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Cell Membrane Lipid Rafts Mediate Caveolar Endocytosis of HIV-1 Tat Fusion Proteins*

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The transactivator protein of human immunodeficiency virus type 1 Tat has the unique property of mediating the delivery of large protein cargoes into the cells when present in the extracellular milieu. Here we show that Tat fusion proteins are internalized by the cells through a temperature-dependent endocytic pathway that originates from cell membrane lipid rafts and follows caveolar endocytosis. These conclusions are supported by the study of the slow kinetics of the internalization of Tat endosomes, by their resistance to nonionic detergents, the colocalization of internalized Tat with markers of caveolar endocytosis, and the impairment of the internalization process by drugs that disrupt lipid rafts or disturb caveolar trafficking. These results are of interest for all those who exploit Tat as a vehicle for transcellular protein delivery.

The Tat protein of the human immunodeficiency virus type 1 (HIV-1)[1] is a powerful transcriptional activator of viral gene expression. At the long terminal repeat (LTR) promoter, the protein binds a cis-acting RNA element (trans-activation-responsive region) present at the 5′-end of each viral transcript (1). Through this interaction Tat activates HIV-1 transcription by promoting the assembly of transcriptionally active complexes at the LTR by multiple protein-protein interactions (for review, see Refs. 2 and 3).

Besides its fundamental role in the control of HIV-1 gene expression, more than 10 years ago it was first demonstrated that Tat also possesses the unusual property of entering the cells and translocating to the nucleus when present in the extracellular environment (4–6). This unusual characteristic depends upon the integrity of the basic region of the protein, a nine-amino acid, arginine-rich sequence that also corresponds to the nuclear localization signal and the trans-activation-responsive region binding domain of the protein (2, 3).

The property of Tat to cross cell membranes is common to a small group of proteins that also includes the herpesvirus structural protein VP22 (7), the homeotic protein of *Drosophila melanogaster* Antennapedia (8), and the fibroblast growth factor (9) as well as several toxins of bacterial and plant origin. In most cases, the cellular mechanisms that these proteins use for cellular penetration are still poorly understood and are most probably different.

The mounting interest in the cell-penetrating capacity of these proteins is due to their ability to drive the internalization of large protein cargoes that are chemically coupled or fused to them. In particular, the addition of an 11-amino acid stretch from the basic domain of Tat (10) or the cross-link of Tat (11) to heterologous molecules, even of a large size, mediates their cellular uptake; this property is currently widely exploited as a biotechnological tool for transcellular protein transduction. In the case of Tat, it has been demonstrated that the intraperitoneal injection of a 120-kDa β-galactosidase/Tat fusion protein results in the transcellular transduction into virtually all tissues in mice, including the passage of the blood-brain barrier (12). Based on these observations, the translocating activity of the Tat basic domain is now being extensively utilized for several therapeutic applications, including protection from apoptosis in the brain (13, 14) and in the heart (15), extension of the cytotoxic activity of herpes simplex virus-1 thymidine kinase for cancer gene therapy (16), improvement of the IκBα activity (17), enhancement of antigen presentation by dendritic cells (18, 19), quenching of the tumor phenotype (20), and enhancement of viral-mediated gene delivery (21), to name but a few of the current applications. In addition, the Tat transduction domain has also been shown to mediate cell internalization of large molecules or particles, including magnetic nanoparticles (22), phage vectors (23), liposomes (24), and plasmid DNA (25).

Despite this variety of successful biotechnological applications of Tat as a delivery vector across cell membranes, the cellular mechanisms involved in the uptake of wild type HIV-1 Tat and of Tat fusion proteins are still obscure. We have previously observed that extracellular Tat specifically interacts with the heparan sulfate chains of cell membrane heparan sulfate proteoglycans (HSPGs) (26) and that this interaction is absolutely required for the uptake process to occur (27). These results together with the observation that the internalization of large molecules fused to Tat is impaired at 4 °C suggest that the interaction of Tat with HSPGs is followed by an active endocytic process.

Endocytosis is a complex mechanism that involves different pathways and a large network of protein-protein and protein-lipid interactions. The first and best-characterized pathway is clathrin-dependent endocytosis, which starts on the plasma

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; HSPG, heparan sulfate proteoglycan; TRITC, tetramethylrhodamine-isothiocyanate; GST, glutathione *S*-transferase; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyl-transferase; MβCD, methyl-β-cyclodextrin.

membrane with the formation of clathrin-coated invaginations that pinch off to make up clathrin-coated vesicles (28). Less defined are the non-classical, clathrin-independent pathways, among which is caveolae-mediated endocytosis.

Caveolae are flask-shaped, small (50–70 nm) invaginations in the plasma membrane that constitute a subclass of detergent-resistant membrane domains enriched in cholesterol and sphingolipids that are called lipid rafts (29). Caveolae are involved in signal transduction and the intracellular transportation of lipid raft-associated molecules, whereas proteins internalized by the clathrin pathway, such as transferrin, are excluded from these membrane domains (30, 31). Several bacterial toxins, including cholera toxin (32), and some viruses, including SV40 (33), make use of caveolar endocytosis to enter the cells.

Here we show that extracellular proteins containing full-length Tat or the Tat transduction domain are internalized through an active endocytic pathway originating from cell membrane lipid rafts and involving caveolar endocytosis. These results are of relevance for all those applications that exploit Tat as a vehicle for transcellular protein transduction.

EXPERIMENTAL PROCEDURES

Antibodies and Fluorescent Markers—Antibodies against early endosome antigen 1 (EEA1) and caveolin-1 were from Transduction Laboratories, Lexington, KY. Secondary Alexa Fluor 568 goat anti-mouse antibody, TRITC-labeled transferrin, Alexa Fluor 594-labeled cholera toxin subunit B, TRITC-labeled M_r 10,000 dextran, and Lysotracker Red were all from Molecular Probes Inc, Eugene, OR. All other reagents were from Sigma unless otherwise specified.

Cell Cultures—HL3T1 cells (a HeLa cell derivative stably transfected with a silent LTR-CAT cassette), a kind gift of B. Felber (34) and HeLa and Cos-1 cells (both obtained from the American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamicin. Jurkat T-cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamicin.

Recombinant Tat Proteins—The plasmid expressing the glutathione *S*-transferase (GST)-Tat11-EGFP fusion protein was obtained by replacing the Tat86-coding region in the GST-Tat86-EGFP plasmid (27) with the sequence encoding for amino acids 48–58 in the basic domain of Tat. The recombinant GST-Tat86-EGFP and GST-Tat11-EGFP fusion proteins were produced and purified as described (27).

Fluorescence Microscopy—For treatment with the different fluorescent proteins, HeLa and Cos-1 cells were grown on chamber slides (Labtech International, Woodside, UK) to about 60% confluence; Jurkat cells were treated in suspension cultures. After a 30-min incubation in serum-free Dulbecco's modified Eagle's medium, fresh, serum-free medium supplemented with Hepes 25 mM (pH 7.2) and containing the different fluorescent molecules was added. Final concentrations were 2 μg/ml recombinant Tat86-EGFP and Tat11-EGFP, 10 μg/ml transferrin-TRITC, 10 μg/ml cholera toxin B-subunit, 50 nM Lysotracker Red, and 0.1 mg/ml dextran-TRITC. At the different time points indicated in each figure, cells were washed twice with PBS and fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. Cells were subsequently washed and incubated with glycine 100 mM in PBS for 5 min and mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). Lysotracker Red was added 30 min before fixation. For immuno-staining, fixed cells were washed twice with 0.1% Triton X-100 in PBS for 10 min and incubated with antibodies in PBS supplemented with 0.15% glycine and 0.5% bovine serum albumin. Images were obtained by confocal microscopy using an Olympus FV300 microscope. For live cell recording, cells plated on 6-cm glass bottom dishes were placed in a humidified Plexiglas chamber and maintained at 37 °C and 5% CO_2 throughout the experiment. For the fast dynamics recording and co-localization experiments cells were imaged using a TCS-SP laser-scanning confocal microscope (Leica Microsystems, Mannheim, Germany).

Treatment with Triton X-100—Cells were incubated with either Tat86-EGFP or Tat11-EGFP together with labeled transferrin for 1 h, washed, and left in ice-cold 1% Triton X-100 in PBS for 20 min and then fixed with 2% paraformaldehyde.

Drug Treatments—Cells were pretreated with the different drugs (5 μM cytochalasin D, 20 μM nocodazole, 20 μM taxol, 10 μM brefeldin A, 10 μg/ml heparin, 5 mM methyl-β-cyclodextrin) for 30 min in serum-free Dulbecco's modified Eagle's medium, and then recombinant proteins in fresh serum-free medium containing the same inhibitors were added. Cells were then processed at different time points (1 h for fluorescence microscopy and 8 h for flow cytometry and competitive reverse transcriptase-PCR analysis).

Flow Cytometry—Quantification of internalized Tat-EGFP recombinant proteins was performed as already described (27). Briefly, cells were plated in 6-well plates to about 60% confluence and incubated with Tat86-EGFP, Tat11-EGFP, or labeled transferrin for the time points indicated in the figures. Cells were then washed twice with PBS, trypsinized, again washed with PBS, washed with 2 M NaCl to completely take off surface-bound proteins, again washed twice with PBS, and finally, analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences).

Competitive PCR—The day before treatment, 2.5×10^5 HL3T1 cells were plated on 6-cm-diameter dishes. Cells were incubated with recombinant Tat86-EGFP protein (or Tat11-EGFP control; 2 μg/ml) as described (27). After incubation, cells were harvested, and RNA was isolated using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). After treatment with DNase I amplification grade (Invitrogen), 1 μg of RNA was reverse-transcribed with SuperScript™ II RNase H reverse transcriptase and random hexamers (Invitrogen) according to the manufacturer's instructions. Competitive PCR was performed by mixing the cDNA obtained in previous step with a fixed amount of a competitor DNA molecule containing the primers for amplification of the β-actin RNA (BA1 and BA4) and of the CAT RNA (CATupper and CATlower) (35). PCR amplification was performed with 1/25 and 1/250 of the cDNA for the CAT and β-actin amplifications, respectively. For both primers, the PCR profiles were denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s for 30 cycles of amplification. Amplification products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified.

RESULTS

To study the molecular pathway involved in the internalization of extracellular Tat, we obtained two recombinant proteins, each one containing the GST protein at the N terminus, the enhanced variant of the green fluorescent protein (EGFP) at the C terminus, and in between, either the 86-amino acid form of Tat or only its 11-amino acid transduction domain (GST-Tat86-EGFP and GST-Tat11-EGFP, respectively, hereafter named Tat86-EGFP and Tat11-EGFP; Fig. 1*A*). We have previously shown that the former protein retains full transcriptional activity on the viral LTR and is efficiently internalized by different cell types when added to the extracellular medium of these cells (27).

Kinetics of Tat Internalization—The kinetics of cellular internalization of the two Tat fusions is shown comparatively in Fig. 1 together with the kinetics of internalization of recombinant TRITC-labeled transferrin. The three proteins were added to the cell culture medium of human HeLa epithelial cells at a concentration of 2 μg/ml, and cellular fluorescence was quantitatively assessed at different time points by flow cytometry after cell treatment with trypsin to remove any protein adsorbed to the cell surface. Analysis of the flow cytometry profiles and of the mean cellular fluorescence values (shown in Fig. 1) clearly indicates that the kinetics of internalization of transferrin and of Tat fusion proteins is remarkably different. Transferrin is internalized rapidly, with >80% of maximum fluorescence already obtained after 15 min of incubation. In contrast, internalization of both the Tat86 and Tat11 proteins is slower and progresses in the first 10 h, after which it reaches a plateau, which persists for at least up to 20 h. There was no difference noticed between Tat86-EGFP and Tat11-EGFP.

A microscopic analysis of cells treated with the three proteins (*inset* in the *graphs* of Fig. 1) indicated that fluorescence was localized to discrete compartments in the cytoplasm, suggestive of internalization by an endocytic process. This observation is consistent with a number of reports from our laboratory as well as from other laboratories which showed that the

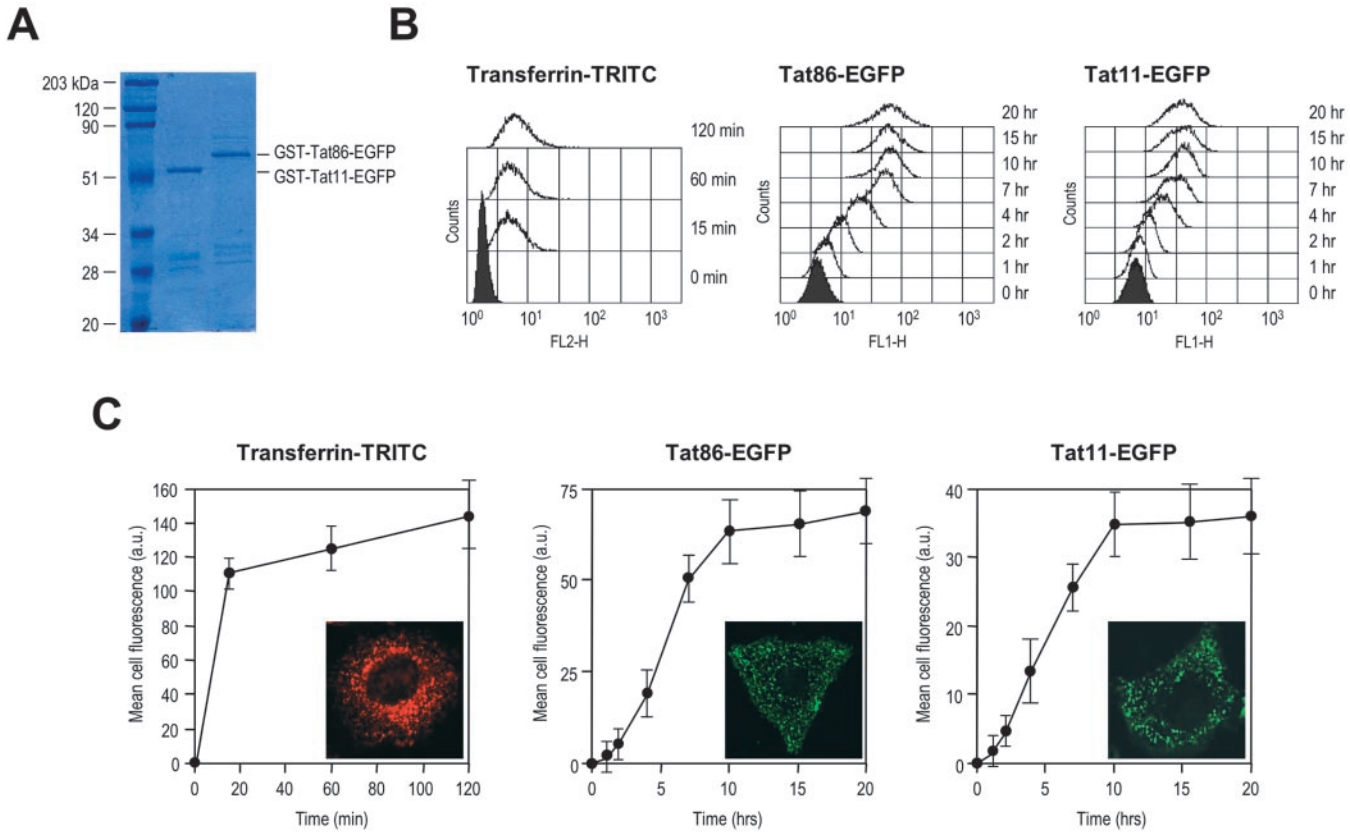

FIG. 1. **Kinetics of extracellular Tat internalization.** *A*, Tat11-EGFP and Tat86-EGFP recombinant proteins were loaded on a 10% SDS-polyacrylamide gel and visualized by Coomassie Blue staining after electrophoresis. *B*, HeLa cells were incubated with transferrin-TRITC, Tat86-EGFP, and Tat11-EGFP for different periods of time as indicated, and then cell fluorescence was analyzed by flow cytometry. The *filled peaks* show fluorescence of cells incubated without recombinant proteins. *C*, mean fluorescence of HeLa cells analyzed by flow cytometry after incubation with the recombinant proteins for the indicated time periods. Experiments were performed in triplicate, and the S.D. is reported. Images in the *insets* show cells incubated for 1 h with the respective fluorescent proteins.

internalization of large protein cargoes fused or conjugated to Tat is an energy-dependent process, that it does not occur at 4 °C, and that is sensitive to drugs blocking endocytosis (36); see also Figs. 5 and 6. The cellular internalization of Tat by endocytosis was further corroborated by the observations that both extracellular Tat11-EGFP and Tat86-EGFPs were found to be internalized in discrete vesicles when mixed together with labeled M_r 10,000 dextran, a fluid phase marker, before the addition to the cell medium (shown in Fig. 2*A* for Tat11-EGFP).

Tat Is Not Internalized by Endocytosis in Clathrin-coated Endosomes—Which is the endocytic pathway involved in Tat internalization? Transferrin is known to be internalized from clathrin-coated invaginations on the plasma membrane that eventually detaches to form clathrin-coated vesicles (28). Maturation and trafficking of a vast proportion of these endosomes eventually leads to their fusion with cell lysosomes. To assess whether this might also be the case of Tat endosomes, we mixed Tat11-EGFP to TRITC-labeled transferrin and added the two proteins to the culture medium of HeLa cells. As shown in Fig. 2*B*, both proteins appeared in discrete intracellular endosomes at different time points after treatment; however, these compartments were clearly distinguishable. Additionally, at earlier time points (10 and 30 min), although transferrin-containing endosomes were dispersed all through the cytoplasm and enriched in the perinuclear recycling compartment, Tat endosomes were still localized to the cell periphery, consistent with the different kinetics of endocytosis shown in Fig. 1. The involvement of clathrin-coated endosomes in Tat internalization was further ruled out by the observation that internalized Tat does not co-localize with the early endosome antigen 1 (EEA1), a protein associated with early endosomes (Fig. 2*C*). Finally, endocytosis of clathrin-coated vesicles is known to involve progressive endosome maturation and eventual fusion with the cell lysosome. Therefore, we tested the possible co-localization of internalized fluorescent Tat with Lysotracker, a marker for cell lysosomes. As shown in Fig. 2*D*, at all the analyzed time points, Tat and lysosome fluorescence appeared in vesicles that were clearly distinct. These results are shown in Fig. 2, *A–D*, for the Tat11-EGFP molecule; those obtained with the Tat86-EGFP protein were superimposable, as shown in Fig. 2, *E–G*, for selected time points. Taken together, these results clearly indicate that Tat is not internalized by endocytosis in clathrin-coated endosomes.

Tat Endocytosis Occurs from Cell Membrane Lipid Rafts—Similar to other microbial toxins (37), the active fraction of cholera toxin is the one internalized by clathrin-independent endocytosis from lipid rafts (38), cell membrane microdomains enriched in cholesterol and sphingolipid. To assess whether this might be the case also for our Tat fusions, we tested their co-localization with the labeled B-subunit of cholera toxin. As shown in Fig. 3, *A* and *B*, respectively, more than 80% of both Tat11-EGFP- and Tat86-EGFP endosomes also contained the cholera toxin.

One group of cell membranes that have lipid raft properties are caveolae, typical flask-shaped membrane invaginations defined by a distinctive membrane coat composed of the cholesterol-binding protein caveolin-1 (39). Therefore, we tested whether Tat endosomes were also positive for the presence of caveolin-1. As shown in Fig. 3*C*, in Cos-1 cells, which are

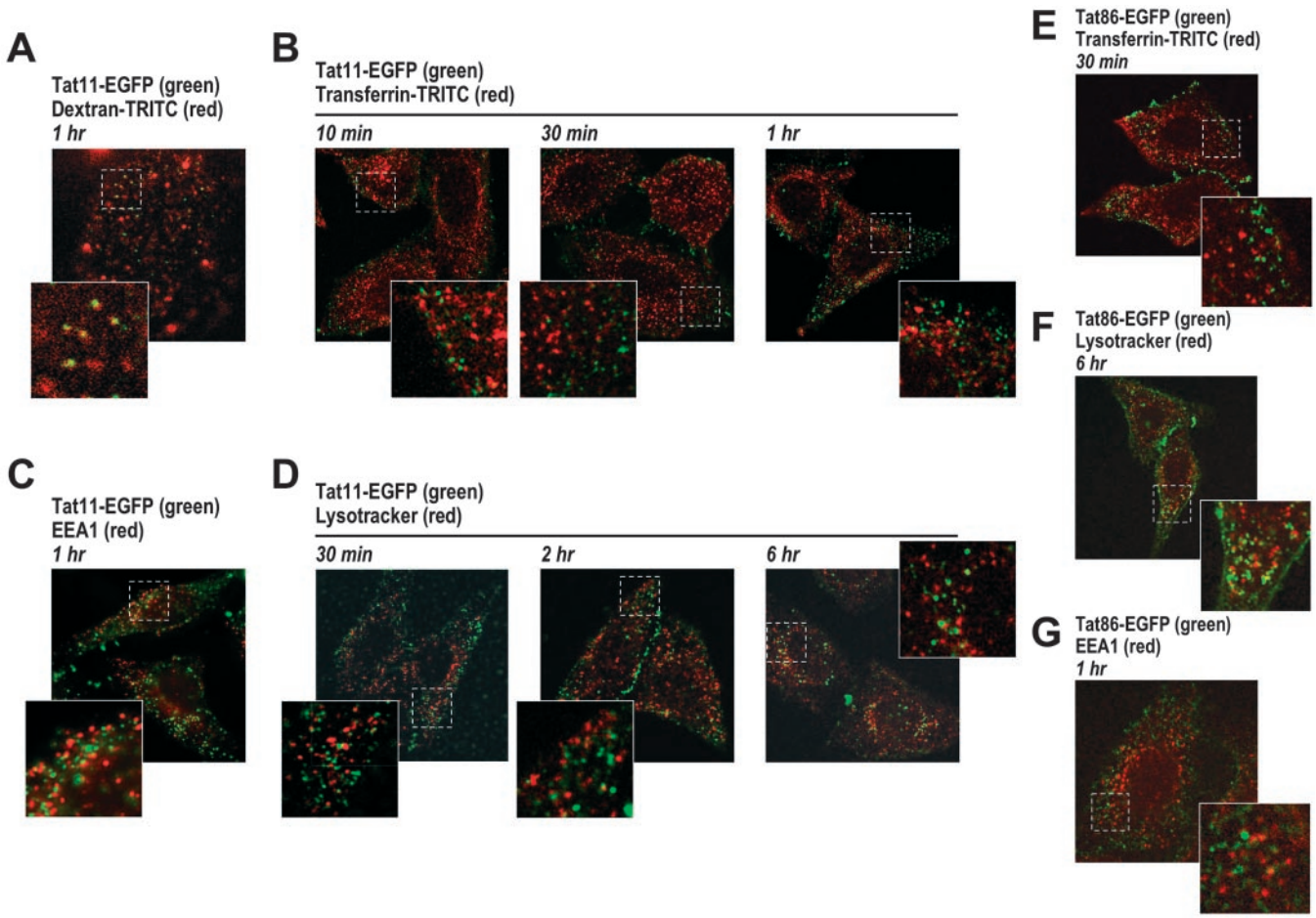

FIG. 2. **Tat and transferrin are internalized through different routes of endocytosis.** *A*, colocalization of GST-Tat11-EGFP and M_r 10,000 dextran-TRITC after 1 h incubation of HeLa cells. *B*, HeLa cells were incubated with Tat11-EGFP and transferrin-TRITC for 10 min, 30 min, and 1 h. Note that the two recombinant proteins are internalized in distinct structures inside the cells. The same localization pattern was also found in the cells incubated with Tat86-EGFP (*E*). *C* and *G*, Tat11-EGFP and Tat86-EGFP did not colocalize with the early endosome marker EEA1 (detected by indirect immunofluorescence) in HeLa cells. *D* and *F*, Tat11-GFP and Tat86-GFP were not present in acidic organelles labeled with Lysotracker Red after incubation with the recombinant proteins for the indicated time points.

known to express caveolin-1, a large proportion of endosomes proved positive for both caveolin-1 and Tat11-EGFP.

Lipid rafts are operationally defined by their insolubility in nonionic detergents. Thus, we tested the effect of Triton X-100 on cells that had previously (1 h earlier) internalized both Tat and transferrin. The detergent completely solubilized the transferrin endosomes, whereas those containing either Tat11-EGFP or Tat86-EGFP remained unaffected (shown in Fig. 3, *D* and *E*, respectively). Finally, to further corroborate the conclusion that the internalization of extracellular Tat occurs from cell membrane lipid rafts, we treated cells with methyl-β-cyclodextrin (MβCD), a drug that extracts cholesterol from cell membranes, thus disrupting lipid rafts. As show in Fig. 3, *F* and *G*, for Tat86-EGFP and Tat11-EGFP, respectively, in cells treated with MβCD, endocytosis of Tat was clearly impaired, and a peculiar fluorescence pattern was visualized marking the cell membrane. No internalized fluorescent endosomes were evident. In contrast, internalization of transferrin was not affected by the drug.

As an additional indication that Tat uptake involves caveolar endocytosis, we comparatively analyzed internalization of Tat, transferrin, and cholera toxin B in living cells by time lapse confocal microscopy. Tat86-EGFP was mixed to either transferrin-TRITC or to CtxB-Alexa 594 and added to the culture medium of HeLa cells. After 30 min, fluorescence of endosomes was visualized at 10-s intervals. Fig. 4 shows one confocal plane for each experiment, with images taken for a total of 40 s. In cells exposed to Tat and transferrin (*panel A*), the localization of the endosomes containing Tat appeared unchanged during this time frame, in sharp contrast to the relative mobility of transferrin-containing endosomes. In cells treated with Tat and cholera toxin B (*panel B*), endosomes containing both proteins again were relatively immobile, whereas the localization of a subset of endosomes containing only cholera toxin B changed over time; internalization of cholera toxin B is known to occur, in part, also in clathrin-coated endosomes (38). These results are in agreement with the notion that the velocity at which caveolar endocytosis proceeds is remarkably slower than that of clathrin-dependent endocytosis (40).

The internalization of fluorescent Tat in the presence of different cell treatments was also quantitatively addressed by flow cytometry and compared with the internalization of labeled transferrin. As shown in Fig. 5*A*, Tat uptake in HeLa cells was blocked at 4 °C (as expected for an active endocytosis process) and by treatment with heparin (a soluble competitor of cell membrane-associated HSPGs), consistent with previous findings (27). The former treatment also inhibited the internalization of transferrin, as expected. Two drugs that affect cellular microtubules (taxol, which stabilizes microtubules, and nocodazole, which disrupts polymerized microtubules) had no apparent effect on either Tat or transferrin internalization. Notably, cell treatment with MβCD drastically reduced Tat uptake, thus indicating that the fluorescence apparent at the cell periphery in cells treated with this drug (Fig. 3, *F* and *G*)

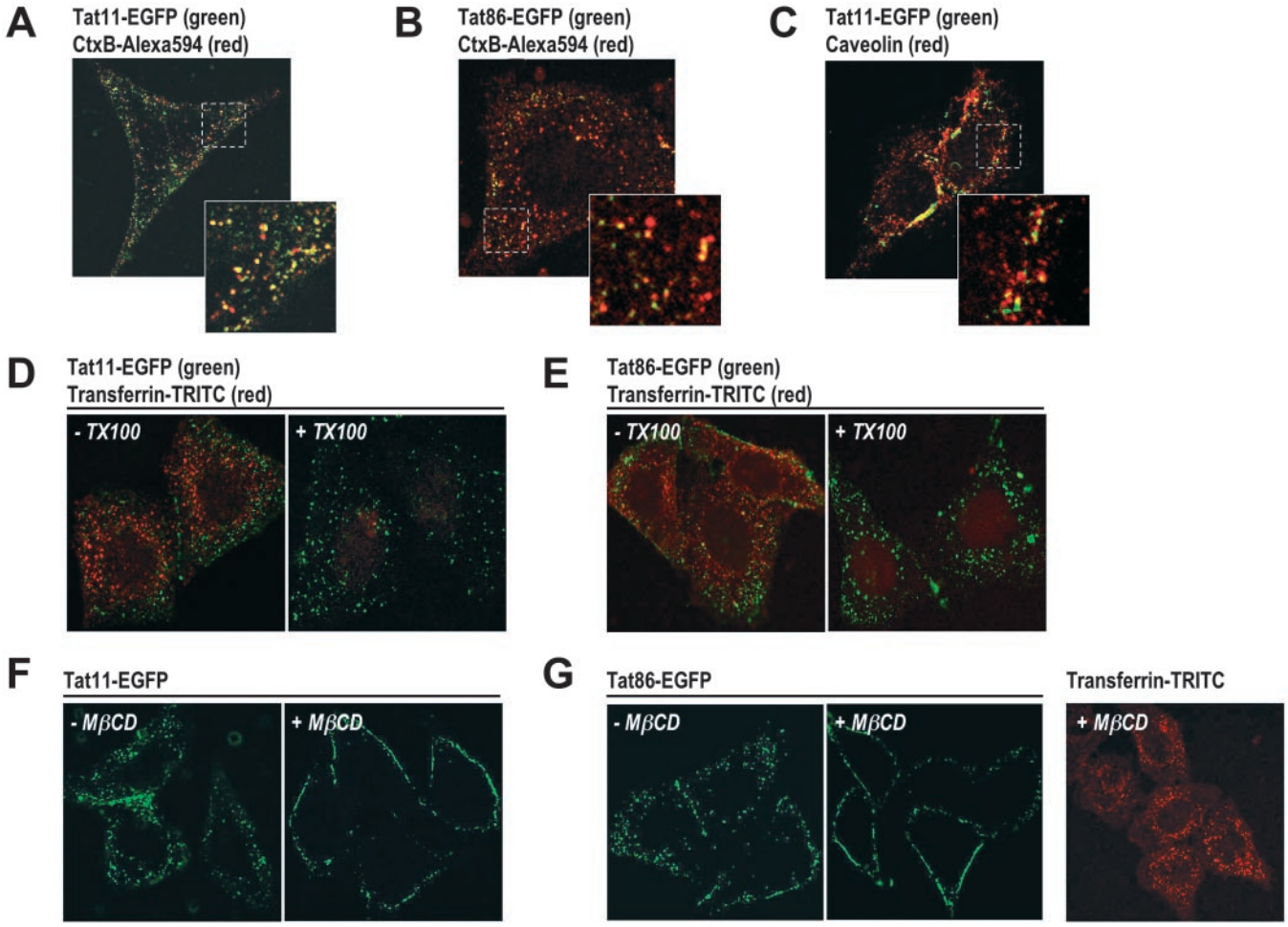

FIG. 3. **Internalization of Tat by caveolar endocytosis from cell membrane lipid rafts.** HeLa cells were incubated with Tat11-EGFP (*A*) or Tat86-EGFP (*B*) and CtxB-Alexa594 for 1 h. Note the extensive colocalization of the recombinant proteins in endocytic vesicles. *C*, colocalization of Tat11-GFP and caveolin-1 detected by immunofluorescence in Cos-1 cells after 2 h incubation with the recombinant protein. *D–E*, Triton X-100 extraction of HeLa cells after 1 h incubation with Tat11-EGFP (*D*) or Tat86-EGFP (*E*) and transferrin-TRITC. Tat11 and Tat86 recombinant proteins were found in detergent-resistant structures, whereas transferrin endosomes were disrupted by Triton X-100 extraction. *F–G*, cells were pretreated with MβCD for 30 min, and then Tat11-EGFP (*F*) or Tat86-EGFP (*G*) was added. Note that internalization of recombinant Tat was blocked by cholesterol depletion, whereas transferrin uptake was not affected.

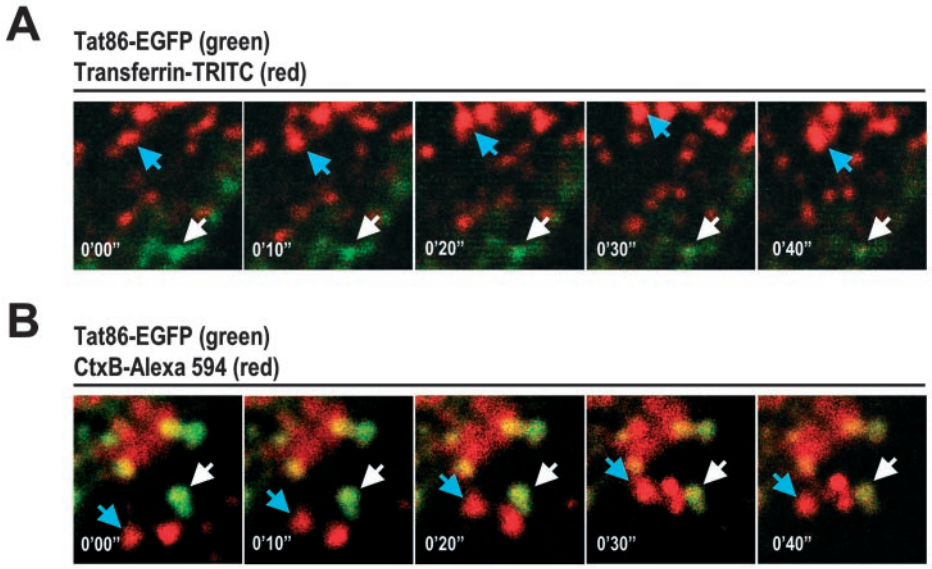

FIG. 4. **Time-lapse imaging of Tat-EGFP endosomes in living cells.** The figure shows the time-lapse imaging of fluorescence co-localization of Tat86-EGFP with transferrin-TRITC (*panel A*) or with CtxB-Alexa594 (*panel B*). Real-time visualization was started 30 min after the addition of the proteins to the cell culture medium. Confocal frames were taken every 10 s. The Tat86-EGFP-positive vesicles (in *green* in *panel A*) and the Tat86/EGFP-CtxB-Alexa594-double positive vesicles (in *yellow* in *panel B*) did not show any appreciable movement during the 40-s time frame of investigation. Representative endosomes are indicated by *white arrows* in the *two panels*. In contrast, transferrin-TRITC endosomes (in *red* in *panel A*) and a subset of cholera toxin-positive endosomes (in *red* in *panel B*) were remarkably mobile (representative endosomes are indicated by *cyan arrows* in *both panels*).

was due to protein interacting with cell surface HSPGs, which eventually were not internalized and were removed by trypsin treatment before flow cytometry. Interestingly, cytochalasin D, a drug causing depolymerization of cell microfilaments, significantly impaired Tat (but not transferrin) internalization, a finding that is consistent with the involvement of microfila-

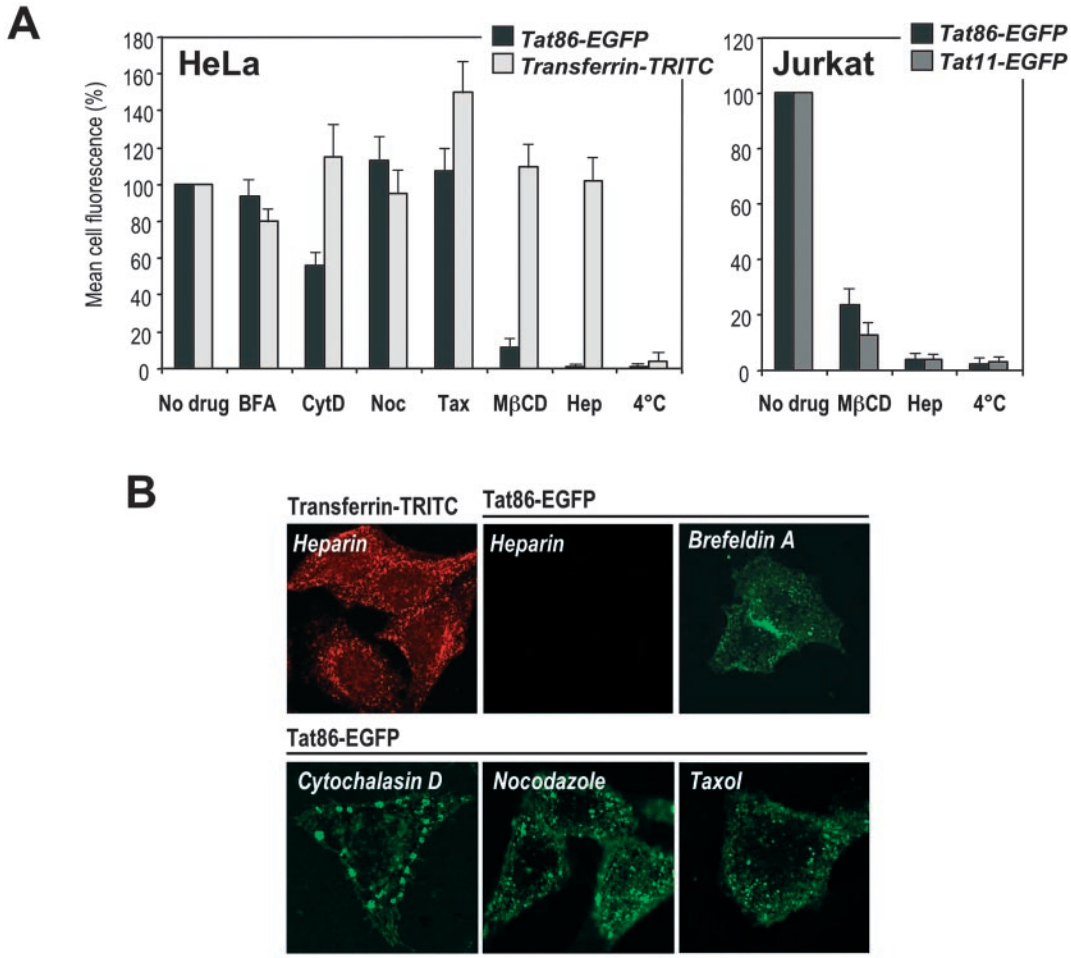

FIG. 5. **Effects of drugs affecting endocytosis on Tat internalization.** *A*, flow cytometry analysis of Tat86-EGFP and Tat11-EGFP uptake in HeLa and Jurkat cells, as indicated, after drug treatment. Treatment with cytochalasin D (*CytD*, a microfilament-disrupting drug), MβCD (a cholesterol-extracting agent), and heparin (*Hep*, a soluble competitor of membrane HSPGs) as well as incubation at 4 °C impaired Tat entry, whereas treatment with nocodazole or taxol (*Noc* and *Tax*; impinging on cell microtubules) did not affect Tat uptake. Flow cytometry analysis of transferrin-TRITC entry after drug treatments is also shown for HeLa cells. Histograms indicate mean and S.D. of three independent experiments for each drug treatment, shown as percent of mean fluorescence over untreated control. *BFA*, brefeldin A. *B*, subcellular localization of Tat86-EGFP in HeLa cells after drug treatment. Note that heparin treatment did not block transferrin entry.

ments in caveolar trafficking (41). Brefeldin A, which impairs Golgi trafficking, had no apparent effect on Tat uptake. Fig. 5*B* shows representative cell images for each of these treatments. Of interest is the pattern of cell fluorescence in cells treated with cytochalasin D, showing the formation of large fluorescent structures at the cell periphery. All data shown in Fig. 4, *A* and *B*, were obtained by using Tat86-EGFP. Superimposable results were also observed with Tat11-EGFP (not shown).

Finally, we tested whether the observed pathway of extracellular Tat internalization also holds true for CD4+ T-cells expressing the CXCR4 chemokine receptor, which besides acting as a co-receptor for HIV-1 infection, has also been shown to be a biologically relevant receptor for extracellular Tat (42, 43). As assessed by flow cytometric analysis (Fig. 5*A*), both Tat86-EGFP and Tat11-EGFP readily entered CD4+ CXCR4+ Jurkat T-lymphocytes, with internalization inhibited by treatment with soluble heparin or at 4 °C. Similar to HeLa cells, treatment with MβCD significantly blocked Tat uptake also in these cells, again indicating the involvement of lipid rafts in the internalization process.

Caveolar Endocytosis Leads to the Translocation of Functional Tat to the Cell Nucleus—We have previously demonstrated that the GST-Tat86-EGFP fusion protein is fully active in transactivation of the viral LTR when exogenously added to the cell culture medium of HL3T1 cells, a cell clone derived from HeLa cells containing an integrated, silent HIV-1 LTR-CAT construct (34). Therefore, we exploited this property to verify that the fraction of protein that was internalized through caveolar endocytosis from lipid rafts was indeed the same one that was eventually translocated to the nucleus and was active in transactivation. To this aim, we developed a quantitative PCR procedure based on competitive PCR to measure the levels of CAT mRNA upon treatment with exogenous Tat86-EGFP. This procedure is based on the co-amplification of the cellular cDNA with a multicompetitor DNA bearing the primer recognition sequences for the CAT transcript and for the reference cellular β-actin mRNA; a representative competitive PCR experiment is shown in Fig. 6*A*. Treatment of HL3T1 cells with Tat86-EGFP for different time periods determined a remarkable increase of LTR-driven transcription, which reached more than 100-fold transactivation after 10 h. Consistent with the fluorescent protein internalization data, when cells were treated with MβCD, heparin, or were kept at 4 °C during internalization, transactivation of the LTR was almost completely abolished (Fig. 6*B*). In contrast, treatment with nocodazole had no effect. Notably, brefeldin A, which had no apparent effect on fluorescent protein internalization, caused a remarkable decrease in the levels of transactivation. As expected, no transactivation was detected in control cells incubated with Tat11-EGFP.

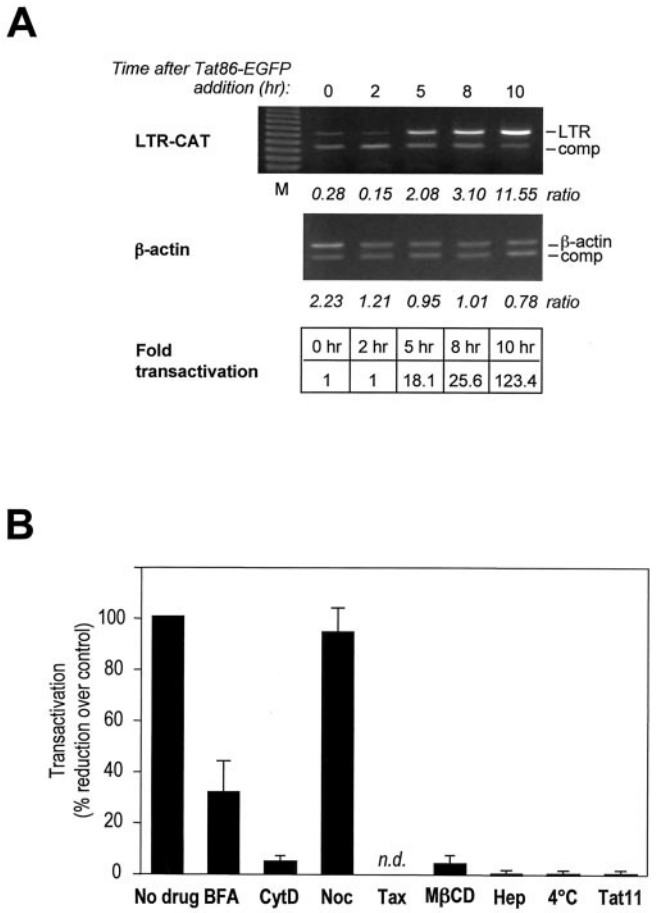

FIG. 6. **Drugs affecting caveolar endocytosis impair Tat transactivation.** *A*, Tat86-EGFP was added to the cell culture medium of HL3T1 cells (bearing silently integrated LTR-CAT construct), and at the indicated time points, the transcripts of the LTR-CAT reporter gene were quantified by quantitative competitive PCR. Cellular RNA was extracted, reverse-transcribed, mixed to a competitor DNA bearing primer recognition sequences for the CAT gene and for the cellular β-actin reference gene, and PCR-amplified. The two figures show representative competitive PCR amplifications for the two cDNA species (*comp*, competitor). On the *bottom* of each *lane*, the ratio between the amounts of the two amplification products is reported. The table on the *bottom* of *panel A* reports the levels of Tat-induced LTR transactivation at the different time points. *B*, the histogram shows the effects of drug treatments on the Tat86-EGFP transactivation activity. The effects were calculated as percent of changes in fold transactivation for each experiment; shown are the means and S.D. of three independent experiments. *n.d.*, not done. *CytD*, cytochalasin D; *BFA*, brefeldin A; *Noc*, nocodazole; *Tax*, taxol; *Hep*, heparin.

These observations clearly indicate that the same drugs or treatments impairing caveolar endocytosis from lipid rafts also block transactivation by exogenously added Tat, thus indicating that this route of internalization eventually leads to the presence of transcriptionally active protein in the cell nucleus.

DISCUSSION

Because of its hydrophobic nature, the cell plasma membrane acts as a selective barrier that physiologically precludes the passage of hydrophilic proteins. This barrier also poses a limit to the use of pharmacologically active macromolecules that are not actively translocated inside the cells. For some time it has been recognized that several toxins of bacterial and plant origin are capable of translocating across cellular membranes to exert their effects in the cytosol or in the nucleus. In most cases, the exploitation of these proteins for the intracellular delivery of other, heterologous proteins has been hampered by their toxicity, large size, and relatively low efficiency. Therefore, the appreciation that a very short amino acid stretch from the basic domain of the HIV-1 Tat could confer translocating capacity even to very large protein cargoes has been met with great enthusiasm and has permitted the development of a number of interesting applications *in vitro* and *in vivo*.

The work described in this manuscript indicates that the cell uptake of large protein cargoes fused to both full-length Tat or to its 11-amino acid transduction domain occurs through a temperature-dependent endocytic pathway that originates from cell membrane lipid rafts and follows caveolar endocytosis. This conclusion is supported by (i) the relatively slow kinetics of internalization, (ii) the finding that Tat endosomes are resistant to the nonionic detergent Triton X-100, (iii) the colocalization of Tat fusion proteins with caveolin-1 in cells that express this protein but not with markers of clathrin-mediated endocytosis, and (iv) the selective sensitivity of Tat internalization to drugs that impair lipid raft formation, such as cyclodextrin, or disturb anchorage of caveolae to the actin cytoskeleton, such as cytochalasin D.

Although this undoubtedly appears to be the route of internalization of large particles fused to Tat (also including a recombinant λ phage particle displaying the Tat transduction domain on its surface (23)), much less clear is whether small peptides encompassing the Tat transduction domain might also enter the cells by alternative pathways. Evidence has been provided to show that short basic peptides, including the Tat basic domain, can directly cross cell membranes by a mechanism that is insensitive to typical endocytosis and metabolic inhibitors (44, 45). When our Tat86-EGFP protein was compared with a fluorescent peptide encompassing the Tat basic domain by simultaneously adding the two molecules to the cell culture medium, it was observed that the peptide entered the cells at 4 °C, whereas the larger recombinant protein did not (36). However, recent experimental evidence seems to indicate that the notion that the Tat basic domain can directly cross the cell membrane might be the consequence of artifacts due to post-fixation diffusion or poor removal of extracellularly bound protein (46). Thus, an active endocytosis process might also be required for the cellular uptake of short, polybasic peptides, as also originally proposed by Mann and Frankel (6). The identification of caveolar endocytosis as the cellular entry pathway of Tat fusion proteins will now permit this issue to be readdressed more specifically.

We have recently observed that Tat internalization requires binding of the protein to cell surface HSPGs, since the uptake process does not occur in cells selectively impaired in HSPG biosynthesis and can be abolished by cell treatment with heparinase III or by competition with soluble, extracellular heparin (26, 27). One of the two families of cell surface HSPGs is the glypicans, which lack a membrane-spanning domain and are anchored to the external surface of the plasma membrane via glycosylphosphatidylinositol (47). Indeed, glycosylphosphatidylinositol-anchored proteins are highly enriched in lipid rafts on the cell surface (32, 48). In addition, glypican-1 is known to be internalized and recycled via the Golgi (49), with the internalization process occurring in caveolin-1-containing endosomes (50). Thus, a testable prediction is that the internalization of Tat might occur after its interaction with the sugar moiety of this HSPG. In this respect, it is of interest to note that the internalization process in CD4+ T-lymphocytes expressing CXCR4, a chemokine receptor that specifically binds extracellular Tat (42, 43) and mainly resides outside of lipid rafts (51–53), is also severely inhibited by cholesterol depletion, indistinguishable from CXCR4-negative cells. This observation indicates that either this receptor is not signifi-

cantly involved in Tat internalization or that its recruitment to lipid rafts is required anyhow for the internalization process to occur, similar to HIV-1 entry.

Several extracellular macromolecules, including bacterial toxins and viruses, are internalized via caveolar endocytosis. Different studies demonstrate that the dynamics of this pathway are relatively slow when compared with clathrin-dependent endocytosis (40, 54). This conclusion is in perfect agreement with the observation made in living cells that Tat-containing endosomes are relatively immobile as compared with transferrin endosomes when analyzed in a 10-s time scale. Consistent with these conclusions, complementary biophysical work performed on living, unfixed cells has shown that endosomes containing an average of ~300 molecules of the Tat86-EGFP molecule proceed from the cell membrane toward the perinuclear region with an average velocity of 3 μm/h and that this translocation process requires the integrity of the actin cytoskeleton (55). As shown in Fig. 5, treatment with cytochalasin D determined the aggregation of Tat-EGFP endosomes in large clusters at the cell periphery. This observation is in perfect agreement with the notion that caveolae are connected with the actin cytoskeleton (41) and that treatment with cytochalasin D leads to clustering of caveolae connected to the plasma membrane (54, 56). In contrast, disruption of microtubules had no effect on Tat-EGFP entry.

Extracellular Tat is internalized by the cells, and eventually it ends up in the nucleus in a transcriptionally active form. One formal possibility existed that the pathway leading to caveolar endocytosis of the protein might be different from the one leading to the presence of the protein in the nucleus. This is clearly not the case, since all the drugs and treatments that inhibited endocytosis also impaired the transcriptional activity of the protein. This observation excludes that there is an alternative pathway for extracellular Tat uptake. In addition, it also implies that the internalized Tat eventually has to egress the endosomes to be transported to the nucleus.

The exit from the endocytic compartment is a molecular process that is likely to require translocation through a cellular membrane. In this respect, it is worth noting that, in the experiments with the Tat fusions presented in this work, at no time did we observe EGFP fluorescence in the nucleus. Still the protein transactivated the LTR and could be evidenced by staining with anti-Tat antibodies (see, for example, Ref. 27). Most probably this observation implies that the Tat-EGFP fusion proteins unfold during membrane translocation, as commonly occurs in this process; once unfolded, an intrinsic property of GFP is to re-fold in an optically active form only at very low efficiency (57). This consideration also implies that the use of the EGFP reporter tag as the sole method by which to study the release of Tat from the cells (another process possibly requiring crossing of a cell membrane), as recently described (58), might possibly bring misleading conclusions.

Bacterial and plant toxins such as cholera toxin and ricin translocate to the cytosol after retrograde transport through the Golgi apparatus and the endoplasmic reticulum (59, 60); consistent with these findings, disruption of Golgi trafficking with brefeldin A inhibits toxin action (61, 62). Of possible interest in this respect is the observation that treatment with brefeldin A did not affect entry of the Tat-EGFP recombinant protein into the cells, as observed by flow cytometry, whereas it did impair its nuclear translocation, as shown by the LTR transactivation data. This observation indirectly implies that the Golgi transport might be required for this process. Despite this suggestion, we have so far failed to visualize Tat in the Golgi or in the endoplasmic reticulum, possibly indicating that the translocation process only occurs in a minority of the internalized molecules, as is also the case for ricin (59). Experiments aimed at identifying specific molecular modification of proteins transiting through the Golgi will directly permit addressing this issue.

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REFERENCES

1. Berkhout, B., Silverman, R. H., and Jeang, K. T. (1989) *Cell* **59,** 273–282
2. Marcello, A., Zoppe, M., and Giacca, M. (2001) *IUBMB Life* **51,** 175–181
3. Jeang, K. T., Xiao, H., and Rich, E. A. (1999) *J. Biol. Chem.* **274,** 28837–28840
4. Green, M., and Loewenstein, P. M. (1988) *Cell* **55,** 1179–1188
5. Frankel, A. D., and Pabo, C. O. (1988) *Cell* **55,** 1189–1193
6. Mann, D. A., and Frankel, A. D. (1991) *EMBO J.* **10,** 1733–1739
7. Elliott, G., and O'Hare, P. (1997) *Cell* **88,** 223–233
8. Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G., and Prochiantz, A. (1996) *J. Biol. Chem.* **271,** 18188–18193
9. Jans, D. A. (1994) *FASEB J.* **8,** 841–847
10. Nagahara, H., Vocero-Akbani, A. M., Snyder, E. L., Ho, A., Latham, D. G., Lissy, N. A., Becker-Hapak, M., Ezhevsky, S. A., and Dowdy, S. F. (1998) *Nat. Med.* **4,** 1449–1452
11. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., and Barsoum, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 664–668
12. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) *Science* **285,** 1569–1572
13. Asoh, S., Ohsawa, I., Mori, T., Katsura, K., Hiraide, T., Katayama, Y., Kimura, M., Ozaki, D., Yamagata, K., and Ohta, S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 17107–17112
14. Cao, G., Pei, W., Ge, H., Liang, Q., Luo, Y., Sharp, F. R., Lu, A., Ran, R., Graham, S. H., and Chen, J. (2002) *J. Neurosci.* **22,** 5423–5431
15. Gustafsson, A. B., Sayen, M. R., Williams, S. D., Crow, M. T., and Gottlieb, R. A. (2002) *Circulation* **106,** 735–739
16. Tasciotti, E., Zoppe, M., and Giacca, M. (2003) *Cancer Gene Ther.* **10,** 64–74
17. Kabouridis, P. S., Hasan, M., Newson, J., Gilroy, D. W., and Lawrence, T. (2002) *J. Immunol.* **169,** 2587–2593
18. Wang, H. Y., Fu, T., Wang, G., Zeng, G., Perry-Lalley, D. M., Yang, J. C., Restifo, N. P., Hwu, P., and Wang, R. F. (2002) *J. Clin. Invest.* **109,** 1463–1470
19. Shibagaki, N., and Udey, M. C. (2002) *J. Immunol.* **168,** 2393–2401
20. Datta, K., Sundberg, C., Karumanchi, S. A., and Mukhopadhyay, D. (2001) *Cancer Res.* **61,** 1768–1775
21. Gratton, J. P., Yu, J., Griffith, J. W., Babbitt, R. W., Scotland, R. S., Hickey, R., Giordano, F. J., and Sessa, W. C. (2003) *Nat. Med.* **9,** 357–363
22. Lewin, M., Carlesso, N., Tung, C. H., Tang, X. W., Cory, D., Scadden, D. T., and Weissleder, R. (2000) *Nat. Biotechnol.* **18,** 410–414
23. Eguchi, A., Akuta, T., Okuyama, H., Senda, T., Yokoi, H., Inokuchi, H., Fujita, S., Hayakawa, T., Takeda, K., Hasegawa, M., and Nakanishi, M. (2001) *J. Biol. Chem.* **276,** 26204–26210
24. Torchilin, V. P., Rammohan, R., Weissig, V., and Levchenko, T. S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8786–8791
25. Sandgren, S., Cheng, F., and Belting, M. (2002) *J. Biol. Chem.* **277,** 38877–38883
26. Rusnati, M., Tulipano, G., Spillmann, D., Tanghetti, E., Oreste, P., Zoppetti, G., Giacca, M., and Presta, M. (1999) *J. Biol. Chem.* **274,** 28198–28205
27. Tyagi, M., Rusnati, M., Presta, M., and Giacca, M. (2001) *J. Biol. Chem.* **276,** 3254–3261
28. Schmid, S. L. (1997) *Annu. Rev. Biochem.* **66,** 511–548
29. Anderson, R. G. (1998) *Annu. Rev. Biochem.* **67,** 199–225
30. Simons, K., and Ikonen, E. (1997) *Nature* **387,** 569–572
31. Simons, K., and Toomre, D. (2000) *Nat. Rev. Mol. Cell Biol.* **1,** 31–39
32. Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D., and Lippincott-Schwartz, J. (2001) *J. Cell Biol.* **153,** 529–541
33. Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001) *Nat. Cell Biol.* **3,** 473–483
34. Felber, B. K., and Pavlakis, G. N. (1988) *Science* **239,** 184–186
35. Demarchi, F., d'Adda di Fagagna, F., Falaschi, A., and Giacca, M. (1996) *J. Virol.* **70,** 4427–4437
36. Silhol, M., Tyagi, M., Giacca, M., Lebleu, B., and Vives, E. (2002) *Eur. J. Biochem.* **269,** 494–501
37. Falnes, P. O., and Sandvig, K. (2000) *Curr. Opin. Cell Biol.* **12,** 407–413
38. Orlandi, P. A., and Fishman, P. H. (1998) *J. Cell Biol.* **141,** 905–915
39. Nichols, B. J., and Lippincott-Schwartz, J. (2001) *Trends Cell Biol.* **11,** 406–412
40. Wacker, I., Kaether, C., Kromer, A., Migala, A., Almers, W., and Gerdes, H. H. (1997) *J. Cell Sci.* **110,** 1453–1463
41. Stahlhut, M., and van Deurs, B. (2000) *Mol. Biol. Cell* **11,** 325–337
42. Xiao, H., Neuveut, C., Tiffany, H. L., Benkirane, M., Rich, E. R., Murphy, P. M., and Jeang, K.-T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 11466–11471
43. Ghezzi, S., Noonan, D. M., Aluigi, M. G., Vallanti, G., Cota, M., Benelli, R., Morini, M., Reeves, J. D., Vicenzi, E., Poli, G., and Albini, A. (2000) *Biochem. Biophys. Res. Commun.* **270,** 992–996
44. Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K., and Sugiura, Y. (2002) *J. Biol. Chem.* **277,** 2437–2443
45. Vives, E., Brodin, P., and Lebleu, B. (1997) *J. Biol. Chem.* **272,** 16010–16017
46. Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., and Lebleu, B. (2003) *J. Biol. Chem.* **278,** 585–590
47. David, G. (1993) *FASEB J.* **7,** 1023–1030
48. Muniz, M., and Riezman, H. (2000) *EMBO J.* **19,** 10–15

49. Fransson, L. A., Edgren, G., Havsmark, B., and Schmidtchen, A. (1995) *Glycobiology* **5,** 407–415
50. Cheng, F., Mani, K., van den Born, J., Ding, K., Belting, M., and Fransson, L. A. (2002) *J. Biol. Chem.* **277,** 44431–44439
51. Popik, W., Alce, T. M., and Au, W. C. (2002) *J. Virol.* **76,** 4709–4722
52. Nguyen, D. H., and Taub, D. (2002) *J. Immunol.* **168,** 4121–4126
53. Kozak, S. L., Heard, J. M., and Kabat, D. (2002) *J. Virol.* **76,** 1802–1815
54. Thomsen, P., Roepstorff, K., Stahlhut, M., and van Deurs, B. (2002) *Mol. Biol. Cell* **13,** 238–350
55. Ferrari, A., Arcangeli, C., Pellegrini, V., Fittipaldi, A., Giacca, M., and Beltram, F. (2003) *Mol. Ther.*, in press
56. Fujimoto, T., Miyawaki, A., and Mikoshiba, K. (1995) *J. Cell Sci.* **108,** 7–15
57. Sacchetti, A., Cappetti, V., Marra, P., Dell'Arciprete, R., El Sewedy, T., Crescenzi, C., and Alberti, S. (2001) *J. Cell. Biochem.* **87,** (Suppl. 36) 117–128
58. Leifert, J. A., Harkins, S., and Whitton, J. L. (2002) *Gene Ther.* **9,** 1422–1428
59. Rapak, A., Falnes, P. O., and Olsnes, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3783–3788
60. Majoul, I. V., Bastiaens, P. I., and Soling, H. D. (1996) *J. Cell Biol.* **133,** 777–789
61. Orlandi, P. A., Curran, P. K., and Fishman, P. H. (1993) *J. Biol. Chem.* **268,** 12010–12016
62. Simpson, J. C., Roberts, L. M., and Lord, J. M. (1996) *Exp. Cell Res.* **229,** 447–451