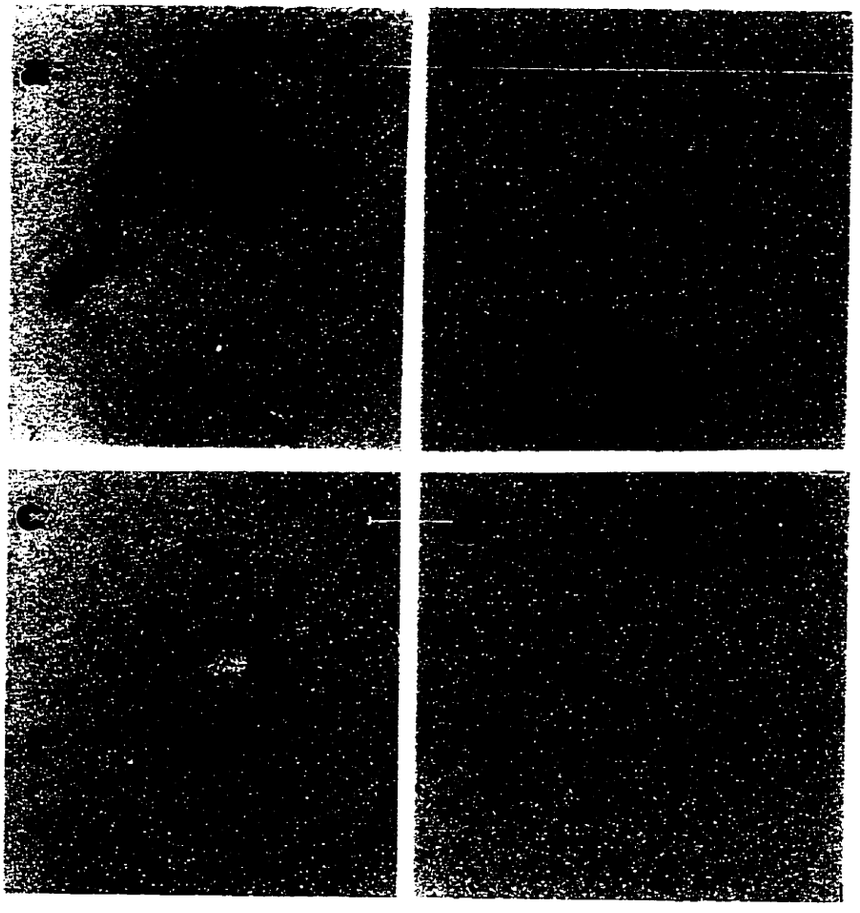
2. Kinetics and Concentration dependency of Tat1-72 binding on astrocytes

To determine the time course of Tat binding on human fetal astrocytes and U373 cells, these cells were incubated with 4 nM ^{125}I-Tat1-72 for 30 min to 5 hours at room temperature. Maximum binding of Tat to both human fetal astrocytes and U373 cells was reached by 2 hours (fig. 27). Similarly, we carried out a dose saturation study in which human fetal astrocytes and U373 cells were incubated with increasing concentrations of ^{125}I-Tat1-72 up to 64 nM for two hours. The binding of ^{125}I-Tat1-72 to human fetal astrocytes and U373 cells was saturated at 24 nM (fig. 28).

3. Specific binding of Tat1-72 on astrocytes

To determine the specificity of Tat binding to the cell membrane, both cell types were incubated at room temperature for 2 hours with 4 nM ^{125}I-Tat1-72 in the presence of

Figure 26. Immunocytochemical detection of Tat1-72 binding to the cell membrane. U373 cells on glass coverslips were treated with 5 μg Tat1-72. After removal of unbound Tat1-72, the cells were fixed with 2% paraformaldehyde and incubated with anti-Tat sera (1:1000) (a) or normal rabbit sera (1:1000) (b) for 90 min. After removal of unbound antibodies by three washes in PBS, goat anti-rabbit IgG conjugated with HRP was added. DAB was used as chromogen. The cells were viewed under a microscope with 400 x magnification. Cells without Tat1-72 treatment were incubated with anti-Tat sera followed by secondary antibody (c) or the secondary antibody alone (d). Only cells treated with Tat-anti-Tat serum (a) showed prominent membrane staining.



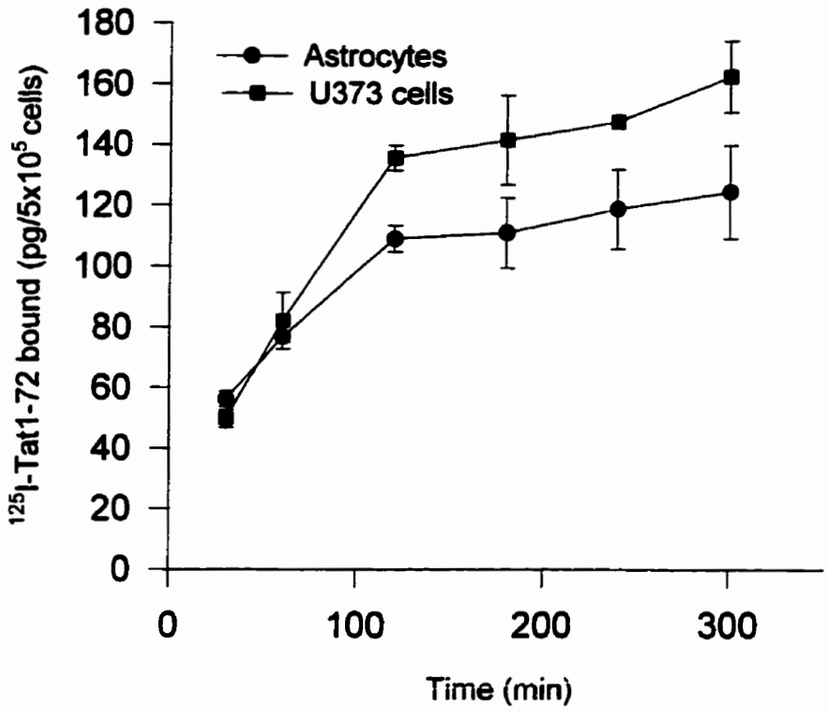


Figure 27. Kinetics of Tat1-72 binding. 5 x 10^5 cells/well were incubated with 4 nM ^{125}I-Tat1-72 for 30 min to 5 hours. After removal of unbound ^{125}I-Tat1-72 by three washes in ice-cold DMEM, membrane-bound ^{125}I-Tat1-72 was obtained by incubation of the cells with trypsin-EDTA for 10 min at 37°C and measured in a gamma counter. The binding on both types of cells was saturated by 2 hours. Each value represents the mean ± standard error of two experiments, each done in triplicate.

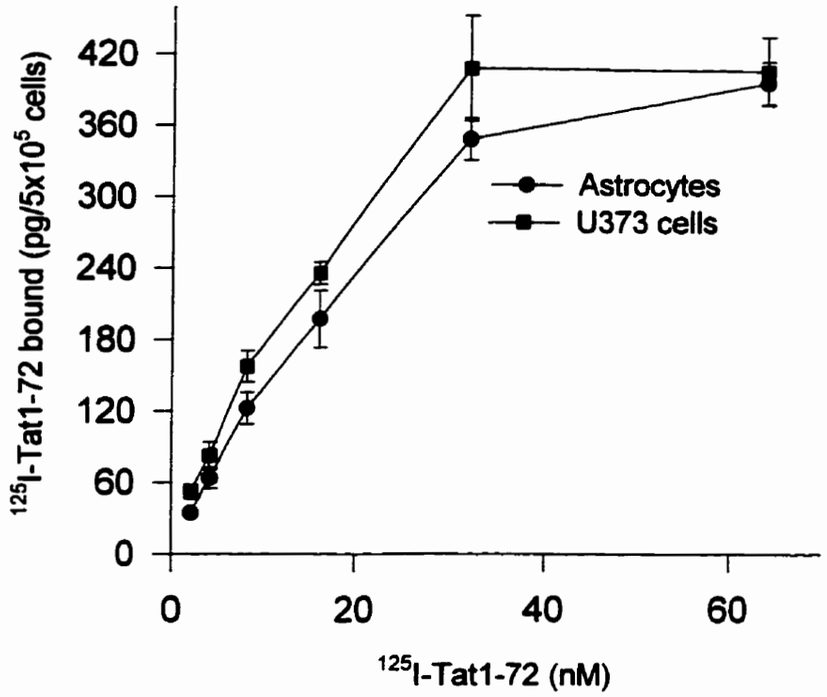


Figure 28. Dose of Tat1-72 binding. 5 x 10^5 cells/well were incubated with increasing concentrations of ^{125}I-Tat1-72 (2 to 64 nM) for 2 hours. After removal of the unbound ^{125}I-Tat1-72 by three washes in ice-cold DMEM, the membrane-bound ^{125}I-Tat1-72 was obtained by incubation of the cells with trypsin-EDTA for 10 min at 37°C and measured in a gamma counter. Tat binding was dose dependent on both human fetal astrocytes and U373 cells and saturated at 24 nM. Each value represents the mean ± standard error of two experiments, each done in triplicate.

increasing concentrations of unlabelled Tat1-72 (0 to 400 nM). In the absence of unlabelled Tat1-72, ^{125}I-Tat1-72 binding was considered to be 100%. Inhibition of Tat1-72 binding on both types of cells was dose-dependent. 75% of ^{125}I-Tat1-72 binding on human fetal astrocytes was blocked by 400 nM unlabelled Tat1-72 (fig. 29) while 50% of ^{125}I-Tat1-72 binding on U373 cells was competed by 400 nM unlabelled Tat1-72 (fig. 29). Thus, the nonspecific binding component on astrocytes was 25% and on U373 cells was 50%. Scatchard analysis of these data revealed that there were $>10^6$ binding sites per cell on astrocytes and U373 cells.

To further confirm that the specific binding of ^{125}I-Tat1-72 on human fetal astrocytes and U373 cells, anti-sera to Tat was pretreated with 4 nM ^{125}I-Tat for 60 min at room temperature. Normal sera were used as a negative control. The mixture of ^{125}I-Tat1-72 and antibodies was then added to the cells for 2 hours. After removal of the unbound ^{125}I-Tat1-72 by three washes in ice-cold DMEM, the membrane-bound ^{125}I-Tat1-72 was obtained and measured as described in Materials and Methods. As shown in figure 30, ~60% of ^{125}I-Tat1-72 binding on human fetal astroyctes and U373 cells was blocked by Tat antibody (1:200) compared to ^{125}I-Tat1-72 binding in cells treated with normal sera ($p < 0.005$).

4. Tat1-72 binding is integrins αvβ3, α5β1 and αvβ5 independent

Integrins αvβ3, α5β1 and αvβ5 are well-known Tat binding proteins. By immunoprecipitation of ^{125}I-surface-labelled human fetal astrocytes and ^{125}I-surface-labelled U373 cells with polyclonal antibodies to αvβ3, α5β1 and β5, we detected

Figure 29. Competition of Tat1-72 binding. Cells were incubated with 4 nM ^{125}I-Tat1-72 in the presence of increasing concentrations of unlabelled Tat1-72 (0 to 400 nM). After removal of unbound ^{125}I-Tat1-72 by three washes in ice-cold DMEM, membrane-bound ^{125}I-Tat1-72 was obtained by incubation of the cells with trypsin-EDTA for 10 min at 37°C and measured in a gamma counter. 75% of ^{125}I-Tat1-72 binding on human fetal astrocytes was inhibited by 400 nM unlabelled Tat1-72 while 50% of ^{125}I-Tat1-72 binding on U373 cells was competed by 400 nM unlabelled Tat1-72. Each value represents the mean ± standard error of two experiments, each done in triplicate.

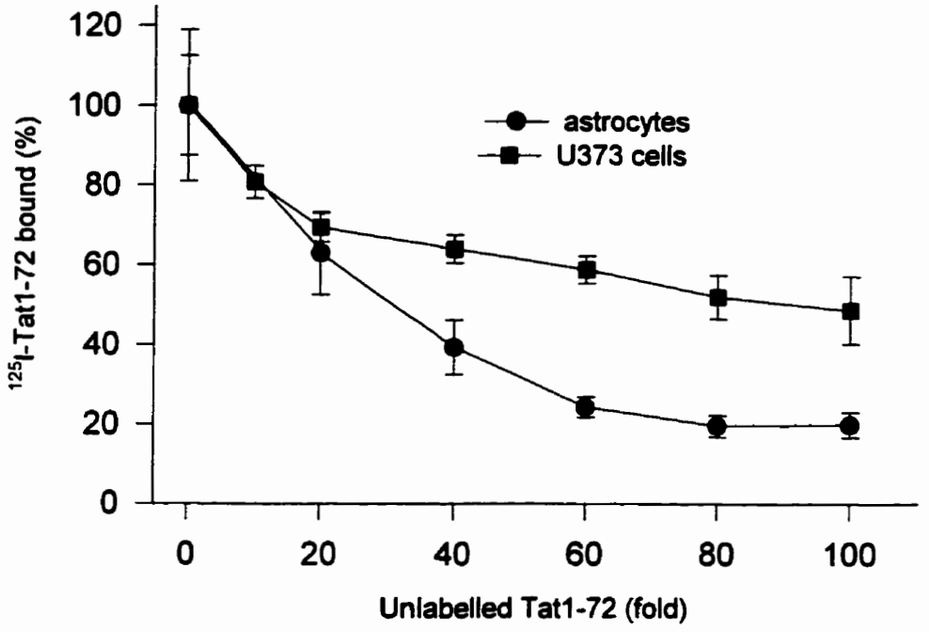

120
100
80
60
40
20
0
^{125}I-Tat1-72 bound (%)
astrocytes
U373 cells
0
20
40
60
80
100
Unlabelled Tat1-72 (fold)

Figure 29

Figure 30. Specificity of Tat1-72 binding. ^{125}I-Tat1-72 (4 nM) was preincubated with polyclonal antibodies (1:200) or normal rabbit sera (1:200) for 90 min. The mixture of ^{125}I-Tat1-72 and antibodies was then added to cells for 2 hours. After removal of unbound ^{125}I-Tat1-72 by three washes in ice-cold DMEM, membrane-bound ^{125}I-Tat1-72 was obtained by incubation of cells with trypsin-EDTA for 10 min at 37°C and measured in a gamma counter as described in Materials and Methods. Anti-Tat antibodies inhibited ~60% of ^{125}I-Tat1-72 binding on both astrocytes and U373 cells compared to normal serum-treated groups (*$p < 0.05$, unpaired Student's *t* test). Each value represents the mean ± standard error of two experiments, each done in triplicate.

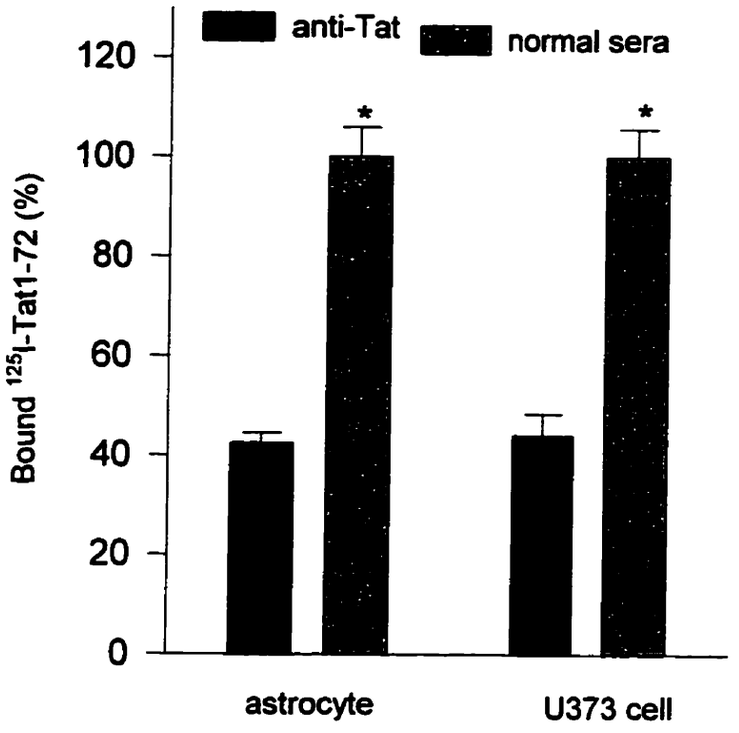

anti-Tat
normal sera
120
100
80
60
40
20
0
Bound ^{125}I-Tat1-72 (%)
*
*
astrocyte
U373 cell

Figure 30

αvβ3, α5β1 integrins on U373 cells (fig. 31A, table 3) and αvβ3 on human fetal astrocytes (fig. 31B, table 3). However, β5 integrin could not be detected on either human fetal astrocytes and U373 cells. αvβ3 is a heterodimer 165/105 kDa. αv subunit has disulphide-linked heavy chain and light chain units with mass of 125 kDa and 24 kDa. α5β1 is a heterodimer with a mass of 160/130 kDa in which the α5 subunit has disulphide-linked heavy and light chains of 135 kDa and 25 kDa, respectively (table 3). Under reducing conditions of SDS-PAGE, the disulphide linkages were broken and no light chain 25 kDa subunits were observed in SDS-PAGE gel because of their molecular mass. Only single bands were observed when U373 cells were immunoprecipitated with ant-α5β1 due to similar molecular masses of two subunits (α5 135/β1 130 kDa) (fig. 31A, table 3). αvβ3 could be observed in bands of 125 kDa and 105 kDa from immunoprecipitation of both U373 cells and human fetal astrocytes (fig. 31, table 3).

Table 3 Tat binding to integrins

Integrins	M.W.(kDa)	U373	Astrocyte	Tat1-72	(RGD)
αvβ3	165/105	+	+	-	+
α5β1	160(135+25)/130	+	-	-	+
αvβ5	125/105	-	-	+	-

It has been shown that the RGD sequence in the C-terminal region of Tat binds to integrins αvβ3 and α5β1. Although we used Tat1-72 for our binding studies which does not contain the RGD sequence, we conducted further studies by pretreating the cells with

antisera to αvβ3, α5β1 and αvβ5 integrins followed by incubation with ^{125}I-Tat1-72. As expected, none of the antibodies inhibited Tat binding on human fetal astrocytes and U373 cells (fig. 32).

5. Enhancement of Tat binding by Tat1-72 peptides

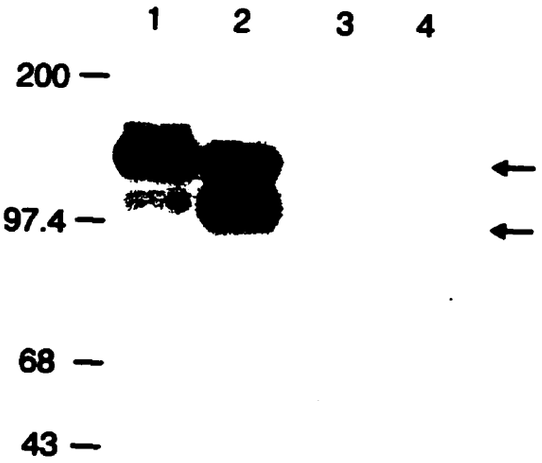
In an attempt to determine the epitope of Tat involved in binding to astrocytes we used 15 mer peptides overlapping by 10 amino acids each and spanning the entire sequence of Tat (1-86 amino acids, table 2) to block the binding of Tat1-72. None of the peptides were able to inhibit Tat1-72 binding even at 600-fold excess concentrations of the peptides. However, ^{125}I-Tat binding on human fetal astrocytes was enhanced up to 4-times ($p < 0.005$) in the presence of Tat28-42 or three-times ($p < 0.005$) in the presence of Tat23-37 (Fig. 33). Tat18-32 and Tat38-52 both produced 2-fold ($P < 0.05$) enhancement of ^{125}I-Tat binding on human fetal astrocytes, while the remaining 15 mer peptides had no significant effect (fig. 33).

6. Immunoprecipitation of Tat1-72 binding proteins

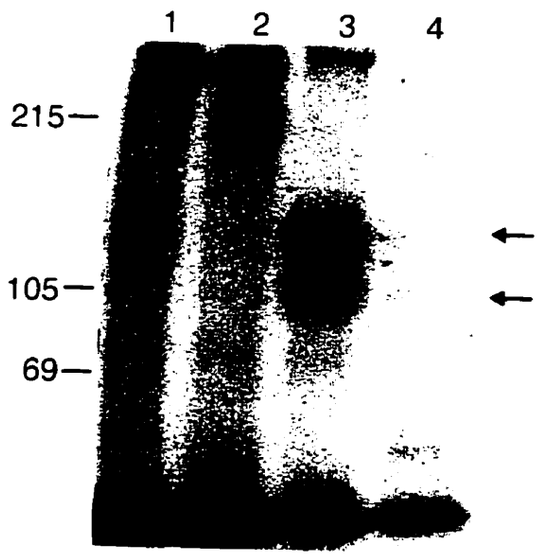
U373 cells were used to isolate the Tat binding protein since the properties of Tat binding on U373 cells were similar to that on human fetal astrocytes. To determine if there were Tat binding proteins on cell surface, U373 cells were surface-labelled with ^{125}I and extracted with cell lysing buffer. The extracts containing labelled surface-proteins were incubated with Tat and immunoprecipitated with anti-Tat sera bound to protein-A agarose and were analyzed by SDS-PAGE. 43 and 35 kDa proteins were detected (fig.

Figure 31. Immunoprecipitation of integrins αvβ3, α5β1 and αvβ5. U373 cells or astrocytes were labelled with ^{125}I and immunoprecipitated with anti-αvβ3 α5β1 or αvβ5 as described in Materials and Methods. Normal rabbit sera was used as a control. (A). Integrins on membranes of U373 cells. α5β1 (lane1) and αvβ3 (lane 2) were detected on U373 cells while β5 (Lane 3) was not detected. Lane 4 represented normal rabbit serum used as a control. (B). Integrins on membranes of human fetal astrocytes. Only αvβ3 (lane 3) was precipitated while α5β1 (lane 2) and β5 (lane 4) were not detected. Lane 1 represented normal rabbit serum control. The numbers indicate molecular weight markers.

A



B



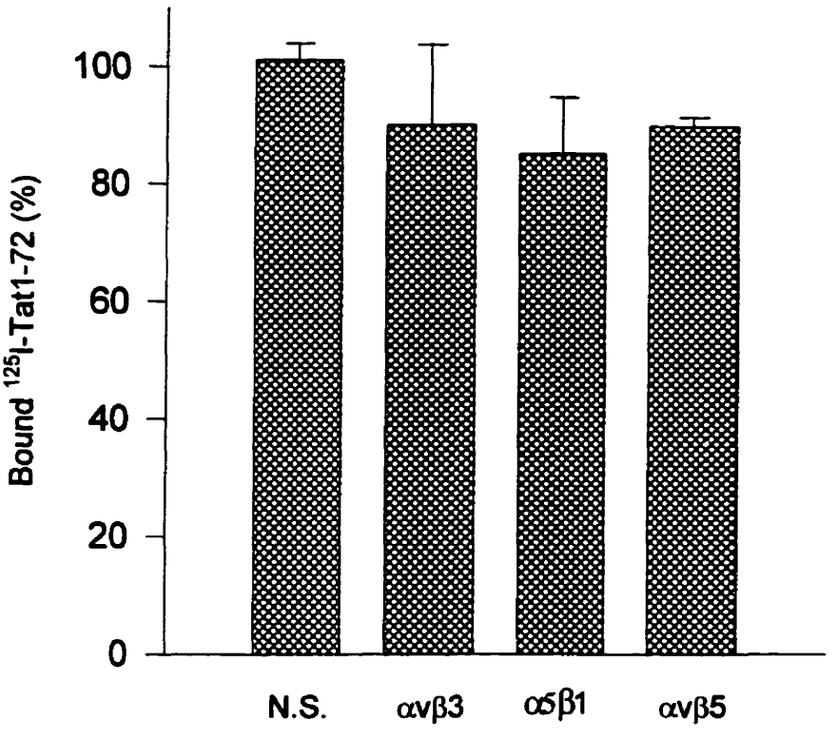


Figure 32. Effects of anti-αvβ3, α5β1, αvβ5 on ^{125}I-Tat1-72 binding. Human fetal astrocytes (5 x 10^5 cells/well) were incubated with 4 nM ^{125}I-Tat in the presence of anti-αvβ3, α5β1, αvβ5 or normal sera (1:200). After removal of the unbound ^{125}I-Tat1-72 by three washes in ice-cold DMEM, membrane-bound ^{125}I-Tat1-72 was obtained by incubation of the cells with trypsin-EDTA for 10 min at 37°C and measured in a gamma counter. None of these antibodies significantly inhibited ^{125}I-Tat1-72 binding. Each value represents the mean ± standard error of two experiments, each done in triplicate.

Figure 33. Effects of 15 mer Tat peptides on Tat1-72 binding. Human fetal astrocytes (5×10^5 cells/well) were incubated with 4 nM ^{125}I-Tat1-72 in the presence of various 15 mer peptides (6 μM each) for 2 hours. After removal of the unbound ^{125}I-Tat1-72 by three washes in ice-cold DMEM, membrane-bound ^{125}I-Tat1-72 was obtained by incubation of the cells with trypsin-EDTA for 10 min at 37°C and measured in a gamma counter. Tat28-42 and Tat 23-37 significantly enhanced the ^{125}I-Tat1-72 binding (**$p < 0.005$) while Tat38-52 and Tat18-32 only slightly enhanced the ^{125}I-Tat1-72 binding (*$p < 0.05$). Each value represents the mean ± standard error of two experiments, each done in triplicate.

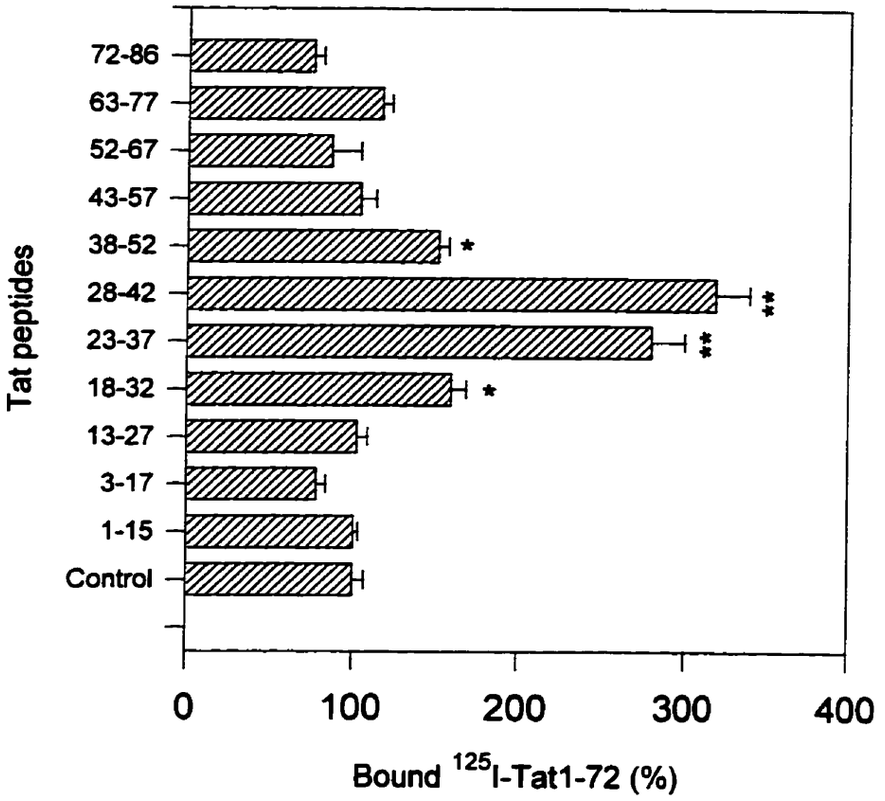

72-86
63-77
52-67
43-57
38-52
28-42
23-37
18-32
13-27
3-17
1-15
Control
Tat peptides
*
**
**
*
0
100
200
300
400
Bound 125I-Tat1-72 (%)

Figure 33

34A). Control samples of the radioiodinated extracts immunoprecipitated with protein A sepharose alone or with protein-A sepharose conjugated to either Tat antisera or normal rabbit sera did not show the above two protein bands. The proteins other than 43 and 35 kDa proteins were also seen in the precipitant with Tat/anti-Tat sera. However, these proteins also appeared on the control groups in the absence of Tat, which were unlikely Tat binding proteins (fig. 34A). To further confirm these two Tat binding proteins, protein extracts of U373 cells were passed over a Tat affinity column. The bound proteins were eluted, lyophilized and radioiodinated with ^{125}I. From ^{125}I-eluted proteins, the 43 and 35 kDa proteins could also be detected by immunoprecipitation with Tat while protein A sepharose alone, Tat/normal sera as controls were not able to detect these two proteins (fig. 34B).

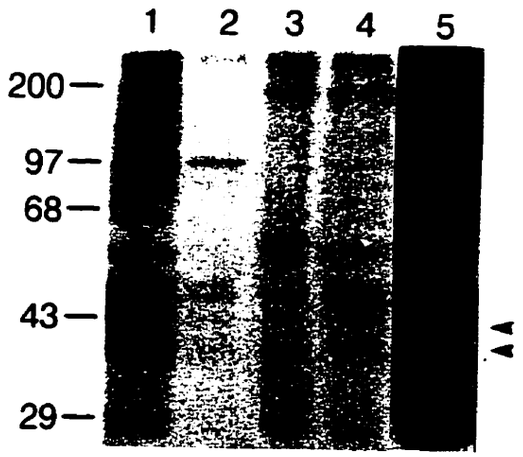
Figure 34. Immunoprecipitation of Tat1-72 binding proteins from U373 cells. The numbers on the left represent molecular mass markers.

(A) Immunoprecipitation of Tat binding protein on surface of U373 cells. The cells were surface-labelled with ^{125}I and lysed with the extraction buffer. The extract was incubated with Tat1-72 followed by addition of anti-Tat antibody (lane 1) or normal rabbit sera (lane 3) pre-conjugated with protein A sepharose beads. The extract was also incubated with protein A sepharose alone in the presence of Tat1-72 (lane 2) or anti-Tat sera conjugated with protein A sepharose beads in the presence of Tat1-72 (lane 4). Lane 5 represents labelled proteins of the extract. Tat1-72 specifically precipitated the 43 and 35 kDa proteins (arrows) (lane 1) which did not appear in the controls (lane 2-4).

(B) Immunoprecipitation of Tat binding protein from the extract of U373 cells. The cell extract from U373 cells was passed through a Tat1-72 column followed by washing the unbound proteins. The bound proteins were eluted, labelled with ^{125}I. The bound proteins were then precipitated with Tat1-72 followed by addition of protein A sepharose beads alone (lane 1), anti-Tat sera (lane 2) or normal rabbit sera (lane 3) conjugated with protein A sepharose beads. The 43 and 35 kDa proteins (arrows) were detected in lane 2.

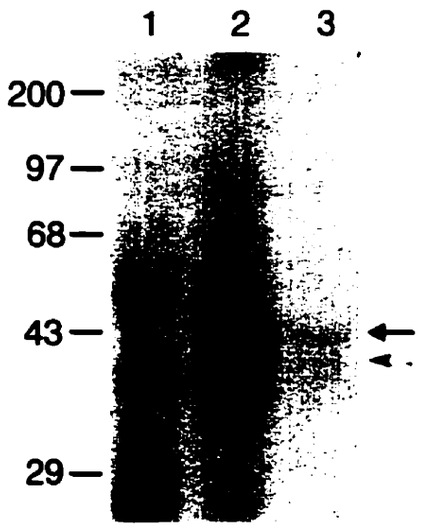
Tat	+	+	+		
anti-Tat sera	+			+	^{125}I cells
normal sera			+		
Protein A	+	+	+	+	

A



Tat	+	+	+
anti-Tat sera		+	
normal sera			+
Protein A	+	+	+

B



V. DISCUSSION

We have studied the interactions of two HIV proteins (gp120 and Tat) with uninfected astrocytes. Both of these proteins are potentially available in the extracellular environment, upon release from infected cells. As discussed earlier, these proteins have been shown in several studies to produce a number of biological effects on brain-derived cells. We have now characterized the membrane interactions of both gp120 and Tat with astrocytes and have shown that they have specific, but distinct mechanisms of interactions with this cell type.

Man is the only natural host for HIV. Hence, the study of interactions of HIV with human astrocytes would be the most applicable to understanding the pathogenesis of HIV encephalopathy. Reliable sources of brain tissue for tissue culture purposes consist of either human fetal brain obtained from therapeutic abortion or adult brain tissue from patients undergoing surgery for epilepsy. However, pure astrocyte culture (> 90%) are best obtained from fetal tissues while adult brain tissues, the source of which is relatively limited, are better used for microglia and oligodendrocyte culture. (Nath and Ma, 1995). Human fetal astrocytes are similar to adult astrocytes in many aspects such as GFAP expression and immune regulation (Furer *et al.* 1993). To exclude the possibility that the small numbers of non-astrocytic cells in our cultures may effect our results, we also used a human astrocytoma cell line for isolated experiments. The observations for both cell types were compared as discussed below.

I. Membrane binding properties of gp120 on astrocytes

1. Recombinant gp120 binds to CD4

The gp120 used in our experiments was expressed and purified from CHO cells. It is important to determine if the molecules were functional prior to binding assay on astrocytes. The best way to do so is to measure the gp120 binding activity to CD4 molecule, since the initial step for HIV-1 infection is gp120 binding to a specific receptor and CD4 is the best characterized receptor for gp120. We employed the two types of cells: SupT cells grown in suspension and HeLa-CD4 cells grown in adhesion, and two different techniques: gp120 binding assay on SupT cells and chemical cross-linking of gp120 to CD4 on HeLa-CD4 cells. The experiments have successfully shown that the gp120 obtained interacted with CD4 molecule expressed on both SupT cells and HeLa-CD4 cells, which indicated that function of gp120 was well retained. However, the ability of gp120 interaction with fusin, a coreceptor for HIV, has not been determined.

2. Gp120 binding specifically on human fetal astrocytes

We have shown that the gp120 binding on astrocytes was both time and dose dependent. To further characterize the gp120 binding site on astrocytes, we used ^{125}I-rgp120 to directly measure the affinity and specificity of binding sites on human fetal astrocytes. Mass law analyses revealed that there was a single high affinity binding site for gp120 on astrocytes with values for the dissociation constant of this binding (Kd) of 26 nM. This Kd of 26 nM is greater than that of gp120-CD4 interaction (2 to 5 nM) (Lasky *et al.* 1987) and gp120-GalC interaction (12 nM) (Harouse *et al.* 1991). These findings suggest that the affinity of gp120 for astrocytes is slightly lower than that for lymphocytes or GalC

expressing cells. Nevertheless, gp120 at nM concentrations has been shown to produce biochemical and morphological changes in astrocytes (Pulliam *et al.* 1993) and at pM concentration causes in intracellular calcium in astrocytes (Nath *et al.* 1996a). Our finding of 4.5×10^5 binding sites on each astrocyte compares favorably to 0.5 to 1×10^4 CD4 receptors per lymphoid cell (Finbloom *et al.* 1991) when taken into account that astrocytes are typically 10-20 times larger than lymphoid cells.

3. Gp120 binding on astrocytes is CD4 independent

The primary receptor for HIV-1 is CD4 (Sattentau and Weiss, 1988). CD4 molecule is expressed on T lymphocytes, monocytes, and macrophages, which serve as targets for HIV-1 infection. Binding of the envelope glycoprotein, gp120 of HIV-1 to CD4 on target cell with strikingly high affinity initiates the cascade of events for viral entry. In brain, infection of microglia has been shown to be mediated by CD4 (Watkins *et al.* 1990; Jordan *et al.* 1991). Chemokine receptors have been discovered as coreceptors of HIV on CD4+ cells. However, the expression and the role of these coreceptors for viral entry in astrocytes have not yet been determined. Nevertheless, HIV-1 infection of astrocytes and other nervous system derived cell lines may proceed via an entry mechanism independent of CD4 (Nath *et al.* 1995; Cao *et al.* 1990; Harouse *et al.* 1989). Several investigators have shown that HIV-1 infection of human glial (Cheng Mayer *et al.* 1987; Bhat *et al.* 1991) and neuronal (Cao *et al.* 1990) cell lines is not blocked by treatment with OKT4a nor is infection blocked by treatment with rsCD4. Thus it now appears unlikely that CD4 antigen is involved in the infection of brain-derived cells except microglia and it has been suggested

that additional HIV-1 receptors are present on neural cells (Lipton, 1991; Kozlowski *et al.* 1991). In the present study using human fetal astrocytes, we were unable to demonstrate the presence of CD4 on human fetal astrocytes by either immunocytochemistry or flow cytometry. Considering the possibility that CD4 may be expressed in levels below the sensitivity of the above techniques, we tried to inhibit the binding of ^{125}I-rgp120 to the astrocyte membrane with OKT4a or rsCD4. In these instances, we were unable to inhibit gp120 binding to astrocytes. We conclude that the CD4 molecule may not be expressed on the cell membrane of human fetal astrocytes and that gp120 binding to astrocytes is not mediated by CD4.

4. Gp120 binding on astrocytes is GalC independent

GalC has been identified as an alternative molecule for gp120 binding. It is expressed on many brain-derived cells such as neurons and oligodendrocytes. HIV-1 infection of a human neuroectodermal tumor derived cell line, SK-N-MC cells, has been shown to occur following binding of gp120 to GalC (Harouse *et al.* 1989; Bhat *et al.* 1991). Here, we were unable to detect GalC on the surface of astrocytes by immunocytochemistry and anti-GalC sera did not inhibit gp120 binding to astrocytes. Further, GalC could not be detected in lipid extracts of human fetal astrocytes by high-performance thin-layer chromatography (Gonzales-Scarano-F, personal communication). These data suggest for the first time that human fetal astrocytes do not express the GalC and the gp120 binding site on astrocytes is not GalC. Therefore, the infection of human fetal astrocytes is most likely mediated by the molecules other than GalC or CD4.

5. Identification of gp120 binding sites on astrocytes

We have shown that the gp120 binding protein on astrocytes has a molecular mass of 260 kDa as opposed to CD4 (58 kDa). In another study, using a glioma cell line, Schneider-Schaulies et al. reported a 180 kDa protein that bound to gp120 (Schneider Schaulies *et al.* 1992). However, the possibility that this protein may have represented a gp120 and CD4 complex (120 + 58 kDa) can not be excluded since glioma cell lines due to malignant transformation may express CD4.

It is generally accepted that a receptor should be saturable and functional. In this study, identification of gp120 binding protein is the initial step to understand the viral entry to astrocytes and gp120 associated alteration of astrocyte functions. However, it was critical to further characterize the 260 kDa protein on astrocytes since exploration of this protein function would strengthen the importance of the findings in this study. In order to do so, generation of antibody to 260 kDa protein or production of recombinant 260 kDa protein would be a key approach. Preliminary data showed that the mouse sera against human fetal astrocytes could inhibit Ca^{2+} influx mediated by gp120 (Nath *et al.* 1996a). However, specificity of the inhibition can not be comfirmed until specific antibody to 260 kDa protein is purified. Further, inhibition of HIV-1 infection by this antibody would conclusively indicate 260 kDa protein on astrocytes as a receptor for gp120. We have thus demonstrated a novel gp120 binding protein on astrocytes which is distinct from previously reported receptors of HIV gp120 (table 3). However, the role of the protein in viral entry into astrocyte needs to be further elucidated. It remains to be determined if there is an

involvement of coreceptor(s). Additionally, since extracellular gp120 has been shown to alter ion transport (Bubien *et al.* 1995; Benos *et al.* 1994; Benos *et al.* 1994), cause an influx of extracellular calcium (J. Geiger, personal communication) and decrease GFAP expression (Pulliam *et al.* 1993), it is possible that some of these effects may be mediated via the 260 kDa molecules identified above.

Table 3 Receptors/coreceptors for gp120

Cell type	Receptor /Coreceptor	Dissociation Constant (Kd)	References
Lymphocyte	CD4 (58 kDa) /CXCR4 (46 kDa)	2 -5 x 10^{-9} M /unknown	Sattentau and Weiss 1988 / Feng *et al.* 1996
Macrophage	CD4 (58 kDa) /CKR5 (41 kDa)	2 -5 x 10^{-9} M /unknown	Sattentau and Weiss 1988 / Cocchi *et al.* 1996
SK-M-NC	GalC (lipid) /unknown	11.6 x 10^{-9} M	Harouse *et al.* 1991
Astrocyte	Protein (260 kDa)* /unknown	26 x 10^{-9} M	Ma *et al.* 1994

* gp120 binding protein

II. Tat can be taken up by astrocytes

1. Recombinant Tat1-72 retains its functional properties

Tat protein purification is difficult because of the unusual features of the protein, it contains a basic region with two lysines and six arginines in a span of 9 amino acids, and seven cysteines within 16 amino acids (fig. 2). The cysteines bind zinc and cadmium ions and may also form disulfide bridges. Purification under reducing conditions prevents oligomers due to disulfide bond formation, but may also prevent metal ion interactions with the cysteine residues. Further, non-specific interaction with the matrix due to the basic properties of Tat also enhances the protein purification difficulty. We have now

developed a purification technique that provides a highly pure Tat protein predominantly in monomeric form as determined by protein electrophoresis and western blot assay. In our purification procedure, DTT was used as a reducing reagent to prevent Tat oxidization. Thus, it is important to determine if Tat purified from *E coli.* remains functional. We employed the HIV-LTR-CAT assay to measure the Tat biological function. The assay showed the purified Tat can efficiently transactivate the integrated HIV-LTR-CAT plasmid in HeLa cells, indicating the Tat is biologically functional.

We chose to synthesize recombinant Tat protein containing only the first exon since we had previously determined that the neurotoxic domain of Tat is contained in this region (Tat31-61) (Nath *et al.* 1996a). Further, the first exon is relatively conserved between different strains of HIV, while the second exon is highly variable in sequence and length (Myers *et al.* 1996). We thus compared the properties of Tat1-72 and Tat1-86 for interaction with astrocytes as discussed below.

2. Tat1-72 uptake is likely receptor mediated

Understanding the precise mechanisms underlying the internalization of extracellular Tat is not only important in determining its role in regulating viral and host function, but this phenomenon could also be potentially exploited to deliver heterologous proteins or drugs into cells. We demonstrate here that both full length Tat (1-86) and to a lesser degree Tat1-72 are internalized by brain cells and following entry, are predominantly localized in the nucleus where they get degraded. The mechanism and significance of this degradation remains to be determined. Consistent with previous

observations that Tat uptake in lymphoid and monocytoid cells could be inhibited by polyanions such as heparin or dextran sulfate (Mann and Frankel, 1991), we found that Tat1-72 uptake in astrocytes could also be blocked by dextran suggesting that internalization of Tat is charge dependent. However, Mann et al. also determined that Tat binds to several cell types with $>10^7$ sites/cell, leading to the conclusion that Tat uptake occurred via a non-specific pathway (Mann and Frankel, 1991). We now demonstrate that uptake of ^{125}I-Tat1-86 and ^{125}I-Tat1-72 by human fetal astrocytes was competitively inhibited by excess of unlabelled Tat1-72 dose dependently, suggesting that uptake of ^{125}I-Tat1-72 may be receptor mediated.

3. Tat uptake is C-terminal region dependent

The *tat* gene has two exons encoding proteins of 72 amino acids and 86 amino acids (Sodroski *et al.* 1985; Arya *et al.* 1985). Transactivation of HIV-2 Tat requires full length protein (Pagtakhan and Tong Starksen, 1995) while the HIV-1 Tat1-72 has the same efficiency for transactivation as HIV-1 Tat1-86 (Green and Loewenstein, 1988). The role of the C-terminal region formed by the second exon of *tat* gene in HIV-1 infection had not been determined. We observed that even though both Tat1-86 and Tat1-72 could be internalized by several cell types, Tat1-86 internalization was up to 10 times more efficient than that of Tat1-72, suggesting that the region encoded by the second exon is important in mediating Tat internalization. However, Tat1-72 uptake was not affected by co-incubating in the peptides derived from the second exon (Tat72-86 or Tat48-86). Thus, peptide bond linkage of the peptides encoded by the two exons is

essential for efficient uptake of Tat. This linkage most likely influences the tertiary configuration of the molecule. The importance of the tertiary configuration for Tat uptake is further supported by the observation that Tat1-86 lost most of its uptake ability following heat treatment. Further, Bonifaci et al. have shown that the Tat molecule unfolds before entering the cells (Bonifaci *et al.* 1995). However, the peptide encoded by the second exon is not exclusively involved in Tat uptake since significant uptake of Tat1-72 was also observed. Previous studies have also suggested that the basic region of Tat may play a role in its internalization (Mann and Frankel, 1991). However, we observed that Tat peptides containing the basic region could not block Tat1-72 or Tat1-86 uptake. Hence, the precise role of this region of Tat remains unclear.

Tat1-72 is highly conserved between different strains of HIV-1. However, the second exon shows heterogeneity in the amino acid sequence and is of variable length. Since this region is a major determinant of Tat uptake into the cell, it may play an important role in regulating the strain to strain variability of the intra- versus extracellular action of Tat on the host cell and hence effect virulence. We have previously shown that extracellular Tat causes neurotoxicity by acting on cell surface excitatory amino acid receptors (Magnuson *et al.* 1995) and the neurotoxic epitope of Tat resides in the first exon (Nath, et al., 1996a). It is thus possible that Tat molecules with low cellular uptake as determined by their second exon might be more neurotoxic since higher levels will be achieved extracellularly.

4. Tat1-72 uptake is independent of integrin $\alpha_5\beta_1$, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ binding

Tat1-86 has an RGD sequence located in the second exon (Barillari *et al.* 1993). Integrins, $\alpha_5\beta_1$ and $\alpha_v\beta_3$, function as receptors for Tat and mediate Tat effects on Kaposi sarcoma cells or cytokine-activated endothelial cells (Ensoli *et al.* 1994) by binding to the RGD sequence of the protein. Further, 12 mer peptides containing the basic region of Tat have been shown to bind to another integrin molecule $\alpha_v\beta_5$ (Vogel *et al.* 1993). However, it is unlikely that binding of Tat to these integrin receptors influences Tat uptake since Tat49-86 containing the RGD sequence, Tat31-62 containing basic domain or antisera to integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ could not inhibit Tat uptake.

5. Enhancement of Tat1-72 uptake by Tat28-42

Tat38-58 and other basic peptides have been shown to significantly enhance Tat uptake and transactivation (Mann and Frankel, 1991). The mechanism of the enhancement has not been understood yet. It was believed that these basic peptides allow Tat to enter cells via unknown pathways. However, our results have shown that Tat28-42 also greatly increased Tat1-72 uptake and transactivation. This peptide contains only four basic amino acids and increased Tat uptake by 35 fold, while Tat38-52 containing five basic amino acids had no significant effect on Tat uptake. This indicates a mechanism of the enhancement other than positive charge dependence might also be involved. The phenomenon by which one protein facilitates internalization of the another protein has also been observed in anthrax toxins. The protective antigen of anthrax toxin can form ion-conductive channels in biological membranes and convey the edema factor and lethal factor of the toxin into the cytoplasm by inducing changes in the membrane permeability

(Milne *et al.* 1994). Presumably, the Tat peptides may use a similar mechanism to facilitate internalization of Tat protein.

One of the unique features of Tat is that Tat can be taken up by many types of cells. This led us to exploit the potential application for Tat-drug delivery into cells (Miyazaki *et al.* 1992; Fawell *et al.* 1994; Frankel *et al.* 1988). Tat1-72 was shown to deliver big proteins such as β-galactosidase, RNAse into cells when chemically conjugated with each other (Fawell *et al.* 1994). However, Tat has a cytotoxic effect to lymphocytes (Benjouad *et al.* 1993) as well as neurotoxicity (Sabatier *et al.* 1991, Nath *et al.* 1996a). These toxic effects will definitely limit the Tat application for drug delivery. We have previously shown that the 15 mer Tat peptides were not neurotoxic (Nath *et al.* 1996a). Much lower amounts of Tat may be used in the presence of Tat28-42, which can enhance Tat uptake 30 fold, to achieve the similar effects on drug-delivery. Subsequently toxicity of Tat may be reduced. Furthermore, Our results have shown that Tat1-86 uptake was not affected by Tat28-42. Tat1-72 uptake into cells, however, could be regulated by Tat peptide (Tat28-42), thus if Tat1-72 were to be used for drug delivery its uptake could be modulated by the presence of peptide 28-42, which make Tat1-72 more favorable for this application. Further studies are required to confirm this hypothesis *in vitro* and *in vivo* since enhancement of Tat uptake may also increase the toxic effects of Tat on cells.

Tat can be released from HIV-1 infected cells. Subsequently the extracellular Tat can be taken up by non-infected cells and localized mainly in nuclei. Some effects of Tat on cellular functions might be mediated by this process. It is important to study the mechanism(s) of Tat uptake which may help in understanding the role of Tat in

pathogenesis of HIV-1 dementia. Previous study has shown that Tat internalization was mediated by endocytosis (Mann and Frankel, 1991). Here, we further conclude that the cellular uptake of Tat is determined by the tertiary configuration of the molecule and is dependent upon the C-terminal region and the basic region of Tat and may be independent of integrin binding. Moreover, Tat uptake may be enhanced by an autologous peptide through yet unknown mechanisms.

III Tat binding to astrocyte surface

1. Specific binding of Tat1-72 on astrocytes

It was found that extracellular Tat released from HIV-1 infected cells specifically affects several functions of astrocytes such as increases in NF-kB binding and protein kinase C activity (Conant *et al.* 1996, Taylor *et al.* 1995), stimulation of transforming growth factor β-1 (Cupp *et al.* 1993), alteration of normal organization (Koken *et al.* 1994) and stimulation of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Chen et al. 1997). It is thus important to understand the mechanism of Tat interaction with astrocytes because of their possible relevance to neurological disorders associated with HIV-1 infection. Naturally, two forms of Tat are produced: major form, Tat1-86 and minor form, Tat1-72. In this study, Tat1-72 was used due to following reasons: first, Tat1-72 is conserved from strain to strain while the second exon of Tat1-86 is variable. So study of Tat1-72 from one strain may help us understand entire picture of Tat from most strains. Second, Tat1-72 has a number of effects on astrocytes by acting on the cell

membrane such as increases in both NF-kB binding and protein kinase C activity, changes in intracellular calcium and stimulation of IL-1β expression in astrocytes. Third, in contrast to Tat1-86 which is predominantly taken up by cells, Tat1-72 is predominantly localized outside cells, which may favor for Tat1-72 action on astrocyte membrane (Ma and Nath 1997). The findings reported here identify a novel interaction between HIV-1 Tat and the astrocyte membrane. We have shown that Tat binding to both human fetal astrocytes and U373 cells is saturable and time dependent. We have also shown that Tat can bind specifically to astrocyte and U373 membranes by immunocytochemistry staining and ^{125}I-Tat1-72 binding assay although there are $>10^6$ binding sites per human fetal astrocyte or U373 cell. Based on our binding data and Scatchard plot analysis, it would seem that these large number of binding sites would suggest that Tat binds "nonspecifically" to the cell membrane. This is predictable due to the positive charge of Tat as reported previously (Mann and Frankel, 1991). The negative charged polysaccharide on the cell surface is not uncommon to serve as receptor for microbial pathogens. For example, heparan sulfate serves as the binding site for Herpes Simplex virus (Wudunn and Spear, 1989, J.V), Bordetella pertussis (Menozzi et al 1991), Chlamydia Trachomatis (Zhang, et al cell 1992) and Leishmania (Mukhopadhyay et al 1989, Butcher et al 1990). Trypanosoma cruzi also express a 60 kDa protein that binds heparan sulfate (Ortega-Barria and Pereira, 1991). We undertook further studies to determine if there might be other molecules on the cell membrane of astrocytes that may interact with Tat.

2. Binding of Tat1-72 on astrocytes is integrins independent

Integrins are a family of cell surface receptors that exist as heterodimers composed of non-covalently associated α and β subunits. Integrins are expressed by a wide variety of cells and bind to a variety of extracellular matrix proteins (eg, collagen, laminin, fibronectin) and other proteins (HIV-1 Tat). Following interaction with a ligand, integrins translate signals outside the cell to alter cellular function. Tat binds to integrin α5β1 and αvβ3 via the RGD sequence and to αvβ5 by its basic region (Vogel *et al.* 1993; Ensoli *et al.* 1990). Interaction between Tat and these integrins may explain some of Tat effects on cells such as enhancement of cell attachment (Vogel *et al.* 1993) and growth of Kaposi's sarcoma cells (Barillari *et al.* 1993; Ensoli *et al.* 1994). We have shown that integrins α5β1 and αvβ3 are expressed on U373 cell surface while αvβ3 is also present on human fetal astrocytes. αvβ5 could not be detected on either fetal astrocytes or U373 cells. These finding clearly indicate that interactions between Tat and astrocytes is not mediated via integrins αvβ5, α5β1 and αvβ3 since the Tat1-72 used in the present study does not have the RGD sequence and integrin β5 is not present on astrocytes. That Tat1-72 binding to astrocyte surface independent on integrins was further confirmed by anti-αvβ5, α5β1 and αvβ3 antibodies. Thus, we conclude that Tat1-72 binding to human astrocytes and U373 cells is integrins independent.

3. **Enhancement of Tat1-72 binding by Tat28-42 and 23-37**

A previous report suggested a role for the basic amino acids in enhancing Tat transactivation of lymphoid cells (Mann and Frankel, 1991). However, in our study,

Tat28-42 (KKCCFHCQVCFTTKA) and Tat23-37 (TNCYCKKCCFHCQVC) which contained only four and three basic amino acids respectively, significantly enhanced Tat protein uptake and transactivation of HIV-LTR. This effect was much greater than that of

Table 4 Tat binding proteins

Names		Location	Molecular Mass (kDa)	References
26S protease	TBP1	Nucleus	45	Nelbock *et al.* 1990
	TBP7	Nucleus	47	Ohana *et al.* 1993
	SUG1	Nucleus	44	Swaffield *et al.* 1992
	MSS1	Nucleus	45	Shibuya *et al.* 1992
Integrins	αvβ3	Membrane	165/105	Viscidi *et al.* 1989
	α5β1	Membrane	160/130	Ensoli *et al.* 1994
	αvβ5	Membrane	125/105	Vogel *et al.* 1993
Tat1-72 binding proteins	43 kDa	Membrane	43	Present Study
	35 kDa	Membrane	35	

Tat38-52 which contained five basic amino acids. However, since Tat28-42 and Tat23-37 also have four and six cysteine residues respectively, it may be possible that oxidization of these peptides facilitates the conjugation of Tat protein to the cell surface. The enhancement of Tat binding by Tat28-42 and Tat23-37 is consistent with our previous observations whereby these peptides also increased Tat uptake by astrocytes.

4. Detection of the novel Tat1-72 binding proteins

Major portion of Tat1-72 binding on astrocytes seems to be mediated by charge-dependent interaction. However, the possibility of a low abundant and high affinity Tat binding protein could not be excluded. Using immunoprecipitation by Tat we were able to detect 43 and 35 kDa proteins on the cell membrane of astrocytes that bound

specifically to Tat. These proteins could also be detected by re-immunoprecipitation with Tat from the eluted proteins of cell extracts bound to Tat affinity column. Since they could be lyophilized and re-immunoprecipitated with Tat, it suggested that the interactions between Tat and these proteins are stable and specific. By the nature of their molecular mass and location in the cells these proteins are distinct from any other Tat binding protein previously reported (table 4). However, the physiological significance of the Tat binding protein remains to be elucidated.

IV. Summary

In these studies, we have characterized kinetically and biochemically binding sites for two HIV-1 proteins gp120 and Tat in primary cultures of human fetal astrocytes. We demonstrated that gp120 binds to a unique 260 kDa protein with moderate affinity. In contrast the binding of Tat to astrocytes was more complex. Tat bound to astrocytes by charge interactions with > 10^6 sites per cell. However, it also bound specifically with low abundant, probably high affinity proteins of 43 and 35 kDa in size.

Tat could also be internalized by astrocytes and localized in the nucleus. Its internalization was confirmation dependent and required the presence of the entire Tat sequence composed of the first and the second exons.

Interestingly, both Tat binding and Tat uptake could be greatly enhanced by Tat peptides containing polycysteine residues. Further characterization of the gp120 and Tat binding proteins on astrocytes will have important implications in our understanding of

the pathogenesis of HIV-associated dementia and will advance our understanding of astrocyte function.

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Molecular Determinants for Cellular Uptake of Tat Protein of Human Immunodeficiency Virus Type 1 in Brain Cells

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We measured the cellular uptake of ^{125}I-labeled full-length Tat (amino acids 1 to 86) (^{125}I-Tat$_{1-86}$) and ^{125}I-Tat$_{1-72}$ (first exon) in human fetal astrocytes, neuroblastoma cells, and human fetal neurons and demonstrated that the uptake of ^{125}I-Tat$_{1-72}$ without the second exon was much lower than that of ^{125}I-Tat$_{1-86}$ ($P < 0.01$). This suggests an important role for the C-terminal region of Tat for its cellular uptake. ^{125}I-Tat uptake could be inhibited by dextran sulfate and competitively inhibited by unlabeled Tat but not by overlapping 15-mer peptides, suggesting that Tat internalization is charge and conformationally dependent. Interestingly, one of 15-mer peptides, Tat$_{28-42}$, greatly enhanced ^{125}I-Tat uptake. These findings are important for understanding the neuropathogenesis of human immunodeficiency virus type 1 infection and in the potential application of Tat for drug delivery to cells.

The Tat protein of human immunodeficiency virus type 1 (HIV-1) is a regulatory protein which transactivates HIV-1 expression (7). However, Tat can be released from productively infected cells (8, 9). Extracellular Tat is internalized by cells and localized in the nucleus (14, 16), where it can affect a variety of cellular functions (3, 10, 13, 17, 18, 27, 31, 33). For example, Tat can also act on neuronal cell membranes to produce neuronal excitation, elevation of intracellular calcium, and toxicity (20, 24, 28). It also causes aggregation of neurons (25). In astrocytes, extracellular Tat increases both NF-κB binding and protein kinase C activity (6). Tat also transactivates JC virus T antigen expression in astrocytes (5). However, it remains to be determined if these actions are due to effects of Tat on the cell membrane or following internalization of Tat.

The ability of exogenous Tat to be taken up by cells has generated considerable interest due to the potential biotechnological applications whereby Tat can be used as a vehicle for delivering heterogeneous proteins and drugs that would otherwise not have access to the intracellular environment (11, 23). In fact, substances conjugated to Tat have been shown to be localized in the nucleus (11). Hence, it is important to determine the regions of Tat that are responsible for its uptake versus those that are responsible for its functional activities following internalization.

Tat is formed from two exons. The first exon encodes amino acids 1 to 72 (Tat$_{1-72}$), and the second is of variable length, encoding another 14 to 32 amino acids. Tat$_{1-72}$ is sufficient for transactivation, which is regulated by the basic region of Tat between amino acid residues 49 and 57 (1, 15, 16, 30). The biological function of the C-terminal region encoded by the second exon is still unclear. However, the C-terminal region has an integrin receptor binding sequence (Arg-Gly-Asp) (3, 32). In this study, we define the regions of Tat that regulate its uptake into brain cells.

Uptake of Tat$_{1-86}$ and Tat$_{1-72}$. Human fetal brain tissue (gestational age, 13 to 16 weeks) was obtained with written consent from women undergoing elective termination of pregnancy and with approval of the University of Manitoba Human Ethics Committee. Human fetal astrocyte and neuron cultures were prepared as described previously (19, 20). Purity of astrocytes (>95%) and neurons (>70%) was determined by immunostaining for glial fibrillary acidic protein and microtubule-associated protein 2, respectively. Prior to the binding assay, the cells were plated into 24-well plates and grown to 100% confluence (5×10^5 cells per well).

The Tat gene encoding the first 72 amino acids (first exon) was inserted into an *Escherichia coli* vector, PinPoint Xa-2 (Promega), expressed as a fusion protein. Tat$_{1-72}$ was enzymatically cleaved from the fusion protein and purified as described previously (6). The Tat protein was >95% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. The purified product was further confirmed by Western blot analysis using polyclonal antisera to Tat (AIDS repository, National Institutes of Health). Recombinant HIV-1$_{BRU}$ Tat$_{1-86}$ was obtained from Repligen and was >98% pure. Purified Tat$_{1-86}$ or Tat$_{1-72}$ was labeled with Na^{125}I by using Iodo-beads (Pierce). The labeled protein was separated from free ^{125}I with a PD-10 column (Pharmacia). The specific activity of ^{125}I-Tat was 2.4×10^4 cpm/ng.

Cellular uptake assays of ^{125}I-Tat were performed as previously described (14, 20). Briefly, cells were incubated with various concentrations of ^{125}I-Tat$_{1-86}$ or ^{125}I-Tat$_{1-72}$ (2 to 64 nM) in 0.2 ml of binding medium (Dulbecco's modified Eagle medium [DMEM] and 0.1% bovine serum albumin) at room temperature for 30 min to 4 h. The cells were washed in ice-cold DMEM and treated with 0.2 ml of trypsin-EDTA (GIBCO BRL) for 10 min at 37°C. The cells were centrifuged, and supernatant was removed as a membrane fraction. The cell pellet was washed twice in DMEM with 10% fetal bovine serum. The nuclear and cytoplasmic fractions were isolated by lysis in 0.2 ml of 0.5% Nonidet P-40 (vol/vol) as described previously (2). The fractions were counted in a gamma counter. Uptake of both ^{125}I-Tat$_{1-86}$ and ^{125}I-Tat$_{1-72}$ was time and dose dependent (Fig. 1). However, the uptake of ^{125}I-Tat$_{1-72}$ was much lower than that of ^{125}I-Tat$_{1-86}$ (0.08 ± 0.01 nM versus 0.42 ± 0.009 nM at 4 h: $P < 0.01$) (Fig. 1A). At the maximal concentration (64 nM) of ^{125}I-Tat$_{1-86}$ or ^{125}I-Tat$_{1-72}$ tested, the uptake of Tat could not be saturated (Fig. 1B).

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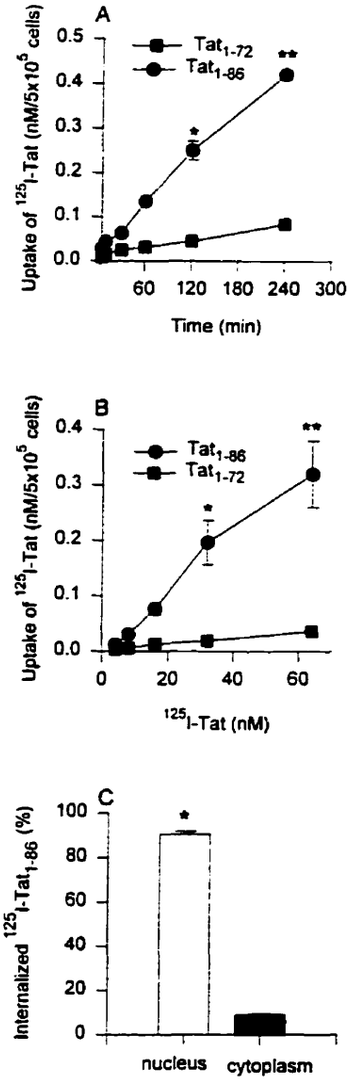

FIG. 1. Uptake of Tat by astrocytes. (A) Astrocytes were incubated with 10 nM ^{125}I-Tat_{1-86} or ^{125}I-Tat_{1-72} in 0.2 ml of binding medium at room temperature for 30 min to 4 h. The cells were washed in ice-cold DMEM and treated with 0.2 ml of trypsin-EDTA for 10 min at 37°C. The cells were centrifuged, and supernatant was removed as a membrane fraction. The cell pellet was washed twice in DMEM with 10% fetal bovine serum. The nuclear and cytoplasmic fractions were isolated by lysis in 0.2 ml of 0.5% Nonidet P-40 (vol/vol). The fractions were counted in a gamma counter. Both ^{125}I-Tat_{1-86} and ^{125}I-Tat_{1-72} were internalized by astrocytes. Uptake of Tat_{1-86} was much more rapid and occurred in larger amounts (*, $P < 0.05$; **, $P < 0.01$). (B) Astrocytes were incubated with various concentrations of ^{125}I-Tat_{1-86} or ^{125}I-Tat_{1-72} (2 to 64 nM) in 0.2 ml of binding medium at room temperature for 2 h and harvested as described above. ^{125}I-Tat_{1-86} and ^{125}I-Tat_{1-72} were internalized dose dependently in astrocytes. Uptake of Tat_{1-86} was more efficient (*, $P < 0.05$; **, $P < 0.01$). (C) Internalized $^{125}Tat_{1-86}$ was predominantly present in the nuclear fraction of astrocytes (*, $P < 0.005$). Values in all panels represent the means ± standard errors (error bars) of two experiments, each done in triplicate.

Further, >90% of internalized ^{125}I-Tat_{1-86} was localized in the nucleus while the cytoplasmic fraction represented only <10% in the astrocytes (Fig. 1C). To further confirm the nuclear localization and the fate of Tat within the nucleus, nuclear fractions were prepared from ^{125}I-Tat_{1-86}- or ^{125}I-Tat_{1-72}-treated astrocytes and analyzed by SDS-PAGE followed by autoradiography. By 2 h, prominent bands for both Tat_{1-86} and

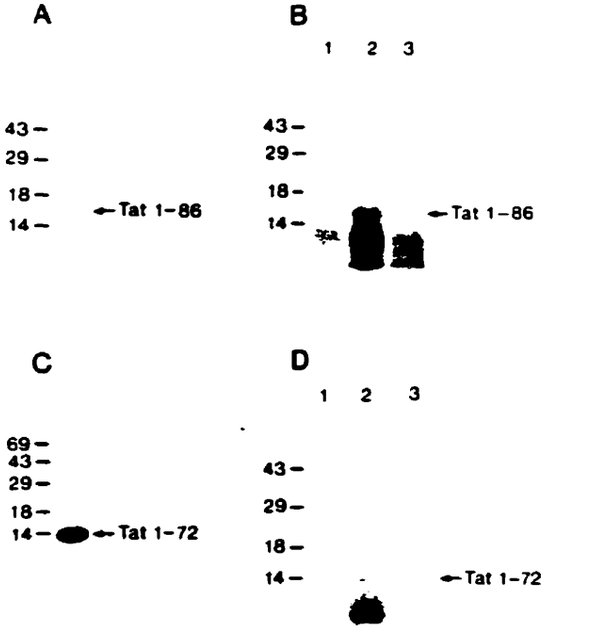

FIG. 2. Analysis of nuclear fractions. Nuclear fractions were prepared from ^{125}I-Tat_{1-86}- or ^{125}I-Tat_{1-72}-treated astrocytes and analyzed by SDS-PAGE and autoradiography. The numbers to the left side of each gel indicate the positions of molecular mass markers (in kilodaltons). (A) Purified ^{125}I-Tat_{1-86} alone shows a single band at 15 kDa. (B) Lanes 1, 2, and 3 represent internalized ^{125}I-Tat_{1-86} in nuclear fractions of astrocytes at 0.5, 2, and 4 h, respectively, which show increasing amounts of Tat_{1-86} degradation products. (C) Purified ^{125}I-Tat_{1-72} alone shows a single band at 14 kDa. (D) Lanes 1, 2, and 3 represent internalized ^{125}I-Tat_{1-72} in nuclear fractions of astrocytes at 0.5, 2, and 4 h, respectively, which show the presence of degradation products.

Tat_{1-72} were detected (Fig. 2B and D). Bands with lower molecular weights were also noted (Fig. 2B and D), and these represent breakdown products since the purified ^{125}I-Tat_{1-86} or ^{125}I-Tat_{1-72} prior to treatment with astrocytes showed a single band in Fig. 2A and C. Similar results were observed in neurons, NB41 cells, and SupT-1 cells (data not shown). All subsequent experiments were done with astrocytes only.

To further determine the specificity of Tat uptake, immunoabsorption of Tat was performed as described previously (20). Briefly, a 1:100 dilution of rabbit anti-Tat serum or normal rabbit serum was bound to protein A-Sepharose (Pharmacia) and incubated with ^{125}I-Tat for 1 h at room temperature followed by centrifugation. The supernatants were collected and used for Tat uptake assays. The uptake of ^{125}I-Tat_{1-86} or ^{125}I-Tat_{1-72} treated with normal rabbit sera was normalized to 100%. As shown in Fig. 3, cellular uptake of ^{125}I-Tat_{1-86} and ^{125}I-Tat_{1-72} treated with anti-Tat sera was decreased by 79% ± 11% and 78% ± 3%, respectively, demonstrating that the uptake of Tat was specific.

Competition of Tat_{1-86} and Tat_{1-72} uptake by unlabeled Tat and dextran sulfate. Astrocytes were treated with 10 nM ^{125}I-Tat_{1-72} in the presence of increasing concentrations of unlabeled Tat_{1-72}, ranging from 200 to 1,000 nM, for 2 h at room temperature. Binding of ^{125}I-Tat_{1-72} was inhibited dose dependently with maximal ^{125}I-Tat_{1-72} inhibition (60%) achieved with 800 nM unlabeled Tat_{1-72} (Fig. 4A). To determine if Tat uptake was charge dependent, human fetal astrocytes were treated with ^{125}I-Tat_{1-86} and ^{125}I-Tat_{1-72} in the presence of increasing concentrations of dextran sulfate (0.25 to 3.2 μM) (Sigma). Dextran sulfate inhibited ^{125}I-Tat_{1-86} or ^{125}I-Tat_{1-72}

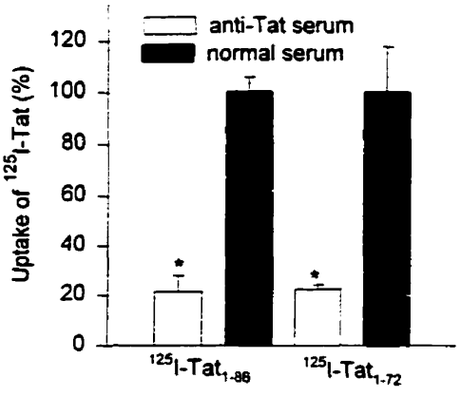

FIG. 3. Specificity of ^{125}I-Tat$_{1-86}$ and ^{125}I-Tat$_{1-72}$ uptake by astrocytes. Rabbit anti-Tat serum or normal rabbit serum was bound to protein A-Sepharose and incubated with ^{125}I-Tat for 1 h at room temperature followed by centrifugation. The supernatants were collected and used for the Tat uptake assay. The uptake of ^{125}I-Tat$_{1-86}$ or ^{125}I-Tat$_{1-72}$ treated with normal rabbit serum was considered to be 100%. Uptake of both of ^{125}I-Tat$_{1-86}$ and ^{125}I-Tat$_{1-72}$ is abolished following preabsorption of Tat with polyclonal Tat antisera conjugated with protein A-Sepharose (*, $P < 0.005$). Each value represents the mean ± standard error (error bar) of two experiments, each done in triplicate.

uptake dose dependently (Fig. 4B). Inhibition of ^{125}I-Tat$_{1-86}$ and ^{125}I-Tat$_{1-72}$ uptake (80 and 75%, respectively) was noted in the presence of 0.4 and 1.5 μM dextran sulfate, respectively (Fig. 4B).

Role of integrin binding on uptake of Tat$_{1-86}$. To determine if the basic region of Tat (Tat$_{49-57}$) or the RGD sequence in the C-terminal region of Tat previously shown to bind to integrins $\alpha_v\beta_5$ and $\alpha_5\beta_1$ or $\alpha_v\beta_3$ (3, 33) mediates cellular uptake of Tat, we treated astrocytes with 10 nM ^{125}I-Tat$_{1-86}$ in the presence of a 600-fold concentration of either Tat$_{31-72}$ containing the basic region or Tat$_{48-86}$ containing the basic region and the RGD sequence (AIDS Reagent Program of the Medical Research Council of the United Kingdom) (Table 1). Both peptides failed to inhibit ^{125}I-Tat$_{1-86}$ internalization in astrocytes (Fig. 4C). Conversely, when astrocytes were treated similarly with ^{125}I-Tat$_{1-72}$ in the presence of Tat$_{31-72}$, a fivefold enhancement of Tat uptake was observed ($P < 0.01$) while Tat$_{48-86}$ had no effect on ^{125}I-Tat$_{1-72}$ uptake by astrocytes (Fig. 4D). The cells were also pretreated with polyclonal antisera to $\alpha_v\beta_3$, $\alpha_v\beta_5$ (kindly provided by J. Wilkins) and $\alpha_5\beta_1$ (Chemicon) at 1:100 and 1:200 dilutions (vol/vol) for 90 min followed by ^{125}I-Tat$_{1-72}$ uptake assay. None of above-mentioned antibodies blocked the uptake of Tat$_{1-72}$ (data not shown).

Effect of 15-mer Tat peptides on Tat$_{1-72}$ uptake. To further determine the region responsible for Tat$_{1-72}$ uptake, we synthesized and purified 15-mer Tat peptides completely spanning the 86-amino-acid sequence of Tat HIV$_{BRU}$ (Table 1) (24). Astrocytes were incubated with ^{125}I-Tat$_{1-72}$ in the presence of various 15-mer Tat peptides (500 M excess) for 2 h at room temperature. None of the peptides blocked Tat$_{1-72}$ uptake. Instead, a 35-fold enhancement of Tat$_{1-72}$ uptake by Tat$_{28-42}$ (KKCCFHCQVCFTTKA) was observed. Tat$_{23-37}$ and Tat$_{18-32}$ also produced a 10-fold and a 2-fold enhancement of ^{125}I-Tat$_{1-72}$ uptake, respectively, while the remaining 15-mer peptides had no significant effect (Fig. 5A). Similarly, Tat$_{72-86}$, which sup-

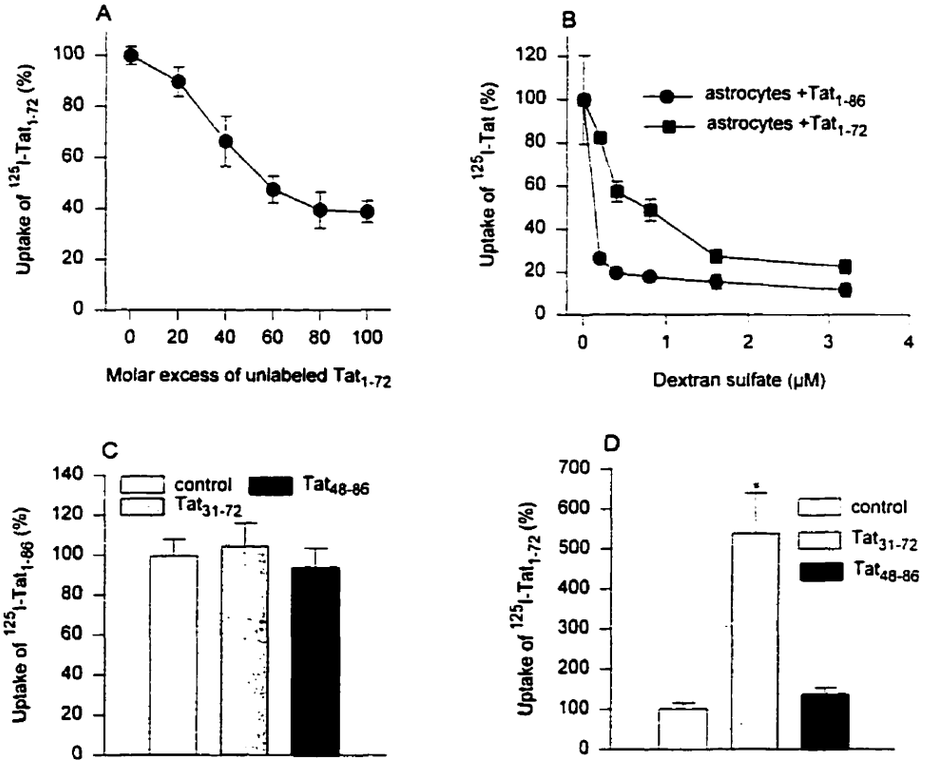

FIG. 4. (A) Competition of ^{125}I-Tat$_{1-72}$ uptake by unlabeled Tat$_{1-72}$ in astrocytes. Increasing concentrations (fold) of unlabeled Tat$_{1-72}$ inhibited ^{125}I-Tat$_{1-72}$ (10 nM) uptake dose dependently. (B) Effect of dextran sulfate on uptake of ^{125}I-Tat$_{1-86}$ and ^{125}I-Tat$_{1-72}$. Increasing concentrations of dextran sulfate (0.2 to 3.2 μM) blocked ^{125}I-Tat$_{1-86}$ (10 nM) or ^{125}I-Tat$_{1-72}$ (10 nM) uptake dose dependently. (C) Internalization of ^{125}I-Tat$_{1-86}$ (10 nM) by astrocytes was not affected by Tat$_{31-72}$ (6 μM) or Tat$_{48-86}$ (6 μM). (D) Uptake of ^{125}I-Tat$_{1-72}$ (10 nM) in astrocytes was enhanced fivefold (*, $P < 0.01$) by Tat$_{31-72}$ (6 μM), while Tat$_{48-86}$ had no effect. Values in all panels represent the means ± standard errors (error bars) of two experiments, each done in triplicate.

TABLE 1. HIV-1 Tat peptides derived from Tat_{BRU} used in this study

Tat peptide	Amino acid sequence
Tat_{31-71}	.....FHCQVCFITTKALGISYGRKKRRQRRRPPQESQTHQVSLSKQ
Tat_{31-61}	.....FHCQVCFITTKALGISYGRKKRRQRRRPPQES
Tat_{48-85}	.....RKKRRQRRRPPQESQTHQVSLSKQPTSQSRGDPTGPKE
Tat_{1-15}	.....MEPVDPRLEPWKHPG
Tat_{1-17}	.....PVDPRLEPWKHPGSQ
Tat_{8-22}	.....LEPWKHPGSQPKTAC
Tat_{13-27}	.....HPGSQPKTACTNCYC
Tat_{18-32}	.....PKTACTNCYCKKCCF
Tat_{23-37}	.....TNCYCKKCCFHCQVC
Tat_{28-42}	.....KKCCFHCQVCFITTK
Tat_{33-47}	.....HCQVCFITTKALGIS
Tat_{38-52}	.....FITTKALGISYGRKK
Tat_{43-57}	.....LGISYGRKKRRQRRR
Tat_{48-62}	.....GRKKRRQRRRPPQGS
Tat_{53-67}	.....RQRRRPPQGSQTHQV
Tat_{58-72}	.....PPQGSQTHQVSLSKQ
Tat_{63-77}	.....QTHQVSLSKQPTSQP
Tat_{68-82}	.....SLSKQPTSQPRGDPT
Tat_{72-86}	.....QPTSQPRGDPTGPKE

plements the deleted region of the second exon, had no effect on ^{125}I-Tat_{1-72} uptake (Fig. 5A).

To determine if internalized Tat_{1-72} retains its functional properties, transactivation of the HIV long terminal repeat (LTR) was determined in HL3T1 cells containing the LTR-chloramphenicol acetyltransferase (CAT) (AIDS Repository, National Institutes of Health) treated with 0.5 μM Tat_{1-72} without scrape-loading in the presence or absence of 6 μM Tat_{1-15}, Tat_{28-42}, or Tat_{72-86}. The Tat transactivation assay was performed as previously described (12, 14), and CAT activity was measured by a simple phase extraction assay (29). As shown in Fig. 5B, Tat_{28-42} produced a ninefold increase in Tat_{1-72}-induced LTR transactivation while Tat_{1-15} or Tat_{72-86} had no effect. Tat_{28-42} alone did not show any transactivation activity.

Not only is understanding the precise mechanisms underlying the internalization of extracellular Tat important in determining its role in regulating viral and host function, but this phenomenon could also potentially be exploited to deliver heterologous proteins or drugs into cells. We demonstrate here that both full-length Tat_{1-86} and to a lesser degree Tat_{1-72} are internalized by brain cells and that following entry, they are predominantly localized in the nucleus. Consistent with previous observations that Tat_{1-86} uptake in lymphoid and monocytoid cells could be inhibited by polyanions such as heparin or dextran sulfate (21), we found that Tat_{1-72} uptake in astrocytes could also be blocked by dextran sulfate, suggesting that internalization of Tat is charge dependent. We demonstrate that uptake of ^{125}I-Tat_{1-86} and ^{125}I-Tat_{1-72} by human fetal astrocytes was competitively inhibited dose dependently by an excess of unlabeled Tat_{1-72}, suggesting that uptake of ^{125}I-Tat_{1-72} is receptor mediated.

Transactivation of HIV-2 Tat requires full-length protein (26), while the HIV-1 Tat_{1-72} has the same efficiency for transactivation as HIV-1 Tat_{1-86} (16). The role of the C-terminal region formed by the second exon of the *tat* gene in HIV-1 infection was not previously determined. We observed that even though both Tat_{1-86} and Tat_{1-72} could be internalized by several cell types, Tat_{1-86} internalization was up to 10 times more efficient than that by Tat_{1-72}, suggesting that the region encoded by the second exon is important in mediating Tat internalization. However, Tat_{1-72} uptake was not affected by

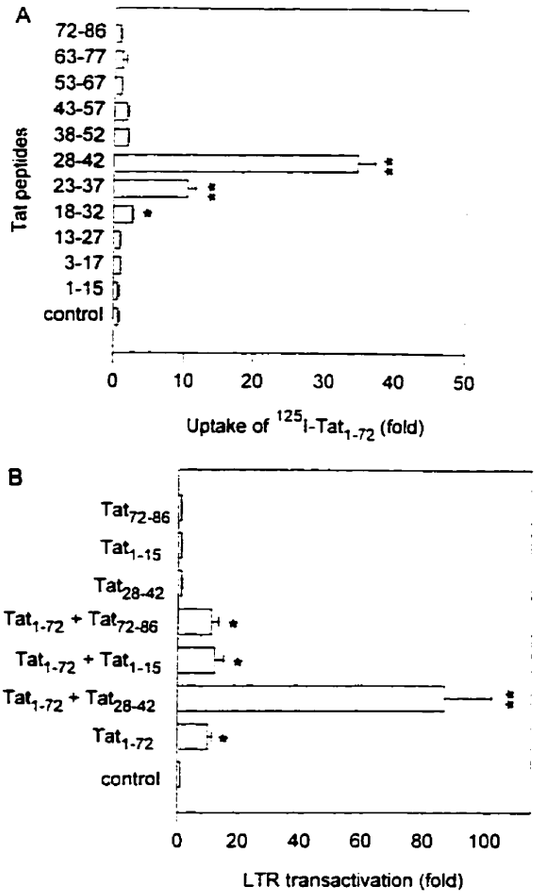

FIG. 5. (A) Effect of 15-mer Tat peptides on Tat_{1-72} uptake. Astrocytes were treated with ^{125}I-Tat_{1-72} (10 nM) in the presence of various 15-mer peptides (6 μM each). ^{125}I-Tat_{1-72} (10 nM) uptake was enhanced 35-fold (**, $P < 0.005$), 10-fold (**, $P < 0.005$), or 2-fold (*, $P < 0.01$) in the presence of Tat_{28-42}, Tat_{23-37}, or Tat_{18-32} respectively, while the rest of the 15-mer peptides had no effect. (B) Effect of 15-mer Tat peptides on transactivation of HIV-1 LTR in HL3T1 cells by Tat_{1-72}. HL3T1 cells were treated with 0.1 to 0.5 μM Tat_{1-72} and 100 μM chloroquine without scrape-loading in the presence or absence of 6 μM Tat_{1-15}, Tat_{28-42}, or Tat_{72-86}. The Tat transactivation assay was performed, and CAT activity was measured by a simple phase extraction assay. Tat_{1-72} transactivated LTR-CAT significantly compared to the control (*, $P < 0.01$). Tat_{1-72}-induced transactivation was enhanced ninefold (**, $P < 0.005$) by Tat_{28-42}. Neither Tat_{1-15} nor Tat_{72-86} had any effect on Tat_{1-72}-induced transactivation. Tat_{28-42} alone did not have any transactivation activity. Values in both panels represent the means ± standard errors (error bars) of two experiments, each done in triplicate.

coincubating with peptides including the second exon (Tat_{72-86} or Tat_{48-86}). Thus, peptide bond linkage of the peptides encoded by the two exons is essential for the efficient uptake of Tat. This linkage most likely influences the tertiary configuration of the molecule. The importance of the tertiary configuration for Tat uptake is further supported by the results of Bonifaci et al., which demonstrate that the Tat molecule unfolds before entering the cells (4). However, the peptide encoded by the second exon is not exclusively involved in Tat uptake since significant uptake of Tat_{1-72} was also observed.

Tat_{1-72} is highly conserved between different strains of HIV-1. However, the second exon shows heterogeneity in the amino acid sequence and is of variable length. Since this region is a major determinant of Tat uptake into the cell, it could play an important role in regulating strain-to-strain variability of

the intra- versus extracellular action of Tat on the host cell and hence effect virulence. We have previously shown that extracellular Tat causes neurotoxicity by acting on cell surface excitatory amino acid receptors (20) and that the neurotoxic epitope of Tat resides in the first exon (24). It is thus likely that Tat molecules with low cellular uptake as determined by their second exon might be more neurotoxic since higher levels will be achieved extracellularly.

Integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ function as receptors for Tat and mediate Tat effects on Kaposi's sarcoma cells or cytokine-activated endothelial cells (11) by binding to the RGD sequence of the Tat protein. Further, 12-mer peptides containing the basic region of Tat have been shown to bind to another integrin molecule, $\alpha_v\beta_5$ (32). However, it is unlikely that binding of Tat to these integrin receptors influences Tat uptake since Tat_{49-86} containing the RGD sequence, Tat_{31-62} containing the basic domain, or antisera to integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, or $\alpha_v\beta_5$ could not inhibit Tat uptake.

Our results show that Tat_{28-42} also greatly increased Tat_{1-72} uptake and transactivation. This peptide contains only four basic amino acids and increased Tat uptake by 35-fold, while Tat_{38-52}, which contains five basic amino acids had no significant effect on Tat uptake, indicating that a mechanism of enhancement other than positive charge dependence might also be involved.

The phenomenon by which one protein facilitates the internalization of another protein has also been observed in anthrax toxins. The protective antigen of anthrax toxin can form ion-conductive channels in biological membranes and convey the edema factor and lethal factor of the toxin into the cytoplasm by inducing changes in the membrane permeability (22). Presumably, the Tat peptides can use a similar mechanism to facilitate the internalization of Tat protein.

We thus conclude that the cellular uptake of Tat (i) is determined by the tertiary configuration of the molecule, (ii) is dependent upon the C-terminal region and the basic region of Tat, and (iii) is perhaps independent of integrin binding. Further, Tat uptake can perhaps be enhanced by an autologous peptide through yet-unknown mechanisms.

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Characterization of a Novel Binding Site for the Human Immunodeficiency Virus Type 1 Envelope Protein gp120 on Human Fetal Astrocytes

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^{125}I-labeled recombinant gp120 bound to primary cultures of human fetal astrocytes with a single class of 260-kDa binding sites, with a K_d of 26 nM and maximal number of binding sites of 29.9 fmol/4 × 10^4 cells. Neither CD4 nor galactocerebroside was detectable on astrocytes, and ^{125}I-labeled recombinant gp120 binding to astrocytes was not blocked by antibodies against galactocerebroside or the gp120 binding domain of CD4.

Infection with human immunodeficiency virus type 1 (HIV-1) frequently results in a dementing illness (25). Since HIV-1 encephalopathy was first recognized, the infection of resident cells with HIV-1 in the central nervous system and the toxic effects of the HIV-1 envelope protein gp120 on neural cell types have been topics of intense study. It has now been firmly established that glial cells (microglia and astrocytes) can be infected in vivo (9, 13, 17, 19, 23, 27, 28, 34) and in vitro (4, 5, 7, 8, 16, 35, 37). As it is now understood, gp120 is responsible for mediating virus entry in various cell types. Infection of microglia is mediated via gp120 binding to CD4 (16). The homologous receptor on astrocytes remains to be determined. It has been shown, however, that gp120 acts directly on astrocytes to (i) alter its transport functions, including Na^+/H^+ exchange, K^+ conductance, and glutamate efflux (2); (ii) induce tyrosine kinase activity (30); (iii) inhibit β-adrenergic regulation of astrocytic function (22); and (iv) inhibit glial fibrillary acidic protein expression (26).

gp120 binds to the CD4 molecule on lymphocytes and macrophages, a membrane-associated C-type lectin in placental cells (7), sulfated oligosaccharides (21, 32, 33), and sulfated glycoconjugates (36). It also binds to glycolipids, galactocerebroside C (Gal C), and sulfatide in neuroblastoma cell lines (3, 14, 15) and binds to a 180-kDa protein on a glioma cell line (18). In this study, we characterize the molecule on the surface of primary human fetal astrocytes responsible for interacting with gp120 and describe specific binding sites for iodinated recombinant gp120 (^{125}I-rgp120).

Binding of ^{125}I-rgp120 to astrocytes. Human fetal brain tissue (gestational age of 13 to 16 weeks) was obtained with written consent from women undergoing elective termination of pregnancy and with approval of the University of Manitoba Ethics Committee. Brain tissue was dissected, and pure (>99%) cultures of astrocytes, as determined by positive staining for glial fibrillary acidic protein, were prepared as previously described (11). HeLa-T4 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 0.2 mg of G418 per ml, and 0.1 mg of hygromycin B per ml. SK-N-MC cells (American Type Culture Collection) were cultured in minimal essential medium with sodium pyruvate and 10% fetal bovine serum. Prior to the binding assay, 2 × 10^4 astrocytes and 3 × 10^4 HeLa-T4 cells and SK-N-MC cells were plated in each well of 96-well plates and maintained at 37°C for 18 h. The final cell number of astrocytes per well was approximately 4 × 10^4 at the time of assay.

HIV-1_{SF2} rgp120 (>90% pure as determined by Coomassie staining after polyacrylamide gel electrophoresis [PAGE]) was labeled with $Na^{125}I$ by lactoperoxidase with Enzymobeads (Bio-Rad). The ^{125}I-labeled protein was separated from free ^{125}I by using a PD-10 column (Pharmacia). The specific activity of ^{125}I-rgp120 was 3.9 × 10^4 cpm/ng. Binding of ^{125}I-rgp120 to cells was performed as described previously (31, 32), with the following modifications. Briefly, astrocytes and HeLa-T4 cells were incubated in binding medium (RPMI 1640, 0.5% bovine serum albumin [BSA], 50 μg of bacitracin per ml) containing ^{125}I-rgp120 (1.0 nM) and unlabeled rgp120 (0 to 120 nM) at 4°C for 5 h. The cells were solubilized in 0.2 N NaOH and were counted in a gamma counter for cell-associated radioactivity. Astrocytes incubated with ^{125}I-rgp120 showed maximal binding at 5 h at 4°C (data not shown), with or without 100-fold unlabeled rgp120. At 1.0 nM ^{125}I-rgp120, the specific binding was 50% of the total binding (Fig. 1), similar to that seen on monocytes (10). Scatchard analysis of these data revealed that ^{125}I-rgp120 bound to a single class of binding molecules on the astrocytes with an apparent K_d of 26 nM and an apparent maximal number of binding sites of 29.9 fmol/4 × 10^4 cells (4.5 × 10^5 binding molecules per cell) (Fig. 1).

^{125}I-rgp120 binding to astrocytes is CD4 and Gal C independent. A monoclonal antibody against the gp120 binding site on CD4 (OKT4a) (Ortho Diagnostics) and rabbit anti-Gal C antibody (Chemicon) were used to determine if these two antibodies could block the binding of rgp120 to astrocytes. HeLa-T4 cells were used as a positive control with OKT4a and SK-N-MC cells as a positive control with anti-Gal C antibody (3, 14). The cells were preincubated with the respective antibodies for 60 min at 37°C, washed twice in ice-cold binding medium, and incubated with 1.0 nM ^{125}I-rgp120 for 5 h at 4°C. The cells were washed and counted as described above. Sixty percent of the total binding on HeLa-T4 cells was inhibited by 1 μg of OKT4a per ml (Fig. 2A), and 70% ^{125}I-rgp120 binding on SK-N-MC cells was seen with anti-Gal C antibody at a dilution of 1:20 (Fig. 2B). Neither of the antibodies (up to 4 μg of OKT4a per ml or 1:20 dilution of anti-Gal C antibody) (Fig. 2) nor recombinant soluble CD4 protein (up to 8 μg/ml) had

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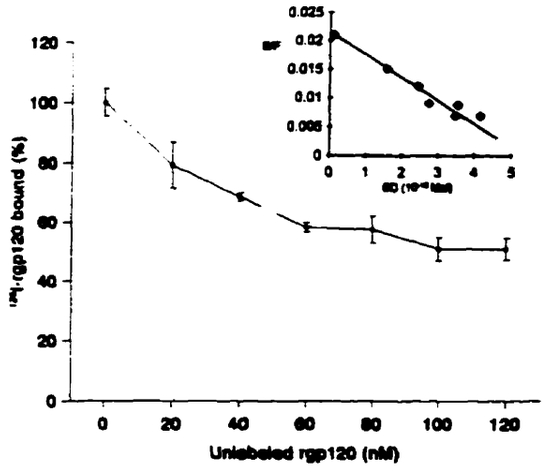

FIG. 1. Binding of ^{125}I-rgp120 to astrocytes. Human fetal astrocytes (4×10^4 cells per well) were incubated with 1 nM ^{125}I-rgp120 alone or with increasing concentrations of unlabeled rgp120 ranging from 0 to 120 nM for 5 h at 4°C. The unbound radioactivity was removed by three washes in ice-cold PBS with 0.5% BSA. The cell-associated radioactivity was measured in a gamma counter after solubilization of the cells in 0.2 N NaOH. The values represent the mean ± standard error of two experiments, each done in triplicate. The insert presents a saturation isotherm plot of the data from above. A single binding site was revealed with K_d of 26 nM and maximal number of binding sites of 29 fmol. B/F, bound/free ratio.

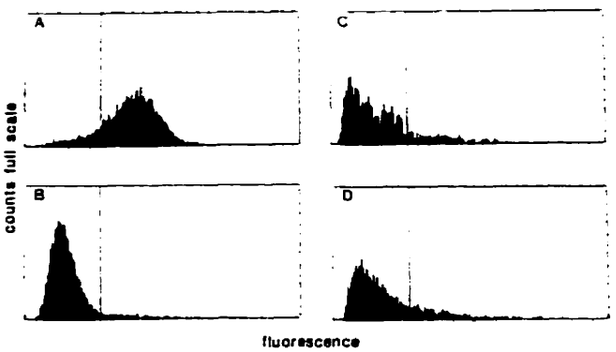

FIG. 3. Expression of surface molecules determined by flow cytometry. (A) As a positive control, CD4 was detected on HeLa-T4 cells by flow cytometry with DAKO-T4-FITC. (B) Background level of fluorescence on HeLa-T4 cells with goat anti-mouse IgG conjugated with FITC alone. (C) CD4 could not be detected on astrocytes by flow cytometry with DAKO-T4-FITC. (D) Background level of fluorescence on astrocytes with goat anti-mouse IgG conjugated with FITC alone.

any effect on the binding of ^{125}I-rgp120 to astrocytes (data not shown).

The presence of CD4 molecules on the cell surface was determined by immunofluorescence flow cytometry (11). The astrocytes were trypsinized briefly (30 s) and removed from the monolayer primarily by mechanical force. Cells were incubated with a monoclonal antibody (1 μg/ml) against CD4 molecule conjugated to fluorescein isothiocyanate (FITC) (DAKO-T4-FITC) (Dakopatts). To ensure that trypsinization did not alter the epitope defined by DAKO-T4, HeLa-T4 cells, which also form adherent monolayers, were used as a positive control. HeLa-T4 cells exhibited strongly positive staining (Fig. 3A). Background levels of fluorescence were determined by using goat anti-mouse immunoglobulin G (IgG) conjugated to FITC (Boehringer Mannheim) (Fig. 3B and D). CD4 could not be detected on the cell surface of astrocytes (Fig. 3C). We simultaneously plated cells on glass coverslips, fixed them in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, and stained them by an immunofluorescence technique (11) with OKT4a (0.5 μg/ml) and DAKO-T4 (1 μg/ml). Goat anti-mouse IgG conjugated with rhodamine (1:50) (Chemicon)

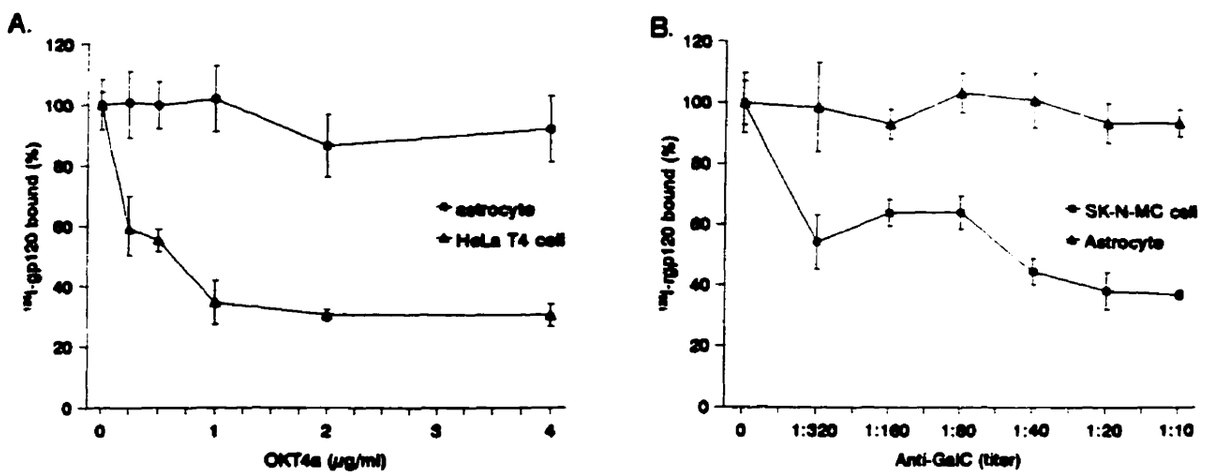

FIG. 2. (A) Effect of OKT4a on the binding of ^{125}I-rgp120 to astrocytes (■) and HeLa-T4 cells (▲). The cells were preincubated with various concentrations of OKT4a for 60 min at 4°C. After removal of unbound antibody by two washes, 1 nM ^{125}I-rgp120 was added for 5 h at 4°C. OKT4a was unable to block ^{125}I-rgp120 binding to astrocytes. The data represent the mean ± standard error of two independent experiments done in duplicate. (B) Effect of anti-Gal C antibody on the binding of ^{125}I-rgp120 to astrocytes (▲) and SK-N-MC cells (■). The cells were preincubated for 60 min at 4°C with various dilutions of anti-Gal C antibody. After removal of unbound antibody by two washes, 1 nM ^{125}I-rgp120 was added and incubated for 5 h at 4°C. The data represent the mean ± standard error of two independent experiments done in duplicate.

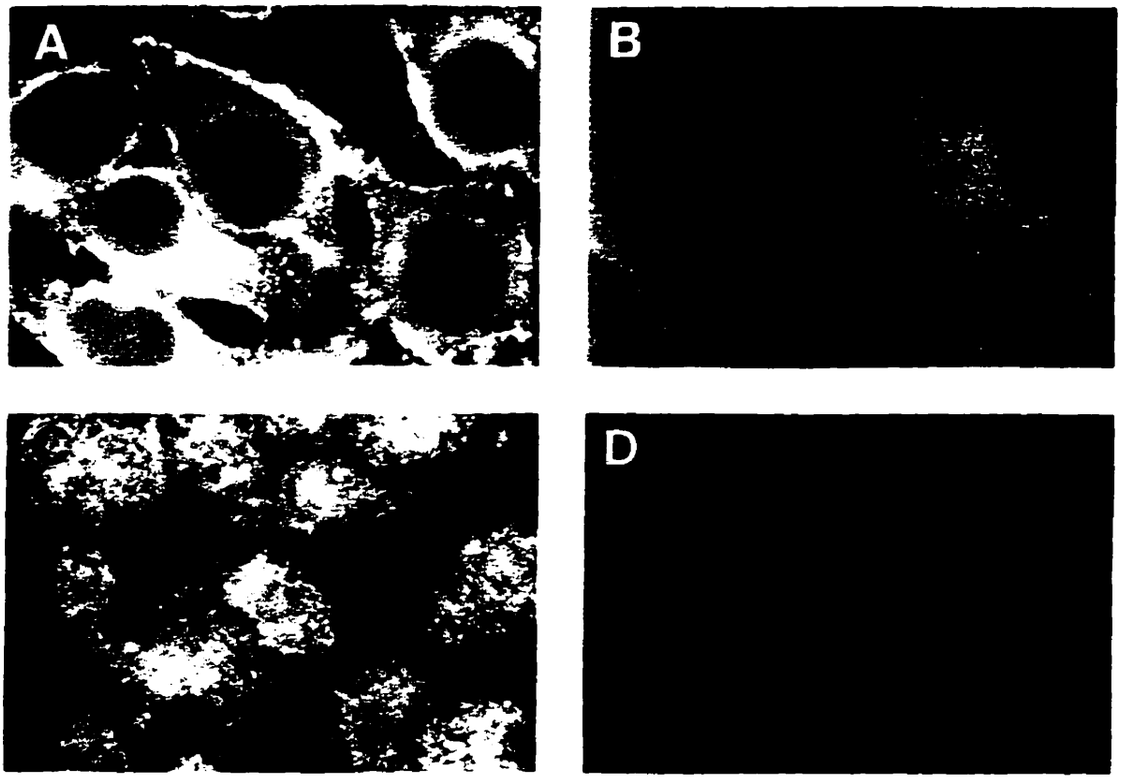

FIG. 4. Immunocytochemistry. (A) HeLa-T4 cells show staining for CD4 on cell membrane. (B) Astrocytes could not be stained with OKT4a. (C) Gal C was detected on SK-N-MC cells by staining with anti-Gal C antibody. (D) Astrocytes were Gal C negative.

was used as a secondary antibody. Secondary antibody alone without incubation in primary antibody was used as a negative control. HeLa-T4 cells were used as a positive control. The CD4 molecule was present on the cell membrane of HeLa-T4 cells (Fig. 4A). However, no specific staining on astrocytes was observed compared with that on controls (Fig. 4B). Similarly, astrocytes and SK-N-MC cells were stained with anti-Gal C antibody (1:100) by using goat-anti-rabbit IgG conjugated with rhodamine (1:50) (Chemicon) as a secondary antibody. SK-N-MC cells showed cell membrane staining with anti-Gal C antibody (Fig. 4C), while astrocytes showed only background levels of fluorescence (Fig. 4D).

Immunoprecipitation of rgp120 binding molecules. Astrocytes were surface radioiodinated by the lactoperoxidase technique (1). Briefly, 5×10^7 cells were suspended in 1 ml of PBS containing 1 mCi of $Na^{125}I$ and 20 μg of lactoperoxidase, and after 0, 1, 5, and 10 min, 10 μl of 0.03% H_2O_2 was added. Reactions were stopped by adding 5 ml of PBS containing 10 mM NaI. Labeled cells were incubated with ^{125}I-rgp120 (0.1 μg) or rgp120 (1 μg) for 5 h at 4°C. The labeled cells without rgp120 were used as a negative control. The cell pellets were lysed by adding detergent lysing buffer, and the nuclei were removed by centrifugation (24). Antiserum from an HIV-1-infected patient predetermined to have a high titer of antibody against gp120 or normal human sera were each bound to protein A-agarose. All lysates were absorbed with the beads for 2 h at room temperature, washed, solubilized, and resolved by 4 to 15% polyacrylamide gradient sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). Dried gels were exposed to X-ray film (Kodak, Rochester, N.Y.) for 5 to 7 days.

A single distinct band at 260 kDa was identified as a putative gp120 binding protein. This protein could not be immunoprecipitated when normal serum was substituted for anti-gp120 serum. It was also absent in cells not treated with rgp120 (Fig. 5A). Because the binding kinetics were determined with ^{125}I-rgp120, we treated ^{125}I-labeled astrocytes with ^{125}I-rgp120 and immunoprecipitated them with anti-gp120 sera. The same 260-kDa protein was again observed, confirming that iodination of gp120 did not alter its binding properties. When ^{125}I-rgp120 was incubated with the anti-gp120–protein A-agarose conjugate and analyzed by SDS-PAGE, a single major band of 120 kDa was seen (Fig. 5B).

The primary receptor for HIV-1 is CD4 (29, 31). In the brain, the infection of microglia has been shown to be mediated by CD4 (16, 37). However, HIV-1 infection of astrocytes and other nervous system-derived cell lines may proceed via an entry mechanism independent of CD4 (3, 4, 7, 8, 14, 18, 35). Furthermore, gp120 can bind to CD4-negative glial cells to activate tyrosine kinase (30) and to human astrocytes, altering ion exchange (2). Thus, it has been suggested that additional HIV-1 receptors are present on neural cells. We were unable to demonstrate the presence of CD4 or Gal C on human fetal astrocytes, and it is doubtful that this was due to CD4 or Gal C expression at levels below the sensitivity of the techniques used, because the binding of ^{125}I-rgp120 to the astrocyte membrane was not inhibited by OKT4a, recombinant soluble CD4, or anti-Gal C antibody. Furthermore, Gal C and sulfatide could not be detected in lipid extracts of human fetal astrocytes by high-performance thin-layer chromatography (12).

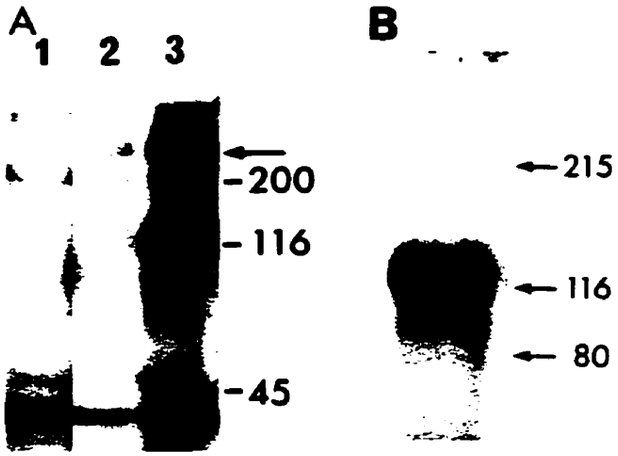

FIG. 5. Immunoprecipitation of gp120 binding sites. (A). Astrocytes were surface labeled with ^{125}I and then incubated with rgp120 or ^{125}I-rgp120 prior to detergent extraction and immunoprecipitation with anti-gp120–protein A-agarose. Samples were analyzed by SDS-PAGE in 4 to 15% polyacrylamide gradient gels followed by autoradiography. Lane 1, ^{125}I-labeled astrocytes only. Lanes 2 and 3, ^{125}I-labeled astrocytes incubated with rgp120 or ^{125}I-rgp120, respectively. A 260-kDa band is seen in lanes 2 and 3 only (arrow). Fivefold more protein was added to lane 3 to detect any minor bands in addition to the 260-kDa molecule. (B). ^{125}I-rgp120 incubated with anti-gp120–protein A-agarose conjugate and analyzed by SDS-PAGE (6% polyacrylamide). A single prominent band at 120 kDa and two minor bands at 95 and 66 kDa representing a breakdown product of gp120 are shown. No polymers of gp120 could be detected.

The K_d of ^{125}I-rgp120 binding to human fetal astrocytes (26 nM) is greater than those of the gp120-CD4 (2 to 5 nM) (20) and gp120-Gal C (12 nM) (13) interactions. These findings suggest that the affinity of gp120 for astrocytes is slightly lower than that for lymphocytes or Gal C-expressing cells. Nevertheless, gp120 at comparable concentrations has been shown to produce biochemical and morphological changes in astrocytes (26). Our finding of 4.5×10^5 binding sites on each astrocyte compares favorably to 0.5×10^4 to 1×10^4 CD4 receptors per lymphoid cell (7) when the fact that astrocytes are typically 10 to 20 times larger than lymphoid cells is taken into account. The size of the gp120 binding site on the astrocytes with an observed molecular mass of 260 kDa was much greater than that of the gp120 binding site to the CD4 molecule, which is a 58-kDa protein. Thus, several lines of evidence suggest that the rgp120 binding site on human fetal astrocytes is distinct from the CD4 molecule and Gal C. However, it remains to be determined if the binding site of HIV-1 gp120 on human astrocytes can act as a receptor for HIV-1.

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Extracellular Human Immunodeficiency Virus Type 1 Tat Protein Is Associated with an Increase in both NF-κB Binding and Protein Kinase C Activity in Primary Human Astrocytes

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Human immunodeficiency virus type 1 (HIV-1) infection has been associated with an increase in the binding of the transcription factor NF-κB to its consensus sequence in the viral promoter. Using cultures of primary human fetal astrocytes, we show that exogenous HIV-1 Tat protein, which has been demonstrated to be released from infected cells, is associated with an increase in the binding of this transcription factor to an HIV-1 long terminal repeat κB sequence. This effect occurs rapidly and is independent of new protein synthesis. We also demonstrate that extracellular Tat protein is associated with an increase in protein kinase C activity. If Tat functions similarly in other cell types, such findings could relate to some of this protein's previously described physiological effects. These effects include Tat's ability to upregulate the synthesis of specific cytokines and to act as a growth factor.

The virally encoded Tat protein interacts with its RNA target sequence (TAR) and, in combination with cellular factors, functions to increase human immunodeficiency virus type 1 (HIV-1) transcription (6, 22, 70). This protein can also be released from infected cells (27). Such extracellular Tat has been associated with toxicity in neural and glial cells (49, 61). Tat can also act as a growth factor (9, 29, 47) and possibly as a tumor promoter. It has been demonstrated that Tat-expressing transgenic mice develop both liver cancer and lesions which resemble Kaposi's sarcoma (75, 76). Recent studies suggest that Tat may also be involved in cellular processes that govern apoptosis (44, 79). Additionally, the production of several cytokines has been demonstrated to be upregulated by Tat (17, 28, 58). Some of these effects could follow the activation of specific transcription factors and/or protein kinases.

NF-κB is a transcription factor that activates a number of cellular and viral promoters, including that of HIV-1 (reviewed in references 36 and 69). Of note is that NF-κB has consensus binding sites in the promoters of a number of inflammatory cytokines and is in turn upregulated by a number of the same cytokines. Its prototypical form, a heterodimer of protein subunits of about 50 and 65 kDa, is sequestered in the cytoplasm by its association with an inhibitor such as I-κBα. Following an appropriate stimulus, however, I-κBα is phosphorylated and degraded (15). The nuclear localization signal on NF-κB is then exposed so that it can translocate to the cell nucleus.

Primary human fetal astrocytes typically demonstrate little NF-κB binding. However, following transfection of these cells with HIV-1 proviral DNA (pNL4-3), we found that NF-κB binding was increased. Because transfection efficiency is low, this effect could have resulted either from the transfection procedure itself or as a result of a diffusable factor that was increased following such a transfection. We found that mock transfection with calf thymus DNA was not associated with a similar increase in NF-κB binding. Because Tat can be released from HIV-1-infected cells (27), we then investigated whether this protein could increase the binding of NF-κB.

Others have previously reported that HIV-1-infected cells demonstrate increased NF-κB binding (59, 60). It has also been shown that in certain Tat transfectants, both NF-κB and nuclear factor interleukin-6 (NF-IL6) binding are increased (63). The underlying mechanism for such observations has not been described. A TAR-like structure in the promoter of at least one cytokine has been proposed as a possible means by which Tat increases the production of this cytokine (17). Increases in NF-κB binding could then follow increased cytokine production. In this study, however, we demonstrate that extracellular Tat protein is associated with an increase in NF-κB binding that occurs rapidly and is independent of new protein synthesis.

While the kinase(s) that directly phosphorylates I-κB-α in vivo is unknown (69), a number of factors that increase NF-κB binding in a protein synthesis-independent manner are associated with the activation of serine/threonine and/or tyrosine kinases (43, 69). Furthermore, it has been suggested that Tat may bind to integrin receptors (9, 14, 74). Activation of such receptors has been linked to an increase in the activity of protein kinase C (PKC) as well as protein tyrosine kinases (19, 40, 64). Integrin receptors have been found on astrocytes (3, 38). Additionally, in astrocytes, Tat has been associated with an increase in intracellular calcium (55). An increase in intracellular calcium is often associated with an increase in the activity of certain PKC isozymes. We therefore examined the effect of Tat on PKC activity in astrocytes and found that Tat was associated with an increase in cytoplasmic PKC activity.

If these findings are common to other cell types, such data suggest at least one mechanism by which some of Tat's pleiotropic effects may be mediated. For example, changes in NF-κB binding and/or PKC activity would be expected to affect both cytokine expression and cell growth.

MATERIALS AND METHODS

Cells. The preparation of astrocyte cultures from human fetal tissue has previously been described (26). Astrocyte cultures were grown in Eagle's mini-

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mal essential medium (MEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and gentamicin (5 μg/ml).

Transfection. Lipofectamine reagent (Gibco-BRL) was used for transfections. A mixture of 5 μg of DNA (pNL4-3 [1] or calf thymus) and 0.8 ml of MEM was added to a second mixture containing 50 μl of lipofectamine with 0.8 ml of MEM. Thirty minutes later, the cells were washed three times with MEM. The above mixture was then added to 6.4 ml of MEM and placed on a culture of 10^7 cells. Cultures were kept at 37°C for 5 h. The transfection solution was then replaced with MEM containing fetal calf serum, L-glutamine, and gentamicin as above. pNL4-3 was obtained from the AIDS Reference and Reagent Catalogue.

HIV-1 Tat. Recombinant Tat was prepared as described before (49). Briefly, the *tat* gene encoding amino acids 1 to 72 (first exon) from HIV-1_{HXB2} was expressed as a fusion protein with a naturally biotinylated protein at the N terminus in *Escherichia coli* DH5αF'IQ (Gibco-BRL). The biotin portion of the fusion protein was first bound to SoftLink soft-release avidin resin (Promega). Tat protein was then cleaved from the resin with factor Xa, a serine endopeptidase (Boehringer Mannheim). Dithiothreitol (DTT) was added in each step of the purification. Finally, Tat protein was suspended in a buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, 1 mM $CaCl_2$, and 0.5 mM DTT. The Tat protein was >95% pure by gel electrophoresis. The purified product was further analyzed by Western immunoblot analysis. Its biological activity was measured by its ability to activate the β-galactosidase (β-gal) gene in an HIV long terminal repeat (LTR)-β-gal plasmid which had been transfected into HeLa cells (AIDS Repository, National Institutes of Health).

Immunoabsorption of Tat. Antiserum to Tat was made following immunization of rabbits with recombinant Tat fusion protein (47a). Immunoabsorption of Tat was performed as described previously (49). Briefly, Tat antiserum was bound to protein A-Sepharose (Pharmacia), washed, and then incubated with Tat for 60 min at room temperature, followed by centrifugation. The supernatant was used in experiments that compared Tat-containing solution with solution from which Tat had been immunoabsorbed.

Nuclear extracts. For each experiment depicted in a single figure, cells were derived from the same tissue specimen and were manipulated similarly prior to treatments. Extracts were made from cells treated with either medium alone, medium with 2 μM Tat, medium from which the Tat had been immunoabsorbed, or medium containing 2 μM Tat in combination with polyclonal Tat neutralizing antibody (kindly provided by Barbara Ensoli). The neutralizing antibody had been in combination with Tat for 15 min prior to administration. Other cells were first treated with either cycloheximide (10 μg/ml; Sigma) for 10 min or 25 μM *n*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma) for 30 min prior to the addition of Tat. Sixty minutes following the addition of Tat-containing medium, control medium, or medium alone, nuclear extracts were prepared by the method of Andrews and Faller (4). This method allows the simultaneous preparation of extracts from multiple samples. Protein concentrations were determined by the method of Bradford (13).

Nucleic acid probes. DNA probes were prepared as described previously (7). The sequences were as follows: $NF\text{-}\kappa B_c$, CAA GGG ACT TTC CGC T; and $NF\text{-}\kappa B_m$, CAA GTT ACT TTA CGC T.

Electrophoretic mobility shift assays. The electrophoretic mobility shift assay was performed as described previously (7). Binding reactions included 10 μg of nuclear proteins and 1 ng of labeled probe. In competition experiments, a 100-fold excess of unlabeled wild-type or mutant competitor was added to the reaction mixture. Supershift studies with anti-p50 (kindly provided by Keith Brown), anti-p65 (Santa Cruz Biotechnology, Santa Cruz, Calif.), or control antiserum (DAKO Corp., Carpinteria, Calif.) were also performed as described previously (7).

Preparation of extracts for PKC assays. For each experiment that compared untreated with Tat- or control-treated astrocytes, cells were prepared from the same tissue specimen. Following 24 h of culture in serum-free medium, cells were incubated for 20 min in the presence or absence of 8 μM Tat in serum-free medium. The cells were then washed quickly in calcium-free phosphate-buffered saline (PBS) containing 2.5 mM EDTA and 2.5 mM EGTA (ethylene glycol tetraacetic acid). Cells were subsequently scraped, centrifuged, and resuspended in homogenization buffer, containing 25 mM Tris-HCl, 4 mM EGTA, 2 mM EDTA, 250 mM glucose, 5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were homogenized by 10 strokes in a Dounce homogenizer and then centrifuged at 100,000 × *g* for 30 min. The supernatant was saved as the cytoplasmic fraction. Homogenization buffer containing 0.5% Triton X-100 was then added to the pellet. Following resuspension by sonication, the suspension was centrifuged at 100,000 × *g* for 30 min. The supernatant was saved as the detergent-soluble membrane or particulate fraction. Using this fractionation method, we were able to detect a decrease in cytoplasmic and an increase in membrane-associated PKC following stimulation of astrocytes with phorbol myristate acetate (PMA).

PKC assay. The PKC assay was performed with the PKC assay kit (Promega). The procedure and the calculation of PKC activity were performed according to the manufacturer's instructions. This assay involves a sample-substrate reaction which is dependent upon the transfer of a radiolabeled phosphate to the PKC-specific substrate $neurogranin_{(28-43)}$ (18, 33). Additionally, the substrate is biotinylated, so that following its transfer to a streptavidin-coated disk, radioisotope that is not substrate associated can be washed from the disk. The amount of neurogranin-associated radioactivity is calculated following scintillation spectrometry.

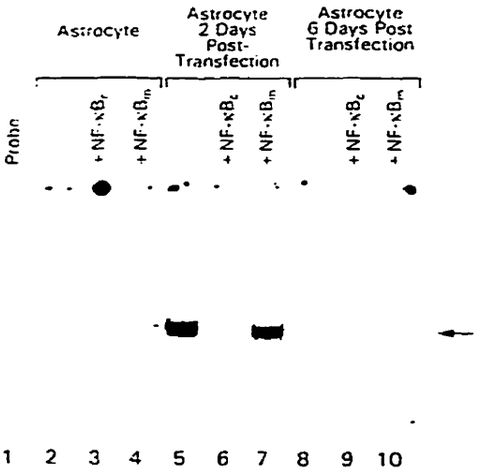

FIG. 1. Competitive gel shift analysis of the binding of astrocyte nuclear proteins to a ^{32}P-labeled NF-κB probe. Lane 1, free probe. Other lanes represent migration of probe that had first been incubated with the indicated nuclear extract. The κB-specific band (arrow) is competed away by an excess of unlabeled κB competitor (lanes 3, 6, and 9) but not by excess mutant κB competitor (lanes 4, 7, and 10).

RESULTS

Transfection of astrocytes with HIV-1 proviral DNA is associated with an increase in NF-κB binding. We have previously demonstrated that stimuli such as tumor necrosis factor alpha (TNF-α) and PMA are associated with an increase in HIV-1 expression in latently infected astrocytes as well as with an increase in p50/p65 NF-κB binding (7, 21). Figure 1 shows an electrophoretic mobility shift assay which demonstrates an increase in NF-κB binding following transfection of astrocytes with pNL4-3, an infectious molecular clone of HIV-1 (71). Lane 1 represents free probe, which has migrated off the gel. Other lanes compare nuclear proteins from astrocytes that were either not transfected (lanes 2 to 4), at 2 days posttransfection (lanes 5 to 7), or at 6 days posttransfection (lanes 8 to 10) for their ability to retard the migration of the radiolabeled κB probe. The band noted by the arrow represents protein bound specifically to the κB consensus site, since it is competed away by excess unlabeled κB competitor (lanes 6 and 9) but not by excess mutant κB competitor (lanes 7 and 10). This band is notably larger when nuclear extracts are made from cells at 2 days posttransfection. By 6 days following transfection, virus production is significantly diminished (71) and so too is NF-κB binding. NF-κB binding was not significantly increased at 2 days after mock transfection (not shown).

Treatment of astrocytes with exogenous Tat protein is associated with an increase in NF-κB binding. Because Tat is released from HIV-1-infected cells (27) and has been demonstrated to have effects which could result from the activation of specific transcription factors, we examined the effect of Tat protein on NF-κB binding in astrocytes. Figure 2A demonstrates that NF-κB binding is increased following stimulation of astrocytes with 2 μM Tat protein for 60 min. Lane 1 represents free probe, while in other lanes, nuclear extracts from untreated astrocytes (lanes 2 to 4) are compared with nuclear extracts from Tat-treated astrocytes (lanes 5 to 7). Again, the arrow denotes the κB-specific band, since it is diminished by excess unlabeled competitor (lanes 3 and 6) but not by excess

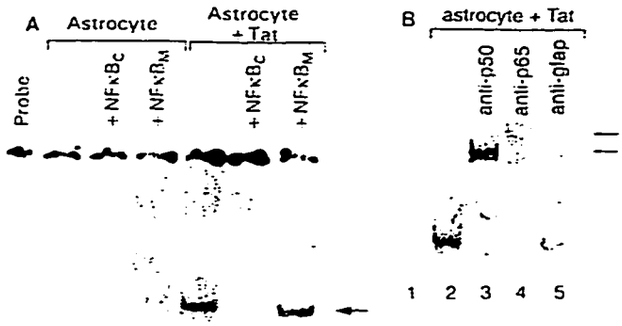

FIG. 2. (A) Competitive gel shift analysis comparing nuclear proteins from untreated astrocytes (lanes 2 to 4) with nuclear proteins from Tat-treated astrocytes (lanes 5 to 7) for their ability to retard the migration of a κB probe. The arrow again denotes the κB-specific band that is competed away by excess unlabeled κB competitor (lanes 3 and 6) but not by excess unlabeled mutant κB competitor (lanes 4 and 7). Lane 1, free probe, which has migrated off the gel. (B) Supershift analysis of Tat-associated κB binding activity. Lane 1, migration of free probe; lane 2, migration of probe with nuclear proteins from Tat-treated cells. Lanes 3 to 5, migration of the probe-protein complex following its incubation with anti-p50 (lane 3, lower arrow), anti-p65 (lane 4, upper arrow), or anti-glial fibrillary acidic protein (lane 5).

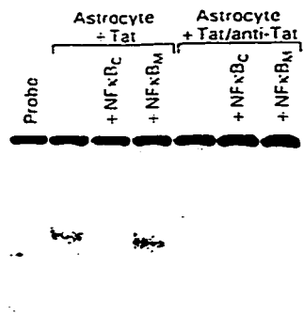

FIG. 3. Competitive gel shift analysis comparing nuclear proteins from astrocytes that were treated with Tat (lanes 2 to 4) with those from astrocytes that were treated with Tat that had first been incubated with a Tat-neutralizing antibody (lanes 5 to 7). The κB-specific band is denoted by the arrow. Free probe was run in lane 1.

mutant competitor (lanes 4 and 7). Full-length, commercially available Tat (Intracel, Cambridge, Mass.) was also associated with an increase in NF-κB binding (not shown). Figure 2B shows a supershift study demonstrating that the Tat-associated κB binding activity reacts with antiserum to either the p50 or the p65 NF-κB subunit. Additionally, this Tat-associated probe-protein complex migrated as did the p50-p65 NF-κB complex that is associated with TNF-α stimulation (not shown).

Tat-specific antibodies inhibit the Tat-associated increase in NF-κB binding. Because of the possibility that a factor other than Tat was responsible for the observed increase in NF-κB binding, we examined whether removal of Tat by immunoabsorption or treatment of Tat with neutralizing antibodies would inhibit the effect. Figure 3 shows a competitive gel shift analysis that compares Tat-treated astrocytes (lanes 2 to 4) with astrocytes which had received Tat in combination with a polyclonal Tat neutralizing antibody (lanes 5 to 7). This figure demonstrates that neutralizing antibody to Tat was associated with an inhibition of the effect of the Tat preparation. Immunoabsorption was also associated with a significant decrease in the intensity of the κB-specific band (not shown). Such data suggest that Tat was responsible for the increased binding activity of NF-κB to its LTR consensus sequence.

Tat-associated increase in NF-κB binding is independent of new protein synthesis. Because the increase in NF-κB binding occurred within 1 h, our observations may have resulted from a protein synthesis-independent increase in NF-κB binding. We therefore examined the effect of cycloheximide, an inhibitor of protein synthesis, on the ability of Tat to increase NF-κB binding. The results are demonstrated in Fig. 4. This competitive gel shift analysis shows that cycloheximide did not block Tat's ability to increase NF-κB binding. Cycloheximide itself was associated with a slight increase in NF-κB binding, an effect which has been observed in other cell types (68).

Chymotrypsin-like protease inhibitor TPCK inhibits Tat's effect. The chymotrypsin-like protease inhibitor TPCK acts on Iκ-Bα to prevent its breakdown and/or modification in response to varied stimuli (30, 43, 48, 69). Figure 5 shows a competitive gel shift analysis which demonstrates that 25 μM TPCK inhibits the Tat-associated increase in NF-κB binding. TPCK at 25 μM did not cause toxicity, as assessed by the trypan blue dye exclusion technique (not shown).

Inhibition of NF-κB binding by TPCK is consistent with the possibility that IκB-α degradation may be required for Tat's effect. However, it must be noted that TPCK could have effects in addition to its inhibition of IκB-α degradation. For example, in certain experimental conditions, this compound may modify NF-κB and thus affect its DNA-binding ability (30).

Extracellular Tat protein is associated with an increase in cytoplasmic PKC activity. Because stimuli which increase NF-κB binding in a protein synthesis-independent manner are often associated with the activation of protein kinases, we examined the effect of extracellular Tat on PKC activity in astrocytes. In four PKC assays which compared PKC activity in untreated and 8 μM Tat-treated astrocytes, treatment with Tat was associated with a 2.3-fold increase in cytoplasmic PKC

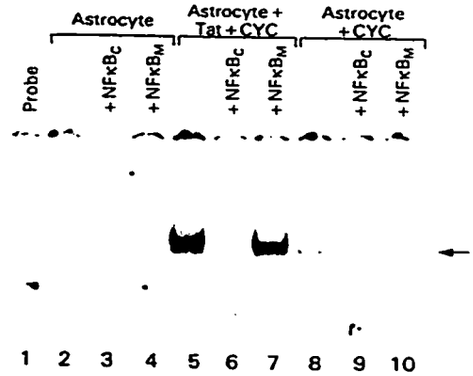

FIG. 4. Competitive gel shift analysis demonstrating that cycloheximide (CYC) did not block the ability of exogenous Tat to increase NF-κB binding. The arrow denotes the κB-specific band. Astrocytes that were treated with Tat and cycloheximide (lanes 5 to 7) demonstrated an increase in NF-κB binding compared with untreated astrocytes (lanes 2 to 4). Cycloheximide alone (lanes 8 to 10) was associated with a lesser increase in NF-κB binding, an effect which has been observed in other cell types (66).

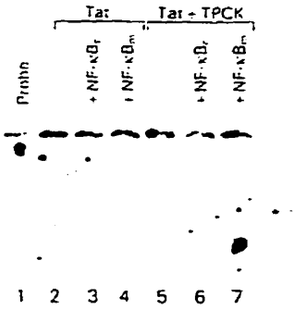

FIG. 5. Administration of TPCK is associated with inhibition of the Tat-related increase in NF-κB binding. Lanes 2 to 4, migration of the κB probe in the presence of nuclear extracts from Tat-treated astrocytes; lanes 5 to 7, migration of this probe in the presence of nuclear extracts from astrocytes that received both Tat and TPCK. The arrow denotes the κB-specific band.

activity (mean values ± standard error, 254.8 ± 67.9 and 592.8 ± 152.3 pmol of [γ-^{32}P]ATP/min/mg of protein, respectively). Immunoabsorption of Tat significantly inhibited the increase (not shown).

DISCUSSION

In the present study, we demonstrate that in cultures of primary human fetal astrocytes, extracellular Tat is associated with a protein synthesis-independent increase in the binding activity of NF-κB. While the mechanism is unknown, possibilities include receptor-mediated kinase activation as well as Tat-associated oxidative stress (31, 65, 66). We also demonstrate a Tat-related increase in cytoplasmic PKC activity. We were not able to demonstrate an increase in membrane-associated PKC activity (not shown). While certain stimuli are associated with an increase in membrane-associated PKC activity, depending on the cell type, the stimulus, and the isozyme examined, an increase in cytoplasmic or nuclear PKC activity may also occur (10, 34, 35, 62, 77). Additionally, we demonstrate that transfection of astrocytes with HIV-1 proviral DNA is associated with an increase in NF-κB binding. While our studies support the possibility that the transfection-associated increase in NF-κB binding could be related to Tat protein, they do not formally demonstrate this. In fact, Tat may not be the only HIV-1-associated protein that can lead to an increase in NF-κB binding and/or the activation of certain kinases. For example, the HIV-1 envelope protein gp120 has been associated with changes in ion conductance in astrocytes (11) as well as with the activation of certain kinases in other cell types (20).

Furthermore, by having demonstrated a protein synthesis-independent increase in NF-κB binding, we do not rule out an effect of Tat at other levels. Tat may also directly activate the promoter of cellular genes (17). Indeed, other stimuli, such as the human T-cell leukemia virus type 1 Tax protein, have been demonstrated to increase NF-κB binding by several mechanisms (5, 41, 45, 69).

Of additional note is that while we demonstrate a Tat-associated increase in PKC activity, we do not demonstrate that increased PKC activity is responsible for the increase in NF-κB binding. In fact, we were not able to block the Tat-associated increase in NF-κB binding with the PKC inhibitors H7 (39) and bisindolylmaleimide 1 (72). The requisite kinase may be activated prior to PKC or via an alternative pathway. Alternately, it may be that a PKC isozyme which is relatively less sensitive to inhibition by these compounds, such as PKCζ, may be involved in the NF-κB translocation (23, 24). However, we cannot conclude from the present study whether the activity of this particular isoform was increased. A PKC-independent increase in NF-κB binding has been described for TNF-α. This cytokine is associated with an increase in PKC activity but can, as a result of its activation of an additional kinase(s) (25, 67), increase NF-κB binding in a PKC-independent manner (53).

The demonstration that extracellular Tat is associated with an increase in both NF-κB binding and PKC activity has several implications. Interestingly, Tat-dependent HIV-1 transcription may depend on the binding of NF-κB to its LTR consensus sequence (2, 46). Additionally, some of Tat's previously described effects may be in some part related to increases in the activity of NF-κB and/or PKC. Tat has been associated with TAR-independent activation of the LTR (8). Tat also affects neuron function (49, 61), cytokine production (17, 28, 58), cell growth (9, 29, 47, 54), and cell death (44, 79). NF-κB is known to increase the expression of a number of cytokines (36, 69), while PKC acts as a growth factor in certain situations (37, 56, 73, 78). Additionally, both NF-κB (12) and PKC (42, 51, 52, 57) may have a role in apoptosis. Some studies suggest that PKC has a protective role, so that an increase in its activity could represent a protective response to an apoptotic signal.

Of note is that the effects that we have observed are dependent on the first 72 amino acids (encoded by the first exon) of the 86-amino-acid full-length Tat protein. This protein includes the domains which are important for HIV-1 replication (32). It does not include the Arg-Gly-Asp (RGD) sequence that may be important for Tat's interaction with certain integrin receptors, such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (9, 14). It does, however, include the basic domain which may mediate Tat's binding to the $\alpha_v\beta_5$ integrin (74). The basic domain has been determined to be responsible for many of Tat's effects, including chondrocyte proliferation and synthesis of transforming growth factor β (47) as well as receptor-mediated neurotoxicity (49, 61). Tat may also enter cells in a receptor-independent manner (50). Whether our observations are receptor dependent is unknown.

In summary, we have demonstrated some previously unreported effects of extracellular Tat protein, defined by the product of the first exon. Indirect effects of HIV-1 infection in the nervous system may be in part related to the action of viral gene products such as Tat on astrocytes. Additionally, the extension of these observations to other cell types and the study of whether such findings are relevant in vivo may ultimately improve our understanding of HIV-1 pathogenesis.

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Human immunodeficiency virus type 1 Tat protein induces death by apoptosis in primary human neuron cultures

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Running Title: Tat-induced apoptosis of human neurons

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Abstract

The neuropathogenesis of human immunodeficiency virus type 1 (HIV-1) infection may result in neuronal loss in the cortex and subcortical gray regions. The mechanisms for neuronal loss remain a mystery since HIV-1 productively infects macrophage and microglia but does not infect neurons in the central nervous system (CNS). Apoptosis is one mechanism that has been suggested for the loss of neurons in HIV-1 infected brain. Putative toxic factors that result in neuronal cell death in HIV-1 infection include the regulatory protein Tat, since this protein is known to be released from HIV-1 infected cells. Tat is neurotoxic in *in vivo* rodent models as well as in human and rodent *in vitro* models, but the mechanism of Tat-induced neurotoxicity remains to be determined. Here we show that Tat induces cell death by apoptosis in cultured human fetal neurons producing characteristic morphological and biochemical features associated with apoptosis. Apoptotic cell death was dose-dependent in cultured human fetal neurons, with an LD_{50} of approximately 0.5μM. These findings suggest that Tat may play an important role as a secreted, soluble neurotoxin in HIV-1 associated dementia.

Introduction

Neurodegeneration associated with infection of the central nervous system (CNS) by HIV-1 results in a CNS dysfunction termed HIV-1 associated dementia (Navia et al., 1986). The pathogenesis of HIV-1 associated dementia is not clear, but it is unlikely that productive infection of neurons with HIV-1 is responsible. In fact, HIV-1 infected neurons have rarely been demonstrated in post-mortem tissue, while macrophages and microglia are primarily found to be productively infected with the virus (Takahashi et al., 1996; Bernton et al., 1992; Wiley et al., 1991; Watkins et al., 1990; Wiley et al., 1986). HIV-1 infection in the brain results in widespread myelin pallor, reactive astrogliosis, alterations of neocortical dendritic processes and neuronal loss without necrosis (Epstein et al., 1993; Sharer, 1992; Wiley et al., 1991). Several studies have demonstrated that HIV-1 infection of macrophages and microglia release soluble toxic factors that mediate neuronal death in *in vitro* models of HIV-1 neurotoxicity (Gelbard et al., 1994; Genis et al., 1992; Merrill et al., 1991; Giulian et al., 1990).

Recent evidence in post-mortem AIDS brain tissue indicates that neuronal apoptosis was present in cases with productive HIV-1 infection (Adle-Biassette et al., 1995; Gelbard et al., 1995; Petito et al., 1995), but the cause of this pathological finding remains to be determined. Several authors have cited indirect mechanisms of neurotoxicity via macrophage-astroglial interactions mediated by a number of soluble factors including the cytokine tumor necrosis factor-alpha, and the phospholipid mediator platelet-activating factor (Gelbard et al., 1994; Genis et al., 1992; Merrill et al., 1991). Also *in vitro* studies indicate that the HIV-1 regulatory protein known as Tat, is released from infected cells (Ensoli et al., 1992). Several studies have shown that Tat is neurotoxic (Nath et al., 1996; Magnuson et al., 1995; Philippon et al., 1994; Hayman et al., 1993; Sabatier et al., 1991). Tat is a viral trans-activator, and is expressed early in the viral life cycle (Sodroski et al., 1985). It activates transcription directed by the HIV-1 long terminal repeat (LTR), which in turn leads to increased expression of all viral genes, (Arya et al., 1985; Sodroski et al., 1985) and some cellular genes (Ensoli et al., 1992; Buonaguro et al., 1992; Helland et al., 1991; Vogel et al., 1988). Neurotoxicity by Tat has been shown *in vivo* (Philippon

et al., 1994; Hayman et al., 1993) as well as *in vitro* (Nath et al., 1996; Magnuson et al., 1995; Sabatier et al., 1991). This protein has been shown to induce neurotoxicity in human fetal neuron cultures which is mediated via excitatory amino acid receptors (Nath et al., 1996; Magnuson et al., 1995). The Tat protein has also been linked to apoptotic cell death in cultured peripheral blood mononuclear cells as well as a CD4-positive ($CD4^+$) T cell line (Li et al., 1995; Purvis et al., 1995; Westendorp et al., 1995a & 1995b). In this study, we demonstrate that recombinant-Tat induces apoptosis in cultured human fetal cortical neurons in a dose dependent fashion.

Results

In situ detection of Tat induced apoptosis in human fetal neurons. Second trimester human cortical neuronal cultures, 2 to 3 weeks post-explantation, were treated with recombinant Tat_{1-86} protein at a concentration of 0.5μM for 18 hours. Utilizing the *in situ* TUNEL stain as described in Methods, neurons undergoing apoptosis were immunocytochemically stained for the free 3'-OH ends of cleaved DNA (Figure 1). 50% or greater TUNEL staining of neurons were consistently noted. Positively stained neurons were observed with chromatin aggregation, nuclear condensation, and apoptotic bodies, features consistent with apoptosis. Neuron cultures were also treated with the vehicle control, with only rare TUNEL staining observed (Figure 1).

Electron microscopy analysis of Tat treated neurons. Morphological indicators of apoptosis in the human fetal neurons treated with recombinant Tat_{1-72} were analyzed by transmission electron microscopy (Figure 2). A number of changes consistent with apoptosis were observed in a subpopulation of cells. Most commonly, condensation of the nuclear chromatin with relative preservation of the cytoplasmic structures, and the nuclear and cell membranes was noted. Blebbing of the cell membrane was seen in some cells. These cells showed only minimal changes in the nucleus and hence cell membrane blebbing was recognized as an early morphological change. Other cells showed partitioning and condensation of cytoplasm and nuclear material into membrane bound apoptotic bodies demonstrating end stage apoptotic changes. No phagocytic cells were seen in these sections. The above morphological

changes were absent in the untreated cultures or in cultures treated with solutions from which Tat had been immunoabsorbed.

Cellular DNA analysis for Tat induced apoptosis. DNA was extracted from Tat_{1-72} treated neurons and analyzed by agarose gel electrophoresis. A characteristic 180 base pair DNA cleavage ladder pattern specific for apoptosis was observed (Figure 3). No laddering was observed in the untreated cultures.

Dose response of Tat induced apoptosis. Tat-induced apoptosis was found to be dose dependent and was detectable at doses as low as 0.125μM and as high as 0.5μM (Figure 4). Doses of 1μM or greater resulted in cytotoxicity of such magnitude that few cells remained attached by 18 hours of treatment (data not shown). The dose response curve generated from our data indicates an LD_{50} for neuronal apoptosis of 0.5μM.

Discussion

The phenomenon of apoptosis is usually associated with development, homeostasis, and aging processes. In HIV-1 infection apoptosis occurs in the $CD4^+$ subpopulation of lymphocytes in the peripheral blood as a consequence of the infection (Meyaard et al., 1992). The protein Tat also induces apoptosis in $CD4^+$ lymphocytes (Ehret et al., 1996; Westendorp et al., 1995a & 1995b). In the CNS it has been suggested that pathological apoptosis occurs resulting in the loss of neurons (Adle-Biassette et al., 1995; Gelbard et al., 1995; Petito et al., 1995). This study focused on whether the HIV-1 regulatory protein Tat may play a role in mediating neuronal apoptosis.

HIV-1 infection of the brain primarily involves direct infection of macrophage and microglia cells (Takahashi et al., 1996; Bernton et al., 1992; Wiley et al., 1991; Watkins et al., 1990; Wiley et al., 1986). Neuronal loss in HIV-1 infection is not directly due to the virus but may be due to toxic viral products that are secreted from infected cells. Tat expression has been observed in HIV-1 infected individuals, therefore Tat is released extracellularly at sometime during HIV-1 infection, but to date the amount of Tat in the CNS has not been determined (Ranki et al., 1995). The HIV-1 regulatory protein Tat is one of many viral and cellular products

secreted from HIV-1 infected cells. It has been suggested that Tat may be transported transcellularly from either infected or transfected cells via direct cell to cell contact in amounts sufficient to transactivate Tat-responsive promoter elements (Helland et al., 1991; Marcuzzi et al., 1992a & 1992b). This suggests that exogenous Tat may affect uninfected cells during the course of HIV-1 infection.

Tat-induced neurotoxicity has been demonstrated in both *in vivo* as well as *in vitro* studies (Nath et al., 1996; Magnuson et al., 1995; Philippon et al., 1994; Dawson et al., 1991; Sabatier et al., 1991). Here we show convincing evidence that the HIV-1 protein Tat induced the morphological and biochemical features of apoptosis in cultured human neurons. Tat-induced apoptosis was demonstrated by several assays. First, we detected the presence of apoptotic neurons by demonstrating chromatin condensation and the formation of apoptotic bodies by *in situ* nick end labeling. We also demonstrated chromatin condensation and aggregation that abuts the inner surface of the nuclear membrane, and blebbing of the cytoplasmic cell membrane along with complete preservation of the integrity of the cytoplasmic organelles in the electron micrographs. Finally we demonstrated the typical DNA fragmentation ladder characteristic of apoptosis in the neuron cultures treated with Tat.

The Tat protein contains a basic region in the first exon which, when infused into the murine lateral ventricle, hippocampus or thalamus produced an inflammatory reaction characterized by macrophage recruitment and astrogliosis, accompanied by loss of neurons in the grey matter (Philippon et al., 1994). Tat-injected brain regions expressed elevated levels of TNF-α, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS). This suggests that Tat mediated toxicity may involve the cytokine TNF-α which can induce neuronal apoptosis (Talley et al., 1995). Blockade of TNF-α by pentoxifylline treatment led to the decrease of IL-1β and iNOS expression accompanied by a reduction of the volume of the lesions indicating that the Tat-induced lesions might be mediated by TNF production (Philippon et al., 1994). Tat-induced toxicity was also arginine-dependent, suggesting involvement of nitric oxide (NO), in NMDA-mediated neurotoxicity (Dawson et al., 1991).

Recently, Tat was shown to activate non-NMDA excitatory amino acid (EAA) receptors and cause neurotoxicity in cultured neurons leading to increases in intracellular calcium and cell death (Nath et al., 1996; Magnuson et al., 1995). The neurotoxic domain resides within a conformationally dependent epitope within the first exon of Tat between residues 31 to 61 (Nath et al., 1996). These increases in intracellular calcium may induce apoptosis by activation of cellular enzymes (Conant et al., 1996).

The full length Tat protein has been shown to suppress the expression of manganese superoxide dismutase (Mn-SOD) and enhance apoptosis in $CD4^+$ T cells (Flores et al., 1993; Westendorp et al., 1995a & 1995b). This is significant because reduced Mn-SOD expression may result in dysfunction of mitochondrial activity and oxidative stress. Oxidative stress has also been shown to induce apoptosis in cultured embryonic cortical neurons (Ratan et al., 1994).

Thus, Tat and basic domain Tat peptides induce death in a subpopulation of cultured human fetal cortical neurons with the characteristic morphological and biochemical features of apoptosis. Tat-induced necrosis of neurons was not observed at the light or electron microscope level. Our studies indicate that the first exon of Tat_{1-72} is sufficient to cause cell death. Tat-induced apoptosis was only observed in cortical neurons and not in astrocytes (New, Angel, unpublished observations). We speculate that Tat-induced apoptosis can occur by more than one pathway, including glutamate receptor activation, oxidative stress and signaling via the pro-inflammatory cytokine TNF-α. The finding that Tat is a potent HIV-1-induced neurotoxin with an LD_{50} of 0.5μM suggests that it may play a highly significant role in mediating neuronal apoptosis.

Materials And Methods

Primary human neuron cultures. Human fetal brain tissue between gestational ages of 13 to 15 weeks were obtained, with consent, from women undergoing elective termination of pregnancy, under the guidelines of the National Institutes of Health, the University of Rochester Human Subjects Review Board, and the Human Ethics Committee at the University of Manitoba. Adherent blood vessels and meninges were removed and the brain tissue was washed in cold

Hanks balanced salt solution (containing Ca^{2+}, Mg^{2+}, HEPES and 50 µg of gentamicin per ml). Brain tissue was cut into 2-mm^3 pieces with scissors then forced through a 230-µm Nitex bag (Tetko, Inc., Elmsford, NY). The cells were centrifuged at 100 x g for 5 min. at 4°C and resuspended in MEM-hipp (2 mM D-glucose, 10 mM HEPES, 1 mM sodium pyruvate, 20 mM KCl) containing N1 components (insulin at 5 mg/liter, transferrin at 5 mg/liter, selenite at 5 µg/liter, progesterone at 20 nM, and putrescine at 100 µM), as well as 5% heat-inactivated fetal bovine serum, PSN antibiotic mix (penicillin at 50 mg/liter, streptomycin at 50 mg/liter, and neomycin at 100 mg/liter), and amphotericin B (Fungizone; 2.5 mg/liter). Cells were plated at a density of 10^5/ml on 12 mm-diameter glass coverslips precoated with poly-L-lysine (70K-150K MW; Sigma), and placed in 24-well culture dishes. Cells were cultured 14 to 28 days at 37°C in a humidified atmosphere of 5% CO_2-95% air, and the medium was changed every 3 days. Sample cultures were stained for the neuroendocrine specific protein, PGP 9.5, a major protein component of neuronal cytoplasm, and glial fibrillary astrocyte protein (GFAP), under these culture conditions neuronal cultures were >70% homogenous for neurons. The remaining cells were predominantly astrocytes and <5% were microglia-macrophages, as determined by RCA-1 lectin and CD68 staining.

HIV-1 Tat. Recombinant HIV-1 Tat_{1-86} was expressed and purified as a glutathione S-transferase fusion protein. The *Escherichia coli* (*E. coli*) strain BL21 harboring the GST-Tat_{1-86} expression plasmid is grown to log phase in Luria Broth containing 50µg/ml ampicillin at 37°C (Herrmann et al., 1993). This clone contains a thrombin proteolytic site between the *Schistosoma japonicum* glutathione S-transferase (GST) sequence and the *tat* insert encoding both the first and second exons (amino acids 1 to 86). The HIV-1 (HXB2) *tat* clone was provided in an ampicillin resistant, transformed BL21 *E. coli* bacteria by Dr. Andrew Rice (AIDS research and reference reagent program, NIAID, NIH; Herrmann et al., 1993). Expression of the fusion protein was induced with Isopropylthio-ß-galactoside. Following lysis and sonication of the bacteria, the crude GST-Tat_{1-86} extract was isolated by centrifugation then bound to equilibrated glutathione-sepharose beads (Pharmacia). A thrombin proteolytic digestion procedure was done

to complete the purification of the Tat_{1-86} from the GST bound to the glutathione coated beads. The preparations were stored at -70°C until use. Purified Tat_{1-86} was further characterized and quantified by Lowry, SDS-PAGE, and Western Blot with a polyclonal antibody (AIDS Research and Reference Reagent Program, NIAID, NIH; Hauber et al., 1987).

To control for the expression of bacterial proteins which could give false positive neurotoxicity, an *E. coli* strain containing a GST expression plasmid without the Tat_{1-86} sequence was also expressed and purified. The sham purified Tat from the GST expression plasmid was used as a vehicle control.

The *tat* gene encoding amino acids 1 to 72 (first exon) from $HIV\text{-}1_{BRU}$ was expressed as a fusion protein with a naturally biotinylated protein at the N-terminus in *E. coli* DH5aF'IQ (Gibco BRL). The biotin portion of the fusion protein was first bound to Softlink™ soft-release avidin resin (Promega). Tat protein was then cleaved from the resin with factor Xa, a serine endopeptidase (Boehringer Mannheim). Dithiothreitol (DTT) was added in each step of the purification. Finally, Tat protein was suspended in a buffer containing 50mM Tris (pH 8.0), 100mM NaCl, 1mM $CaCl_2$ and 0.5mM DTT. The Tat protein was 95% pure by gel electrophoresis. The purified product was further analyzed by Western immunoblot analysis. Its biological activity was measured by its ability to activate the ß-galactosidase (ß-gal) gene in an HIV-1 long terminal repeat (LTR)-ß-gal plasmid which had been transfected into HeLa cells (AIDS Repository, NIH) (Conant et al., 1996).

In situ detection of apoptotic neurons by TUNEL stain. Human fetal neurons cultured on 12 mm poly-L-lysine coated coverslips were treated with Tat at doses ranging from 0.125µM to 1µM for 18 hours. The Tat_{1-86} protein at 0.5µM (as determined from dose response curve) was used as a standard dose for expressing apoptosis following 18 hours of Tat exposure. The cultures were assessed for apoptotic expression and apoptotic neurons were counted from 16 randomly selected fields. Each field of at least 100 cells was counted for positively stained versus negatively stained cells. The apoptotic cells were stained with an *in situ* terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling (TUNEL) assay

which was supplied as a kit (Oncor, Gaithersburg, MD). Neurons stained by the TUNEL assay were first rinsed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde. The paraformaldehyde was removed and the neurons were rinsed again with PBS then post-fixed with 100% ethanol: acetic acid solution (2:1) and rinsed with PBS. Neurons were pretreated with 2% H_2O_2 to quench endogenous peroxidase prior to the addition of the terminal deoxynucleotidyl transferase (TdT), an enzyme which catalyzes a template independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA. Following the addition of TdT and the resulting incorporation of nucleotides that form heteropolymers of digoxigenin-11-dUTP and dATP, anti-digoxigenin-peroxidase is added which binds to the heteropolymers. The peroxidase on the anti-digoxigenin-peroxidase antibody was then catalytically reacted with 0.05% diaminobenzidine (DAB) in phosphate buffered saline, the result was an intense precipitate signal that was visible by light microscopy.

Electron microscopy analysis of neurons. Human fetal brain cultures were treated with 1μM Tat for 18 hrs. The cells were scraped and fixed in 2% glutaraldehyde for 60 min. at room temperature. A 0.1M sodium cacodylate solution was used between each step for washing. The cells were suspended in agarose at 40°C for 5 min. The gel was cut into $1mm^3$ blocks and fixed in 1% osmium tetroxide for 1 hour on ice. The cells were then dehydrated in successively increasing concentrations of acetone followed by propylene oxide and then embedded in Epon 812/ araldite 502 (Marivac, Halifax, NS). Sections were made with an ultra-microtome and placed on nickel grids. The cells were stained with Reynolds lead citrate and viewed on a transmission electron microscope (Hayat 1981). Control cultures which had been treated with solution following immunoabsorbtion of Tat as previously described or untreated cultures were similarly processed for electron microscopy.

DNA extraction and electrophoresis. Human fetal neuronal cultures (4×10^6 cells) were treated with 2.4μM Tat_{1-72} for 18 hrs. The neurons were separated by vigorous shaking and harvested by centrifugation at 400 g for 5 min. The cell pellet was lysed in 0.5 ml lysis buffer (20mM Tris-HCl pH 7.5, 4mM EDTA, 3% SDS, 0.5 mg proteinase K per ml) at 50°C for 1 hour.

RNase H (20g/ml, Boehringer Mannheim) was then added and the incubation continued for another 1 hour. The lysate was centrifuged at 12,000 x g for 10 min. and the supernatant was extracted with phenol followed by chloroform-isoamyl alcohol (24:1) and precipitated with ethanol at -70°C. The DNA pellet was dried and resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA). The DNA was analyzed by 1.8% agarose gel electrophoresis.

Computerized morphometry and statistical analysis. Digitized images of TUNEL stained neurons in 16 or greater microscopic fields were analyzed for numbers of positively stained neuronal nuclei divided by total numbers of neurons per 50x field using computerized morphometry (Imaging Research Inc., Ontario, Canada). Data were expressed as means ± SEM, with significance determined by one-way ANOVA.

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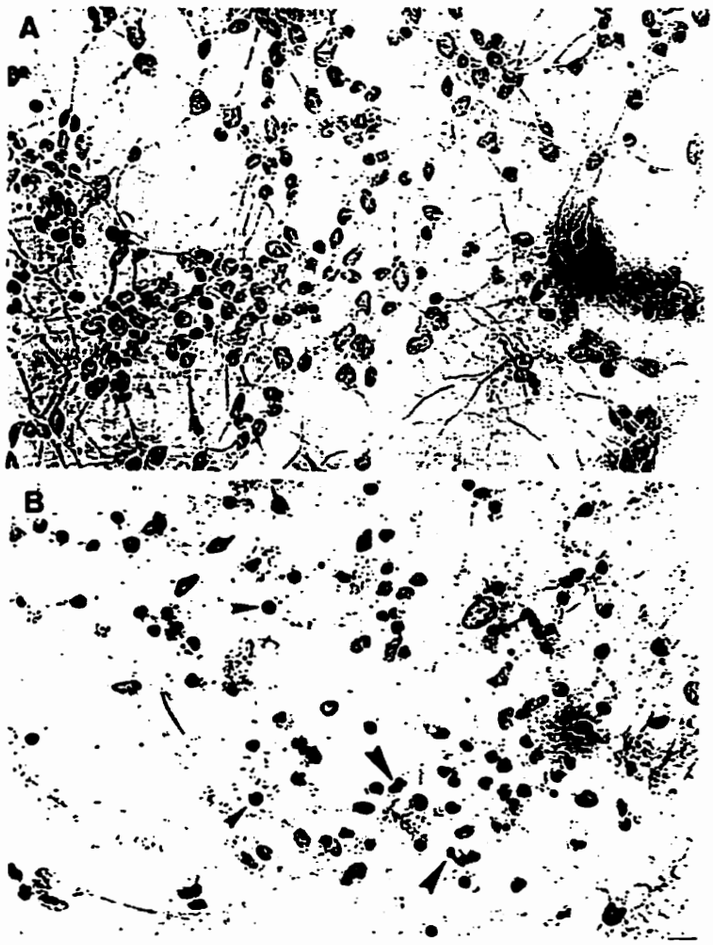

Figure 1: Primary human fetal neurons cultured for 4 weeks were treated with vehicle (A) or 0.5μM of Tat (B) for 18 hours. Neurons were subsequently fixed and stained *in situ* for new 3'-OH DNA ends generated by DNA fragmentation that results in chromatin condensation, a morphologic feature of apoptosis. The TUNEL assay or active labeling of cells by end labeling utilizing the "Apoptag kit" (Oncor, Gaithersburg, MD) is indicated by the black precipitate seen as chromatin condensation (small arrows), and apoptotic bodies (large arrows).

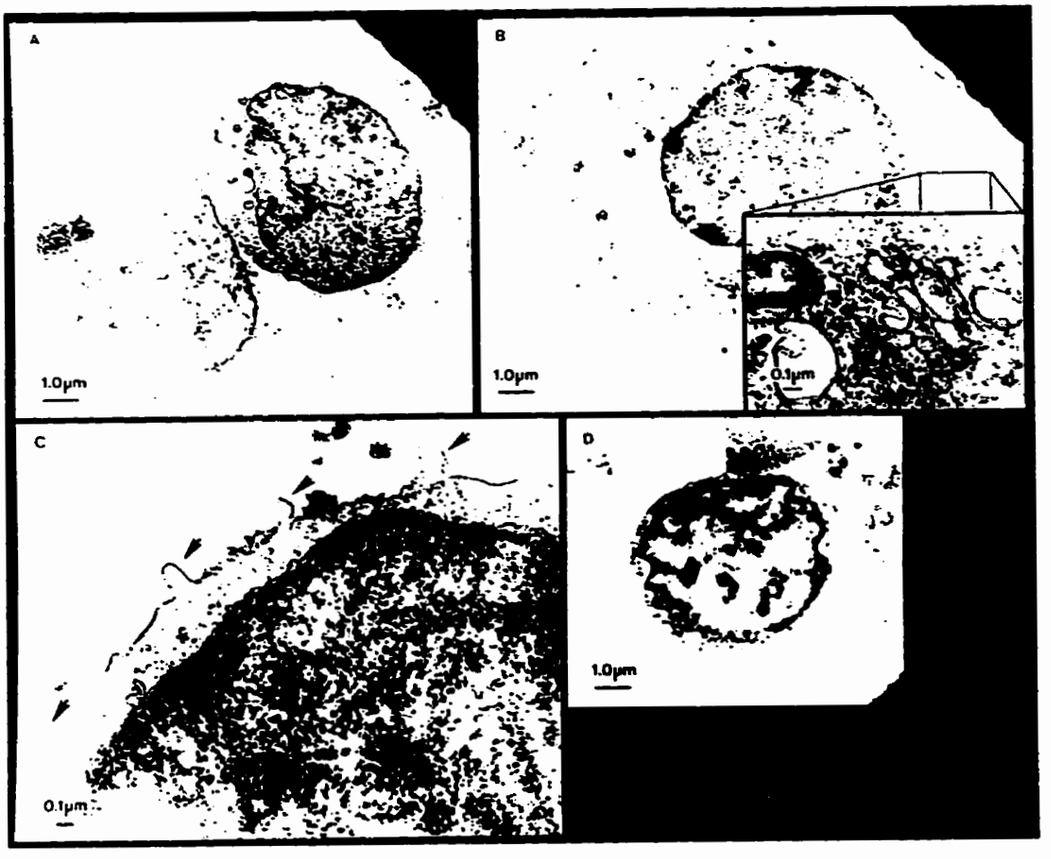

Figure 2: Morphological characteristics of Tat-induced apoptosis. (A) Two neurons are seen in close proximity. The one on the right shows normal morphological features while the other neuron shows degradation and condensation of the nuclear chromatin with preservation of the nuclear and cell membrane. (B) Condensation of nuclear chromatin with relative preservation of the cytoplasmic structures, Insert shows normal mitochondria and golgi apparatus. (C) Arrows show typical blebbing of the cytoplasmic cell membrane. (D) Another cell with clumping of the chromatin, degradation of cytoplasmic structures, but relative preservation of the nuclear and cell membranes.

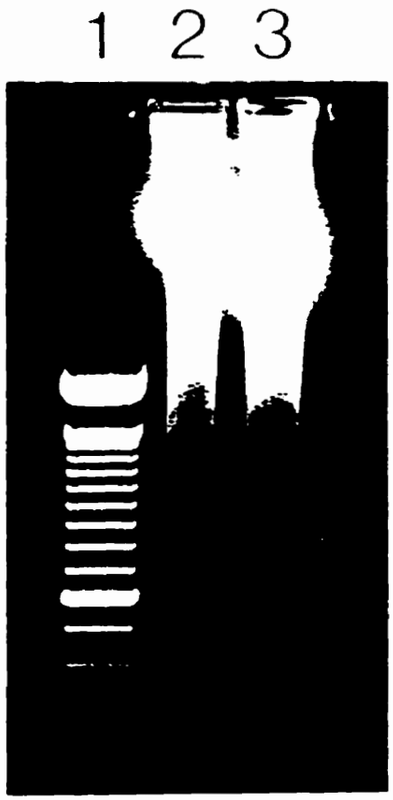

Figure 3: Tat induced DNA laddering. Lane 1. Molecular weight markers (1kb) Lane 2. DNA extracts from Tat treated human fetal neurons analyzed by agarose gel electrophoresis show a 180 base pair ladder. Lane 3. Untreated neurons show absence of laddering.

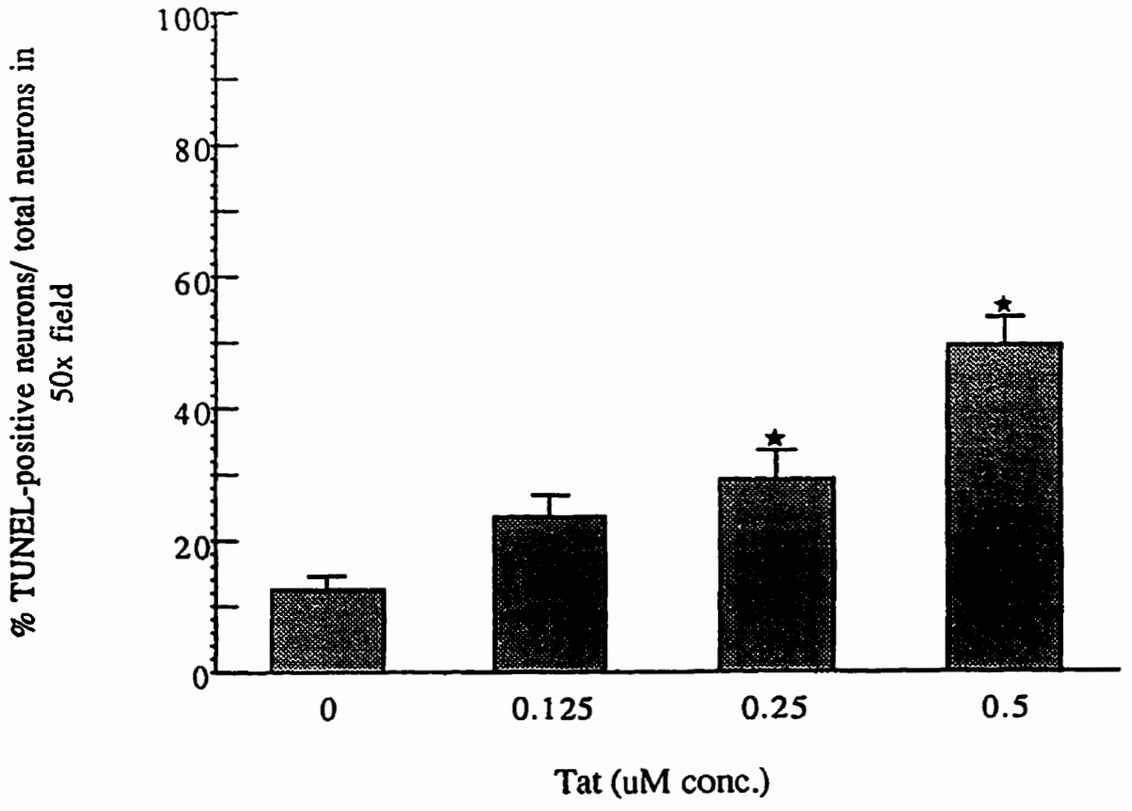

Figure 4: Primary human fetal neuron cultures were prepared as described in Methods. Neuron cultures were treated with Tat at the following doses; 0, 0.125, 0.25 and 0.5 μM for 18 hours. Cultures were fixed and TUNEL stained as described in Methods, the positive apoptosis immunostained neurons were analyzed in 16 random fields of each treatment by computerized morphometry. The histogram bars represent the average percent of TUNEL-positive apoptotic cells per total neurons per 50x field, SEM is shown by vertical lines (★ = $p<0.001$ vs. control). This initial dose response curve at 18 hours indicates an LD_{50} at 0.5μM, a higher dose at 1μM was also analyzed, but the toxic response resulted in detached cells.