Evidence of Protein Transduction but Not Intercellular Transport by Proteins Fused to HIV Tat in Retinal Cell Culture and *in Vivo*

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The human immunodeficiency virus type-1 Tat protein is known to exit virally infected cells and enter the nucleus of adjacent uninfected cells. This property has been mapped to an 11-amino-acid protein transduction domain (PTD). When the PTD of Tat is fused to heterologous proteins and added exogenously to cells, the fusion peptide is able to demonstrate protein transduction across plasma membranes. Recent reports indicate that endogenously expressed Tat fusion peptides can demonstrate intercellular transport and improve biodistribution of therapeutic protein in the context of adenovirus vectors. Intercellular transport and protein transduction have not been observed in some studies and in the former have been attributed to an artifact of fixation. We have attempted to resolve these studies using an approach that unambiguously distinguishes cells that express Tat fusion protein from those that receive it from their environment. We find no evidence of intercellular transport in the context of an adenovirus vector in cell culture or *in vivo*. Instead, we find that Tat fusion peptides are down regulated in terms of expression not only in the context of adenovirus vectors, but also when expressed from transfected plasmid DNA. However, when Tat fusion peptides are released from cells by degradation of the plasma membrane, the fusion peptides demonstrate protein transduction without the need for cell fixation, indicating a unidirectional transport of Tat fusion proteins across the plasma membrane. Our data are consistent with previously reported studies and help to explain the apparently different results obtained from several different laboratories.

**INTRODUCTION**

Human immunodeficiency virus type-1 (HIV-1) expresses an 86-amino-acid (aa) *trans*-activating protein (Tat) involved in the regulation of transcription [1]. The first 72 amino acids of Tat are involved in *trans*-activation of the HIV LTR [2] and cellular genes such as tumor necrosis factor, interleukin-2, and interleukin-6 [3–5]. The C-terminal 14 aa contain an RGD motif that binds αvβ3 and αvβ1 integrins [6]. Tat has the unusual property of being able to leave HIV-infected cells and cross the plasma membrane of adjacent uninfected cells, where it localizes in the nucleus in an active form [7]. The minimal region required for intercellular transport has been mapped to residues 47–57 and is generally referred to as the “protein transduction domain” (PTD) [8–10]. Significantly, Tat maintains its nascent properties post-intercellular trafficking. This observation led to the hypothesis that the PTD of Tat might be useful as a molecular tool for achieving widespread distribution of recombinant proteins in gene therapy applications [11]. Several research groups have pursued this idea.

The 11-aa PTD of Tat has been previously used to deliver a wide variety of proteins ranging in size from 15 to 120 kDa across the plasma membrane by a mechanism referred to as “protein transduction” [1,2,8]. These studies include proteins involved in cell cycle regulation [12,13], regulation of the cytoskeleton [14,15], apoptosis [16–18], mRNA editing [19], and oxidation [20–22]. Some of these Tat fusion proteins have also been shown to transduce tissues *in vivo* [23]. Other molecules that have been trafficked via Tat include DNA [24] and antibody fragments [25]. The ability of the Tat PTD to deliver the 120-kDa β-galactosidase protein across the blood–brain barrier [23] has generated considerable interest in the context of gene therapy. In each of these studies the recombinant fusion peptides were synthesized exogenously and added directly to the cells or tissue where protein transduction was observed.

Recently there have been some reports demonstrating
in vivo endogenous expression of a Tat fusion peptide followed by intercellular trafficking [26,27]. In these studies the PTD of Tat was fused to β-glucuronidase (β-gluc) and expressed from a recombinant viral vector. These studies found a significantly enhanced “biodistribution” of β-gluc in vitro and in vivo if the PTD of Tat was fused to β-gluc. Also, the fusion protein retained its enzymatic activity and ability to be targeted to the lysosome. Inter- cellular transport of β-gluc–Tat fusion peptide is not completely surprising given that both endogenously expressed and exogenous Tat protein can trans-activate the HIV-1 LTR in human neuronal cell lines [28].

The validity of intercellular transport and protein transduction properties of Tat fusion peptides has recently been questioned by Leifert and colleagues [29]. From their studies the authors conclude that the PTD of Tat does not act to enhance release from or uptake into cells but merely increases binding to the cell membrane. Evidence for this was provided through their observation that “intercellular transport” is observed only post-cell fixation. In addition, Falnes and colleagues [30] recently examined the ability of Tat to translocate diphtheria toxin across cell membranes. Although import of only a few toxin molecules is sufficient to cause cell death, very little additional toxicity was observed. These authors also suggested that any effect of Tat on diphtheria toxin-mediated cytotoxicity was due to an enhanced binding but not uptake of the fusion peptide.

Because of the significant promise HIV Tat holds for gene therapy, it is important to resolve these apparently contradictory results. Our interest lies in the treatment of genetic disorders of the retina such as retinitis pigmentosa (RP) and macular degeneration, two of the most common causes of blindness in humans [31]. We have previously demonstrated partial rescue of retinal degeneration in animal models of RP through the use of “gutted” adeno- virus vectors [32,33]. To enhance rescue, we are exploring the therapeutic potential of PTD-fused proteins. Previously we have shown that the full-length (300 aa) herpes simplex virus (HSV) VP22 protein fused to green fluorescent protein (GFP) exhibits intercellular transport in retinal cell culture and in murine retina [34]. The well- characterized PTD of Tat has the potential of enhanced utility relative to VP22 due to its smaller size of 11 aa. Hence, we began our studies by testing the ability of GFP to be trafficked intercellularly when fused either to Tat (hereafter referred to as tat-GFP) or to the PTD of Tat (tats-GFP) in the context of a viral gene expression system. Our results indicate that endogenously expressed tat-GFP or tats-GFP fusions do not exhibit intercellular trafficking of GFP in cell culture or in vivo. However, tat-GFP or tats-GFP fusion proteins prepared from virally infected cells retain the property of protein transduction if added exogenously. For protein transduction to be definitively observed, cell fixation was not required. Hence we conclude that Tat and Tats fusion peptides are limited to unidirectional transport across the plasma membrane. We attribute the observation of intercellular transport of β-gluc to a combination of its own property of secretion as well as Tat-mediated uptake. Our results are hence consistent with the apparently contradictory results obtained from some previously published studies.

RESULTS
Protein Transduction by Tat Conjugated to FITC
We tested the ability of the 11-aa HIV-1 PTD of Tat conjugated to FITC (tats-FITC) to be taken up by Y79 and RPE-J cells, ocular cell lines derived from the retina [35,36] and retinal pigment epithelium (RPE), respectively [37]. We found that, whereas there was no uptake of FITC by either Y79 or RPE-J cells, the tats-FITC conjugate was taken up by these cells within 30 min (Fig. 1A). To distinguish uptake of tats-FITC from cell membrane-bound tats- FITC, we incubated RPE-J cells in suspension with the peptide at 4 or 37°C, followed by incubation with trypsin at 37°C. RPE-J cells were also incubated with a control peptide, RGD-TRITC, under the same conditions. RGD-containing peptides are known to bind plasma membranes and become internalized in a receptor-mediated manner involving integrins [38]. We determined that uptake of tats-FITC at 4°C was not sensitive to trypsin (69.6 ± 4.0% with and 72.9 ± 0.3% without trypsin) (Fig. 1B). Similarly, uptake of tats-FITC at 37°C was not significantly sensitive to trypsin (87.2 ± 1.2% with and 81.0 ± 1.9% without). In contrast, uptake of RGD-TRITC peptide incubated at 37°C was enhanced following treatment with trypsin (64.4 ± 1.2% with and 31.7 ± 0.5% without). Uptake of RGD-TRITC incubated at 4°C was also enhanced following treatment with trypsin (30.3 ± 2.1% with and 12.5 ± 2.1% without). In addition, whereas uptake of RGD-TRITC was reduced twofold at 4°C (and possibly greater given that incubation of RGD-TRITC at 37°C was performed with half (2.5 nM) that used at 4°C—5.0 nM), the uptake of tats-FITC at this temperature was not significantly reduced. We made similar observations

FIG. 1. (A) Protein transduction exhibited by FITC when fused to the 11-amino-acid protein transduction domain of Tat (tats-FITC) in human retinoblastoma (Y79) or rat retinal pigment epithelium (RPE-J) cells 30 min postincubation. (B) FACS analysis of the uptake of tats-FITC and RGD-TRITC by RPE-J cells at 4 and 37°C with (+T) and without (−T) trypsin treatment. The blue plots correspond to cells incubated in the absence of peptide and the green plots correspond to cells incubated in the presence of either tats-FITC or RGD-TRITC. The appropriate control plot (blue) was subtracted from each of the experimental plots (green); FL-1 Height, FITC; FL-2 Height, TRITC. (C) Uptake of tats-FITC by murine retina and cornea. R, retinal pigment epithelium; O, outer nuclear layer; I, inner nuclear layer; G, ganglion cell layer; L, lens. Original magnification ×200; BF, bright field.
for tats-FITC uptake by Chang C cells and with the use of proteinase K instead of trypsin (data not shown).

To examine the potential of Tats in vivo, we injected tatas-FITC conjugate into the vitreous of 3-month-old C57BL/6J mice. We found that, whereas there was no uptake of FITC by any ocular tissues, tatas-FITC conjugate was taken up by ganglion cells, cells of the inner nuclear layer of the retina, and cells of the cornea (Fig. 1C). Hence, we conclude that exogenously added Tats is sufficient for protein transduction of FITC across plasma membranes in vitro and in vivo.

Tat Fusion Peptides Expressed from Adenovirus Vectors

To determine if endogenously expressed Tat or Tats fusion proteins can exit cells and be taken up by neighboring cells (intercellular trafficking), we constructed DNA expression cassettes expressing tat-GFP or tatas-GFP regulated by the CMV promoter (Fig. 2). These expression cassettes were individually cloned into the E1 region of a first-generation (E1/E3-deleted) adenovirus (Ad) vector, similar to previous studies involving H9023 [26]. To unambiguously differentiate cells that are GFP-positive due to an infection by the recombinant Ad from ones that contain trafficked GFP protein produced by neighboring cells, we incorporated an expression cassette coding for red fluorescent protein (RFP) in the same recombinant viral vector. To achieve equal levels of expression of GFP and RFP (to the extent possible), each expression cassette was regulated by the same (CMV) promoter and cloned in the same (antisense) orientation in the (deleted) E1 region of Ad (Fig. 2A). To confirm that GFP fusion protein and RFP were expressed from each virus, we infected human embryonic kidney 293 cells with each of the recombinant viruses and observed concomitant expression of GFP and RFP (data not shown). We confirmed the size of the GFP

FIG. 2. (A) Structure of viruses constructed for this study. All expression cassettes were cloned in the antisense orientation (with respect to the E1 enhancer) into the E1 region of an E1/E3-deleted Ad5 virus. Each cDNA is regulated by the cytomegalovirus (CMV) promoter/enhancer. tat-GFP, full-length HIV tat protein fused to green fluorescent protein (GFP); tatas-GFP, 11-aa protein transduction domain of HIV Tat fused to GFP. RFP, red fluorescent protein; pA, bovine growth hormone polyadenylation signal; ITR, adenovirus inverted terminal repeat; ¥, Ad packaging signal; MLT, major late transcription unit; E, early region. (B) Western analysis of GFP, tat-GFP and tatas-GFP fusion proteins, and LacZ (negative control) using anti-GFP antibody.
fusion peptides by Western analysis (Fig. 2B). For each recombinant virus, the expressed fusion peptide was of the size predicted by protein sequence analysis (MacVector).

No Detectable Levels of Intercellular Transport in Living Cells

To quantify the intercellular trafficking effects of full-length Tat or the PTD of Tat when fused to GFP, we infected RPE-J and Chang C cells with equivalent titers of the recombinant viruses described above and measured the relative numbers of GFP- and RFP-positive cells by FACS. In each of the experiments involving expression of tat-GFP or tats-GFP there were fewer GFP-positive cells relative to RFP-positive cells. Specifically, in RPE-J cells, there were 28.5 ± 6.2 (tats-GFP) and 43.3 ± 13.4% (tat-GFP) fewer GFP-positive cells relative to RFP-positive cells. For Chang C cells there were 27.6 ± 13.8 (tats-GFP) and 52.5 ± 6% (tat-GFP) fewer GFP-positive cells relative to RFP-positive cells. In contrast, when we infected cells with virus coexpressing GFP and RFP, there were 11.5 ± 2.2 and 12.3 ± 0.7% more GFP-positive relative to RFP-positive cells for RPE-J and Chang C cells, respectively (Fig. 3). The results presented here were surprising to us as they were contrary to expectation given previous studies using viral vectors [26,27]. These results suggest that not only is there an absence of intercellular trafficking (or no observed increase in biodistribution) of GFP in the context of a viral infection, but the GFP seems to be down regulated in terms of expression when fused to either Tat or Tats. The down regulation was greater for Tat than for Tats. A greater number of GFP-positive cells compared to RFP-positive cells in GFP- and RFP-expressing virus is possibly due to slightly enhanced expression or detection of GFP relative to RFP. As each virus was plaque purified and analyzed for structure postamplification we do not attribute this difference to rearrangements (loss of the RFP transgene). This latter result also suggests that we may be underestimating the total amount of down regulation attributed to Tat or Tats.

Another possibility to consider is that RFP expression might be involved in down regulation of Tat or Tats fusion proteins through cellular toxicity or competition for protein synthesis machinery. In previous studies performed by others, RFP was not expressed in the viral constructs. Hence, we constructed recombinant adenoviruses expressing tat-GFP and tats-GFP fusions that did not contain RFP expression cassettes (Fig. 2A). To overcome possible variations in the infectivity of the virus prepara-

FIG. 5. (A) Detection of GFP by fluorescence microscopy using an anti-GFP antibody on fixed cells. No enhancement of GFP expression is observed in vitro. (B) Injection of AdcmvGFP, AdtatGFPRFP, or AdtatsGFPRFP into the subretinal space of 2-month-old C57BL/6J mice. In each case the retinal pigment epithelium (R) is the primary cell type infected and no intercellular transport of tat-GFP or tats-GFP is evident in vivo. O, outer nuclear layer.
tions, we developed a more sensitive method with which to normalize the different viral constructs. This was not of significant concern in our previous experiment as RFP was available as an internal control. Specifically, we first infected 911 cells with each of the recombinant viruses and probed with antibody that would detect adenovirus DNA binding protein (DBP) and counted phycoerythrin- (PE; conjugated to secondary antibody) positive 911 cells by FACS. Since 911 cells allow a productive Ad infection, this is a rather sensitive way to detect single infectious viral particles (Fig. 4A) as long as the cells are infected at low multiplicities of infection and counted prior to cell burst. Results from this experiment were used to determine the infectious titer of each virus independent of particle number. RPE-J and Chang C cells were infected with the normalized tat-GFP-, tats-GFP-, and GFP-expressing viruses that did not contain RFP. Measurement of GFP expression again revealed a down regulation in the number of GFP-positive cells for tat-GFP and tats-GFP relative to GFP (Fig. 4B). Specifically, in RPE-J cells there were 40.8 ± 2.3 and 74.5 ± 1.0% fewer GFP-positive cells for tat-GFP and tats-GFP, respectively, in comparison to GFP. Similarly, in Chang C cells there were 41.2 ± 4.1 and 66.7 ± 10.9% fewer GFP-positive cells for tats-GFP and tat-GFP, respectively, in comparison to GFP. These experiments indicate that down regulation of Tat or Tats fused to GFP is independent of the expression of RFP. Moreover, with this approach the observed down regulation is greater than...
that observed with RFP-containing viruses, supporting our suggestion that in previous experiments we were underestimating the true total down regulation associated with Tat or Tats because detection of GFP is more sensitive than that for RFP.

**No Detectable Levels of Intercellular Transport in Fixed Cells or Tissue**

Some previous studies investigating intercellular trafficking of Tat or HSV VP22 have claimed that the phenomenon can be observed only postfixation and by the use of an antibody as a detection method [29,39,40]. Hence, we infected Chang C cells with recombinant virus expressing tat-GFP, tats-GFP, or GFP, fixed these cells, and probed with a monoclonal GFP antibody. We determined by fluorescence microscopy that there was an approximately one-to-one correlation of GFP fluorescence with anti-GFP antibody complexed with TRITC (Fig. 5A). We conclude that this more sensitive method of detection postfixation does not reveal any additional GFP-positive cells, supporting the data from our previous experiments indicating absence of any detectable intercellular transport of tat-GFP or tats-GFP fusion proteins. FACS analysis of these cells confirmed the observation of fluorescence microscopy (data not shown). In contrast to Leifert and colleagues [29], we observed no fixation artifact in our experiment, which is possibly due to the use of a different

![Graph 1: RFP Fluorescence](image)

![Graph 2: GFP Fluorescence](image)
fixative, i.e., paraformaldehyde (PFA) instead of methanol. Other studies have demonstrated a “leaching” effect of methanol fixation that does not occur with PFA fixation [41].

We considered the possibility that observation of intercellular transport might be restricted to experiments in vivo. To examine this, we injected 2-month-old C57BL/6J mice with viruses expressing GFP, tat-GFP, or tats-GFP into the subretinal space. We found that, consistent with previous results [34], the major cell type infected was the RPE. Furthermore, both tat-GFP and tats-GFP expression was restricted to the RPE as was shown for GFP (Fig. 5B).

In previous studies [34] we found that if GFP was fused to HSV VP22, cells throughout the inner retina in addition to the RPE were GFP-positive. Hence we conclude that there is no observable intercellular transport of Tat fusion peptides in vivo.

**Protein Transduction in Living Cells**

Results presented above independently confirm that there is no detectable intercellular trafficking of Tat or Tats GFP fusions in the context of a recombinant viral vector in cell culture or in vivo. However, improved biodistribution of β-gluc was previously observed in endogenously expressed Tats–β-gluc fusion peptides in at least two independent studies [26,27]. However, as the authors note, β-gluc has its own trafficking properties and that trafficking might be augmented by Tat. Is it possible perhaps that the secretory signal of β-gluc allows exit of the fusion peptide and then Tats potentiates its uptake by a mannose-6-phosphate-independent mechanism? We wished to test the hypothesis whether Tat or Tats movement across plasma membranes might be unidirectional, i.e., that Tat or Tats fusion peptides may be taken up by cells but may not be secreted. To address this, we infected 911 cells with recombinant viruses expressing tat-GFP or tats-GFP fusions, prepared homogenates from these cells, in-}

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that 27.5 ± 5.0 and 0% of cells were GFP-positive for tat-GFP and tats-GFP, respectively, relative to GFP. For cells in the media we found that 38.9 ± 7.0 and 21.2 ± 1.8% were GFP-positive for tat-GFP and tats-GFP, respectively, relative to GFP. The proportion of cells with propidium iodide staining above background was 27.3 ± 0.9% for GFP, 35.0 ± 1.5% for tats-GFP, and 62.3 ± 0.5% for tat-GFP (Fig. 6B). Any cell death occurring as a result of the addition of homogenate was subtracted from the tats-GFP- and tat-GFP-positive cells by subtraction of the GFP.

We attribute the additional toxicity of tats-GFP and tat-GFP to the Tat portion of the GFP peptide. These experiments suggest that although tat-GFP and tats-GFP fusion proteins can enter cells, in doing so they have a toxic effect on the cells they enter. As this toxicity may be responsible for making those cells more vulnerable to further uptake, Tat may be more efficient at protein transduction than Tats because it is considerably more toxic. These data corroborate the observation that endogenous expression of Tat or Tats fusion proteins is insufficient for intercellular trafficking and hence might not have general application beyond those proteins that contain a secretory signal or another method to exit the cell.

Leifert and colleagues reported [29] that intercellular transport could be observed only after fixation and that Tat fusion proteins were membrane bound rather than intracellular. In the case of protein transduction observed in our experiments, Tat or Tats fusion proteins were not restricted to the plasma membrane (Fig. 6C).

**Down Regulation of Tat Fusion Protein Expressed from Plasmid DNA**

Finally, we postulated whether the down regulation of tat-GFP or tats-GFP was possibly happening only in the context of the recombinant Ad vector. It has previously been shown that E1/E3-deleted Ads do in fact express low levels of some viral proteins in cell culture and in vivo [reviewed in 42], despite the absence of the region E1. Adenovirus 12S E1A has previously been shown to down regulate HIV Tat [43]. We transfected DNA expression cassettes (the shuttle plasmids used in generation of the adenovirus constructs) for tat-GFP, tats-GFP, and GFP into Chang C cells. Each plasmid also expressed RFP, allowing us to compare our DNA transfection results directly with those obtained with Ad. Experimental results indicate down regulation of tat-GFP or tats-GFP fusions in the context of DNA expression cassettes (Fig. 7). In experiments described above we had noted a slightly higher expression/sensitivity of detection of GFP than RFP in Ad-infected cells of about 12.3 ± 0.7% in Chang C cells. In the DNA transfection experiments we noted a difference of approximately 16.6 ± 2.9% more GFP-expressing than RFP-expressing Chang C cells. For tats-GFP and tat-GFP expression cassettes, reduced GFP expression was observed at levels of 18.6 ± 1.0 and 9.9 ± 1.2%, respectively. Hence, we conclude that similar to Ad-mediated expres-
sion of Tat or Tats, there is significant down regulation of Tat or Tats in the context of a DNA expression cassette. These data argue against the interaction of some unknown Ad protein with HIV Tat and Tats leading to the down regulation of those fusion proteins, although some minimal Ad sequences are present in the shuttle plasmids.

**DISCUSSION**

Several groups have previously demonstrated the utility of the HIV Tat protein in the context of gene therapy. Almost all of these studies added exogenously prepared Tats fusion peptides to cells in culture or in vivo. This approach has significant utility for enhancing the biodistribution of a therapeutic protein for the treatment of diseased tissue. Protein transduction can, however, provide only transient therapy without continuous infusion of protein. Hence, the preferred approach would be the use of vectors that are stable in vivo and produce therapeutic proteins such as Tats fusion for the lifetime of the patient. Viral vectors have previously been shown to achieve long-term correction of disease through persistence of transgene expression, for example, hypercholesterolemia in apoE-deficient mice has been corrected for the natural “lifetime” of the animal [44]. Recently some studies have demonstrated improved biodistribution of Tats–gluc fusion peptide expressed from a viral vector, suggesting the possibility that endogenously expressed Tats fusion peptides may have significant utility in long-term gene therapy. However, recent studies have found no evidence of intercellular transport of Tats fusion peptides. Since an intercellular trafficking ability of HIV Tat could provide a solution to some of the current limitations of gene therapy, it is imperative to resolve the differences between studies before significant progress can be made.

We first validated the utility of Tats by demonstrating uptake of tats-FITC conjugates in ocular cell lines by fluorescence microscopy and FACS analysis. We addressed the issue of membrane binding versus protein transduction by the treatment of tats-FITC-positive cells with trypsin. We found that trypsinization of cells did not have a significant effect on the transduction properties of tats-FITC, supporting the hypothesis that tats-FITC is rapidly internalized rather than bound to the plasma membrane. Our results suggest that tats-FITC uptake occurs via an energy- and endocytosis-independent mechanism, as transduction is equally efficient at 4 and 37°C. This is unlike the uptake of RGD-TRITC, which is reduced 2- to 2.5-fold at 4°C and possibly more because twice as much RGD-TRITC was used at 4°C than at 37°C. Alternatively, uptake of tats-FITC at 4°C might possibly be reflective of the rapid uptake kinetics of tats-FITC following transfer of the cells to 37°C for treatment with trypsin. This hypothesis is supported by the incomplete ablation of RGD-TRITC uptake at 4°C. Our results are in contrast to those of Richard and colleagues [45], who concluded an endocytic- and energy-dependent pathway for the uptake of Tat peptide. Those authors found that in the presence of trypsin, there was complete ablation of Tat-mediated uptake of FITC at 4°C but not at 37°C. The discrepancy in the two results is probably not due to the different cell lines being used (Jurkat compared to RPE-J), as we found consistent results in other cell lines, such as Chang C, compared to RPE-J. In our experiments we observed a difference in fluorescence between trypsin-treated and untreated control cells. This difference was taken into account during the measurement of uptake of tats-FITC in our experiments. It is possible that this may account for the contrasting results between our experiments and those of Richard and colleagues [45]. The increased uptake of RGD-TRITC following trypsin treatment could potentially be explained by exposure of additional integrins on the cell surface or increased cycling of integrins in response to the protease treatment. For example, increased binding of adenovirus to cells after trypsin treatment has previously been observed for certain serotypes [46].

When adenovirus expression constructs were developed expressing either Tat or Tats fusions, we did not observe any intercellular transport. Our approach of incorporating an RFP expression cassette in the same viral construct allowed us to determine quantitatively the effect (if any) of intercellular transport, an approach not previously used in this context. Unexpectedly, not only was there no observable intercellular transport, but the experimental results indicated a down regulation of Tat or Tats fusion peptides. We confirmed that this down regulation was not attributable to a potential toxic effect of RFP, as down regulation was also observed when RFP was removed from the viral vector. Down regulation was also observed when DNA expression constructs for tat-GFP or tats-GFP were utilized instead of Ad as a backbone, arguing against the involvement of adenovirus proteins in down regulation. We have not excluded the possibility that fusion of Tat or Tats to proteins such as GFP targets such proteins for proteosomal degradation at a rate greater than the native protein. Observation of fewer GFP-positive cells than RFP-positive cells could possibly be due to toxicity associated with Tat. However, if toxicity was an issue, then we would also expect a concomitant down regulation of RFP in those cells expressing tat-GFP or tats-GFP, which was not observed.

More sensitive methods such as antibody-based detection of GFP also failed to reveal evidence of intercellular transport of endogenously synthesized Tat or Tats fusion peptides. These studies are relevant because there has been significant debate regarding the issue of intercellular transport properties of both HSV VP22 and HIV Tat. Some studies have suggested that intercellular trafficking is an artifact of fixation and can be observed only post fixation. All of our FACS analyses except antibody staining were
performed on live cells. Our studies did, however, support the idea that protein transduction is observable if the fusion peptide is artificially released from cells. Exogenously added Tat or Tats fusion peptides harvested from virally infected cells are able to display transmembrane transport, suggesting a unidirectional movement (uptake of Tat or Tats fusions) across the plasma membrane.

The uptake of tat-GFP was considerably more efficient than that of tats-GFP, although the toxicity associated with Tat was also greater. The toxicity observed for cells administered the tat-GFP homogenate is not unexpected given previous reports of toxicity associated with Tat in vitro and in vivo [47,48]. In some studies no significant toxicity of the Tat amino acids 49–58 was observed [49]. It is possible that Tat-associated toxicity increases the permeability of cells to further uptake of the Tat fusion peptide.

Although not much is known about how Tat exits cells, it has previously been shown that Tat uses heparan sulfate transporters [50]. Our experiments, in which tat-GFP and tats-GFP fusions are released from cells and added exogenously, demonstrate uptake of GFP, indicative of the presence of receptors required for uptake of Tat on the cells being tested. We performed experiments similar to those described above in HeLa cells, a cell line used in previous studies to demonstrate protein transduction [51], and again we did not observe any intercellular transport (data not shown). Absence of in vivo intercellular transport in the retina is also probably not due to absence of receptor, as heparan sulfate is abundantly present in the retina [52].

We cannot exclude the possibility of very small amounts of Tat-mediated intercellular trafficking, levels below those detectable by the anti-GFP antibody or FACS. The histochemical and enzyme assays for β-gluc activity are likely more sensitive than the GFP-fluorescence assay used in our study. However, other explanations are more likely to explain increased biodistribution of β-gluc. β-Gluc, as for other lysosomal hydrolases, has its own intercellular trafficking properties [53]. Consistent with our observations would be the hypothesis that Tats contributes to improved biodistribution not by intercellular trafficking but by enhancing uptake of β-gluc postsecretion.

Although our results help to explain many of the previous studies employing Tat for protein transduction and intercellular transport, some questions remain unanswered. For example, Falnes and colleagues [30] concluded that the Tat PTD fused with diphtheria toxin (DT) did not lead to enhanced cytotoxicity, even though a single molecule of DT is sufficient to cause cell death. However, these researchers did observe an increase in the total amount of Tat–DT in the cytosol, as well as that bound to the plasma membrane. This would suggest that Tat–DT fusion protein has a reduced activity compared with DT. It is quite likely that more proteins will be identified for which Tat fusion perturbs function significantly enough to make Tat-mediated protein transduction not useful.

In summary, we have shown that endogenously synthesized Tat or Tats fusion peptides do not exit cells and hence do not display any intercellular transport properties in cell culture or in vivo. Furthermore, tat-GFP and tats-GFP fusion proteins are down regulated in the context of both adenovirus and plasmid vectors. Exogenously added protein harvested from such cells is able to display transmembrane transport, suggesting a unidirectional movement (uptake of Tat or Tats fusions) across the plasma membrane. Whereas Tats–β-gluc seems to increase biodistribution of enzyme, this property is most likely due to a combination of the lysosomal enzyme’s intrinsic trafficking properties as well as the protein transduction properties of Tats. Hence, in limited cases Tat and Tats fusion peptides may have utility in gene therapy applications.

**Materials and Methods**

**Protein transduction by tats-FITC.** The 11-amino-acid PTD of HIV-1, YGRKKRRQRRR, was conjugated with fluorescein isothiocyanate at the N-terminus via a linker of four glycines. The RGD-TRITC peptide consists of the 6 amino acids GRGDSP labeled at the N-terminus with carboxytetramethylrhodamine (Molecular Probes). Both peptides were synthesized by the University of Utah DNA/peptide core facility. A total of 4 × 10⁸ RPE-J cells and 1.5 × 10⁸ Y79 cells were incubated with 0.95 nmol of either tats-FITC or FITC equivalent (determined using a Bio-Rad VersaFluor fluorometer) for 30 min at 37°C and 5% CO₂. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. For measurement of peptide uptake following treatment with trypsin, 1.5 × 10⁸ RPE-J cells were resuspended in 200 µl of serum-free medium containing 2.0 nmol of tats-FITC and incubated for 15 min at either 4°C or 37°C. The cells were washed twice with PBS prior to incubation with trypsin (2.5 mg/ml) for a further 12 min at 37°C. The cells were resuspended in PBS for 10 min at 37°C and analyzed by flow cytometry. The RGD-TRITC, the conditions were the same except that 2.5 and 5.0 nmol of the peptide were used at 37°C and 4°C, respectively. For in vivo experiments 1.7 nmol of either tats-FITC or FITC equivalent was resuspended in 10 µl of PBS and injected into the intravitreal space of 3-month-old C57BL/6 mice as described previously [54]. Errors were calculated as the standard deviation from the mean percentage of tats-FITC/RGD-TRITC-positive cells of three different experiments.

**Construction of tat fusion expression cassettes.** All recombinant viruses were constructed in principle as described previously [34]. Each expression cassette was first cloned into pShuttle prior to recombination with a circularized adenovirus genome [54]. To construct pShuttle-GFP we first annealed the oligonucleotides TatU (TTTGGGCCCGGTATGGCAGGAAAGACCGGAGACAGCGACGAAGAAATTCGGG) and TatL (CCCGAAATTCTCGTCGCTGTCTCCGCTTCTTCCTGCCATACCGGGCCCAAA), digested with XmnI/BglII and circularized adenovirus genome [54]. To construct pShtats-GFP we digested pShuttle-GFP with ApaI/EcoRV to remove the IRES element, generating pShuttle-GFP-IRES. This plasmid was then digested with SmaI and EcoRI, and cloned it into ApaI/EcoRI-digested pQBI-25f1 (QiBiogene, Carlsbad, CA), generating pQBI-GFP. The integrity of the tats-GFP fusion was confirmed by sequencing. A 2.5-kb BglII/XmnI fragment from pQBI-GFP was cloned into BglII/EcoRV-digested pShuttle [54], generating pShuttle-GFP. To generate a shuttle expressing the full-length Tat peptide fused to GFP, we first digested pRESNeo (Clontech, Palo Alto, CA) with SmaI and EcoRV to remove the IRES element, generating pRESNeoΔIRES. This plasmid was then digested with BglII and XhoI and the 1.8-kb poly(A)-containing fragment was cloned into BglII/XhoI-digested pShuttle [54] to produce pShneo. The 2-kb BglII/XhoI fragment
Protein transduction by tats-GFP or tat-GFP prepared from infected cell homogenates. Human embryonic retinoblast 911 cells were infected at a multiplicity of infection of 1.6 and the infection was allowed to proceed to complete cytopathic effect. The cells were harvested, washed with PBS, and homogenized in 50 mM Tris–HCl, pH 8.0/150 mM NaCl containing leupeptin (10 μg/ml), aprotinin (10 μg/ml), and PMSF (0.1 mM). The homogenate was separated from cell debris by centrifugation at 1700 rpm for 15 min. GFP concentrations were determined in each sample using a VersaFluor fluorometer (Bio-Rad, Hercules, CA) with GFP filters (excitation/emission: 490 nm/510 nm) and a standard curve generated using purified EGFP (Clontech). Homogenate containing 260 pmol each of GFP, tats-GFP, or tat-GFP was added to 1.5 × 10⁶ Chang C cells and incubated at 37°C and 5% CO₂. After 1 h, the live cells were photographed. Cells were treated with trypsin and resuspended in PBS with 5 μg/ml propidium iodide for FACS analysis. Errors were calculated as the standard deviation from the mean percentage of GFP/propidium iodide-positive cells above background.

Western blot analysis. 911 cells were infected as described above. Cells were harvested, washed with 1× PBS, and resuspended in 50 mM Tris–HCl, pH 8.0/150 mM NaCl/0.1% SDS/1% Triton X-100 containing leupeptin (10 μg/ml), aprotinin (10 μg/ml), and PMSF (0.1 mM). A total of 12 μg of protein was loaded on a 12% denaturing gel (BMA, Rockland, ME) and GFP detected using a 1/500 dilution of the monoclonal GFP antibody 11E5 (QiBiogene) followed by a 1/1000 dilution of an HRP-conjugated anti-mouse antibody (Jackson Immunoresearch). Chemiluminescent signal detection was performed using an ECL kit (Bio-Rad) followed by exposure to Kodak BioMax ML film.

Calcium phosphate transfection. A total of 1 × 10⁶ Chang C cells were transfected with 3.5 μg of pAdGFP, pShcmvGFPRFP, pShatsGFPRFP, or pShatatGFPRFP in 2% FBS using calcium phosphate. The transfection was allowed to proceed overnight at 37°C and 5% CO₂ after which the medium was replenished with 10% FBS. The cells were analyzed by FACS for 48 h. Errors were calculated as for FACS analyses of virus infections.

Intravitreal and subretinal injections. All animals were cared for in accordance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research and in compliance with federal, state, and local regulations. C57BL/6j mice were maintained under 12-h cycles of light and dark. Intravitreal and subretinal injections were performed as previously described [32,34]. Briefly, mice were anesthetized by intraperitoneal injection of 2.5% Avertin (0.015 ml/g body weight). Transcral trans-scleral injections were given into the subretinal or intravitreal space of 2-month-old mice using a 30-gauge needle. Virus (2–4 × 10⁸ particles) was injected into one eye while the control solution (FICt) etc. was injected into the contralateral eye. Eyes were analyzed 7 days postinjection. Histology and immunofluorescence microscopy of frozen sections were performed as described previously [34].

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