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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 IκB-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_{BRU} 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 cyclohexamide (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-$κB_c$, CAA GGG ACT TTC CGC T; and NF-$κB_m$, CAA G**TT** ACT TT**A** 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.

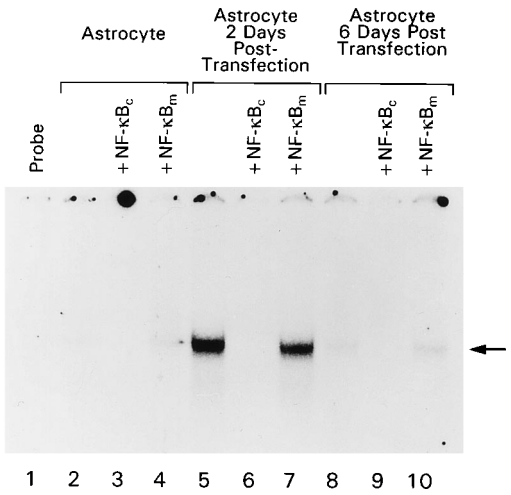

FIG. 1. Competitive gel shift analysis of the binding of astrocyte nuclear proteins to a ^{32}P-labeled NF-κB probe. Lane 1, free probe. Other lanes represent migration of probe that had first been incubated with the indicated nuclear extract. The κB-specific band (arrow) is competed away by an excess 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

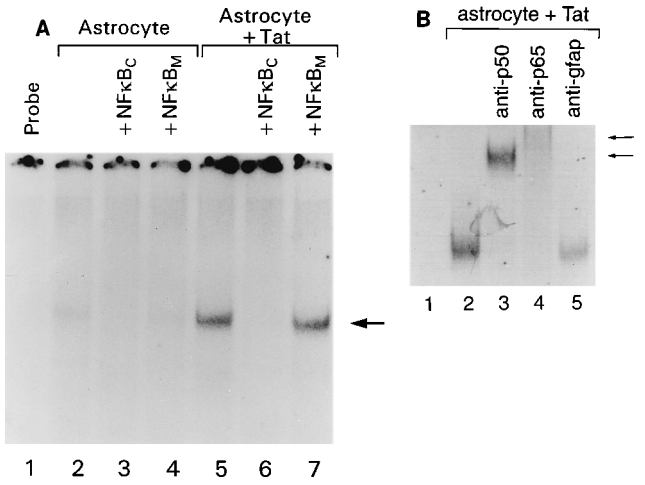

FIG. 2. (A) Competitive gel shift analysis comparing nuclear proteins from untreated astrocytes (lanes 2 to 4) with nuclear proteins from Tat-treated astrocytes (lanes 5 to 7) for their ability to retard the migration of a κB probe. The arrow again denotes the κB-specific band that is competed away by 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).

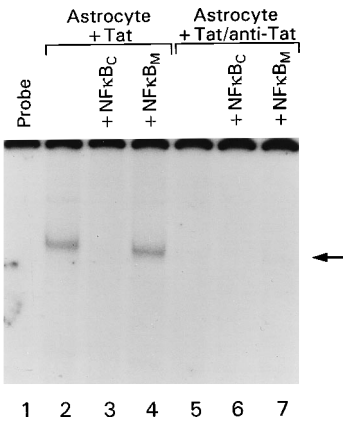

FIG. 3. Competitive gel shift analysis comparing nuclear proteins from astrocytes that were treated with Tat (lanes 2 to 4) with those from astrocytes that were treated with Tat that had first been incubated with a Tat-neutralizing antibody (lanes 5 to 7). The κB-specific band is denoted by the arrow.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

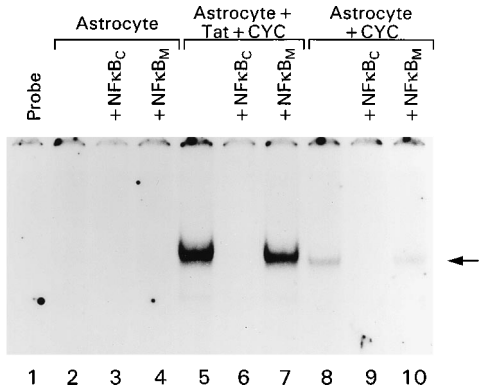

FIG. 4. Competitive gel shift analysis demonstrating that cycloheximide (CYC) did not block the ability of exogenous Tat to increase NF-κB binding. The arrow denotes the κB-specific band. Astrocytes that were treated with Tat and cycloheximide (lanes 5 to 7) demonstrated an increase in NF-κB binding 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).

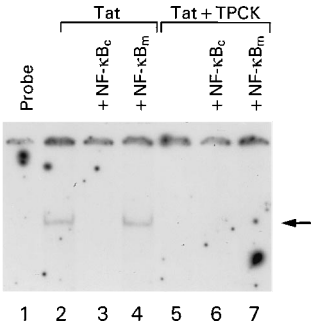

FIG. 5. Administration of TPCK is associated with inhibition of the Tat-related increase in NF-κB binding. Lanes 2 to 4, migration of the κB probe in the presence of nuclear extracts from Tat-treated astrocytes; lanes 5 to 7, migration of this probe in the presence of nuclear extracts from astrocytes 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. Alternatively, 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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