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Endosome disruption enhances the functional nuclear delivery of Tat-fusion proteins

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Abstract

Tat protein from human immunodeficiency virus can deliver biologically active proteins in vivo and is of considerable interest for protein therapeutics. The mechanism responsible for Tat-fusion protein internalization is still poorly understood and controversial. The punctuate distribution, timing, and temperature sensitivity observed in our experiments with Tat-fusion proteins are consistent with endocytosis. After a few hours, Tat-fusion proteins accumulated around the nucleus without any significant visible nuclear targeting. Using a Cre/Lox based functional assay, lysosomotropic agents known to disrupt endosome integrity, increased by up to 23-fold the nuclear delivery of functional Tat-Cre recombinase without increasing cell uptake in a similar fashion. This shows that endosome disruption can significantly increase Tat-fusion protein access to the cytosol and nucleus. In addition, we found that internalized Tat-fusion proteins persisted several hours and that inhibitors of lysosome acidification did not increase functional nuclear delivery of Tat-Cre. This suggests that Tat-fusion proteins enter via the endosomal pathway, circumvent lysosomal degradation, and are then sequestered in the periphery of the nucleus. Most importantly, our work indicates that an inadequate intracellular trafficking is the main factor limiting the efficiency of protein cargo delivery using Tat.

Keywords: Tat; Endocytosis; Uptake; Transduction; Cre recombinase; Lysosomotropic agents; Perinuclear accumulation; Chloroquine

The Tat protein from human immunodeficiency virus (HIV-1) was shown to be taken up by cells when added exogenously and to trans-activate the HIV-1 LTR promoter in the nucleus. Full-length proteins can also be delivered inside cells or tissues when conjugated or expressed in fusion with the basic domain of Tat [1,2]. The basic domain (RKKRRQRRR) extending from residues 49 to 57 is generally considered to be the minimal sequence responsible for the internalization. Since the early 1990s, other small regions of proteins such as VP-22 [3], ANTP [4], and Kaposi FGF [5] or arginine/lysine-rich peptides [6] were shown to enter cells to carry diverse bio-molecules in the cytoplasm and the nucleus. The underlying mechanism of cellular internalization and intracellular trafficking of cell-penetrating peptides (CPPs) remains misunderstood [7].

An early study concluded that the full-length Tat protein entered cells via adsorptive endocytosis [8]. Since the observed uptake of CPPs is relatively cell-type independent and not completely inhibited at 4 °C, these CPPs were first called "protein transduction domains" (PTDs) and assumed to be internalized using a novel and unknown mechanism involving translocation through the cell membrane [5,9]. Recently, controversy has spurred when groups showed that fixation could lead to artifactual uptake and nuclear localization of Tat, arginine-rich peptides [10] or VP-22 fusion proteins [11]. Interestingly, the exogenously added Tat-eGFP fusion protein accumulated outside the nucleus, whereas the same fusion protein expressed using a transfection system had a nucleolar localization [12,13]. Tat peptide internalization was not abolished at 4 °C, while the uptake of Tat-GST-eGFP was totally inhibited [7].

Although several full-length Tat-fusion proteins (TFPs) can be internalized in cultured cells following their addition in the culture medium, it is often assumed

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that they are internalized in the same manner as short CPPs using an unknown receptor/endosome/energy-independent mechanism. However more recently, TFPs were shown to be internalized by the cells through a temperature-dependent endocytic pathway that follows caveolar endocytosis [14]. In this study, we sought to reexamine the contribution of the endocytic pathway in Tat-fusion protein internalization and to better understand the events implicated in the intracellular trafficking. To do so, we monitored the subcellular localization of a variety of TFPs using fluorescence microscopy against markers relevant to targeted cell compartments. Also, using a novel reporter system based on the in vivo quantification of functional nuclear Cre recombinase, we tested the effects of compounds known to destabilize endosome integrity and acidification or block protein degradation, to better assess the factors influencing the fate of internalized proteins.

Materials and methods

Constructs and protein purification. The pTat-eGFP.4 expression vector was constructed by inserting eGFP from pIRES-eGFP (Clontech) in pTat vector (from Dr. S.F. Dowdy) to produce an in-frame fusion protein [13]. The pTat vector contains a 6-histidine leader followed by the PTD of Tat and a polylinker [1]. The plasmid pTat-Myc was obtained by PCR amplification from pcDNA-Myc (from Dr. D. Jung). The pTat-βGal plasmid was obtained through cohesive (*Kpn*I–*Bst*BI) ligation of the LacZ gene from pEF6/V5-His-Topo/LacZ (Invitrogen) in pTat. Negative control pβGal.1 was generated by *Bam*HI digestion excising the Tat peptide from pTat-βGal. SSR 69 is a polycistronic retroviral vector that contains the *hygro-tk* fusion and the SV40T antigen flanked by LoxP sites and followed by *neo* [15]. The pTriEx-HTNC plasmid coding for a 6×His-Tat/PTD-SV40/NLS-Cre (HTNC) fusion protein (from Dr. F. Edenhofer) [16]. The HTNC, Tat-Myc, Tat-β-Gal and β-Gal [16], and Tat-eGFP [13] fusion proteins were purified as previously described. In short, BL21 Plus RL (Stratagene) bacteria transformed with proper construct were inoculated in LB medium, grown at 37 °C to reach 0.8 $OD_{595\,nm}$, and induced using 0.5 mM IPTG for 3 h. Cells were spun down and sonicated in buffer A (100 mM Hepes, pH 8.0, 300 mM NaCl, and 10 mM imidazole) and centrifugation cleared cell lysates were loaded on a Ni–NTA agarose (Qiagen). Afterwards, the resin was washed twice in buffer A + 20 mM imidazole and eluted in buffer A + 250 mM imidazole. The eluate was dialyzed against 50 mM NaH_2PO_4, 5 mM Tris, 450 mM NaCl, and 5% glycerol overnight at 4 °C. Rhodamine labeling and gel filtration were conducted as previously described [13]. Purified fusion protein concentrations were determined by Coomassie blue staining of SDS–PAGE compared to BSA standards.

Cell culture. NIH 3T3, NIH SSR, and Psi2 (ecotropic retrovirus producing cell line) were grown in DMEM HG supplemented with 10% newborn calf serum, 10,000 U/ml penicillin/streptomycin in 5% CO_2 at 37 °C. Psi2 SSR 69 are Psi2 cells stably transfected with the SSR 69 construct using Metafectene lipofectant (Wisent) and selected for 21 days in a culture medium containing 400 μg/ml hygromycin B (Roche). The NIH SSR cell line was established by infecting NIH 3T3 cells with Psi2 SSR 69 supernatant with 8 μg/ml polybrene and selecting with 400 μg/ml hygromycin for 21 days. DTT (DMD-TAg-*hTERT*) were derived from the myoblasts of a Duchenne muscular dystrophy (DMD) patient and proliferated in MCDB120 as previously described [17].

Functional nuclear Cre recombinase assay. Prior to treatment, NIH SSR were seeded at 10,000 cells/well in 24-well plates (Corning). After 24 h, cells were washed twice in HBSS before they were co-incubated in serum-free DMEM HG containing HTNC for 1 h. Cells were co-incubated with chloroquine (Sigma), sucrose (Lab Mat), bafilomycin A1 (Sigma), concanamycin A (Sigma) or MG 132 (Calbiochem) in some experiments. The incubation mix was removed and cells were then proliferated overnight in complete medium. Twenty-four hours later, treated NIH SSR cells were trypsinized and plated 1:2 or 1:4 in T-25 flasks in complete medium containing 800 μg/ml G418. Cells were grown until distinct colonies appeared (9 days). Cells were rinsed twice in HBSS and then colored with Coomassie blue and colonies were counted.

Immunoblotting. After rinsing three times with PBS, cells were recovered directly from culture dishes by adding sample buffer (50 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and were then frozen at −20 °C immediately until analysis. Sample protein concentration was established using protein dot densitometry stained with amido black 10B curve fitted against a BSA standard curve [18]. Samples were subjected to 10% SDS–PAGE and transferred on Transblot nitrocellulose membrane (Bio-Rad). Membranes were washed once in water and then blocked in PBS containing 10% dried milk for 1 h at room temperature. Membranes were blotted with an anti-polyhistidine mouse antibody (Amersham Biosciences) or an anti-Cre rabbit antibody (Novagen) according to the manufacturer's protocol in PBS containing 10% dried skim milk. Membranes were washed three times in PBS containing 0.05% Tween 20 between each step. Afterward, they were blotted with an appropriate secondary HRP-conjugated antibody (Dako Diagnostics) and later visualized with Western Lightning Chemoluminescent Plus (Perkin–Elmer) and Hyperfilm ECL films (Amersham Biosciences) according to the manufacturer's protocol. Images were digitized with a Nikon CoolPix 4500 camera. Spot densitometry of the 45 kDa HTNC band was performed using NIH Image 1.62.

In vitro intracellular internalization assays. Intracellular delivery assays were performed in 8-well Lab-Tek chamber slides (Nalge Nunc) or with cells adhered on a glass coverslip in a 35 mm dish. For fluorescence microscopy analysis, cells were treated with rhodamine-labeled proteins for 2 h (unless stated otherwise) at indicated concentrations. Cells were then kept in complete medium for the remainder of the experiment, washed three times in HBSS before they were fixed in 4% paraformaldehyde in PBS for 10 min, rinsed once in PBS, stained with 100 ng/ml DAPI for 2 min, rinsed twice in PBS, and mounted under PBS/glycerol (1:1) for observation. Phase contrast and fluorescence images were digitized with a Nikon CoolPix 4500 camera mounted on a Zeiss Axiophot microscope at 100× and 200× magnifications.

Statistical methodology. Statistical analysis of the data was performed using analysis of variance (ANOVA) test and $P < 0.05$ was selected as significant threshold. Data were drawn as means ± SD for each time point or concentration.

Results

Tat-fusion protein uptake is temperature sensitive and does not lead to rapid nuclear accumulation

Recently, it was shown that incubation at 4 °C strongly inhibited uptake of Tat-fusion proteins (TFPs), suggesting that unlike short cationic peptides, full-length protein internalization requires energy [7]. In order to assess the participation of the endosomal pathway in TFP internalization, we first evaluated the effects of low

temperatures on protein uptake in NIH SSR mouse fibroblasts. As shown previously by our group and others, uptake occurs in nearly 100% of cells treated with TFPs even at relatively low concentrations [1,13]. When applied on cells at 4 °C, the detection of Tat-β-Gal is reduced to a level comparable to the β-Gal control (Fig. 1A). Because mild fixation procedures, such as glutaraldehyde fixation, can lead to artifactual cellular uptake of highly charged peptides [10,11], we examined TFP internalization using fluorescence microscopy. Incubation at 4 °C also resulted in a strong inhibition when the uptake was examined using rhodamine-labeled Tat-β-Gal, illustrating even more clearly that the uptake of TFPs is reduced at low temperatures. In contrast to enzymatic detection, when the uptake of TFPs was followed by fluorescence microscopy, there was no detectable binding of the fluorescently labeled proteins to the external cell surface at 4 °C (Fig. 1B).

To examine the kinetics of TFP internalization, cells were incubated with rhodamine-labeled Tat-eGFP for times varying from 10 to 60 min (Fig. 1C). Fluorescence microscopy clearly indicated that in contrast to short Tat peptides that rapidly accumulated in the nucleus [19], full-length TFPs were not rapidly internalized in the cytosol and nucleus. After 10 min, Tat-eGFP generally localized to the cell surface. After 30 min, Tat-eGFP further proceeded inside the cell clearly demonstrating a punctuate appearance, indicative of localization in transport vesicles like endosomes or lysosomes. After 60 min, Tat-eGFP started accumulating around the nucleus. Interestingly, even if the TFPs used in this study contained the strong nuclear localization signal of Tat, none were detected in the nucleus using fluorescence microscopy.

TAT-fusion proteins accumulate in the perinuclear region

To better characterize the final subcellular localization of TFPs, NIH SSR fibroblasts were pulse treated for 2 h with rhodamine-labeled Tat-β-Gal or Tat-eGFP, rinsed, and then proliferated for 4 h in regular culture medium. Rhodamine-labeled Tat-β-Gal and Tat-eGFP accumulated in the periphery of the nucleus (Fig. 2A). Since some endocytic pathways are known to target the ER and Golgi [20], we sought to establish if TFPs co-localized with these compartments. The cell region where TFPs accumulated was stained by concanavalin A (con A), a lectin that recognizes sugars commonly found in the ER and Golgi. While con A and TFPs were detected in the same general intracellular region, they did not co-localize. Perinuclear accumulation was not restricted to Tat-eGFP and Tat-β-Gal (Fig. 2A). Tat-Myc and Tat-Cre, a fusion protein further investigated below, also accumulated around the nucleus (Fig. 2B). This was also the case for Tat-eGFP in human myoblasts (Fig. 2B). In our hands, all TFPs showed perinuclear accumulation, without any significant visible nuclear targeting in all investigated cell types, showing that this phenomenon is not restricted to mouse fibroblasts. These results confirm previous work with Tat-eGFP indicating that internalized TFPs demonstrate an intracellular distribution different from what is observed with similar constructs expressed through transfection [12].

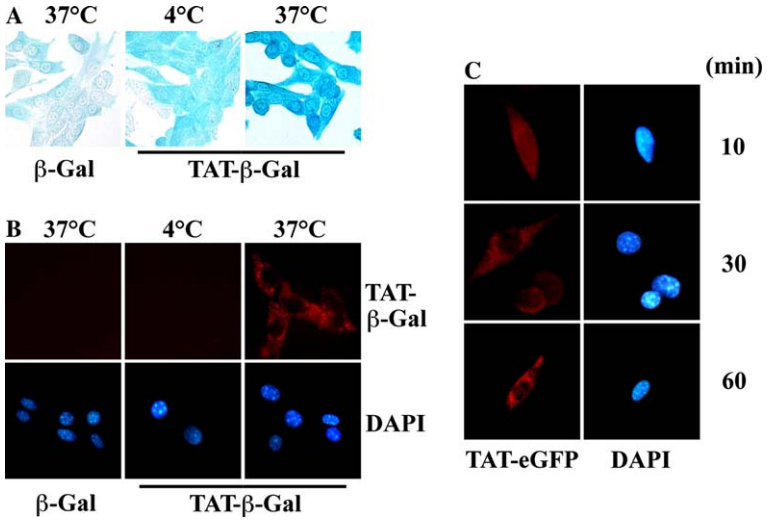

Fig. 1. Effects of incubation at 4 °C and early intracellular distribution of Tat-fusion proteins. (A) Phase contrast microscopy analysis of Tat-β-Gal uptake and influence of low temperature on the internalization process. NIH SSR fibroblasts were treated with Tat-β-Gal or β-Gal (250 nM) for 2 h in complete medium, rinsed three times, fixed in glutaraldehyde, and stained with X-Gal. (B) Fluorescence microscopy analysis of NIH SSR treated with rhodamine-labeled Tat-β-Gal or rhodamine-labeled β-Gal (500 nM) for 2 h in complete medium, rinsed three times, fixed in 4% paraformaldehyde, and stained with DAPI. (C) Fluorescence microscopy analysis of the early subcellular localization of Tat-eGFP in NIH SSR. NIH SSR treated with rhodamine-labeled Tat-eGFP (150 μg/ml) at 37 °C for 10, 30, and 60 min, paraformaldehyde fixed, and stained with DAPI.

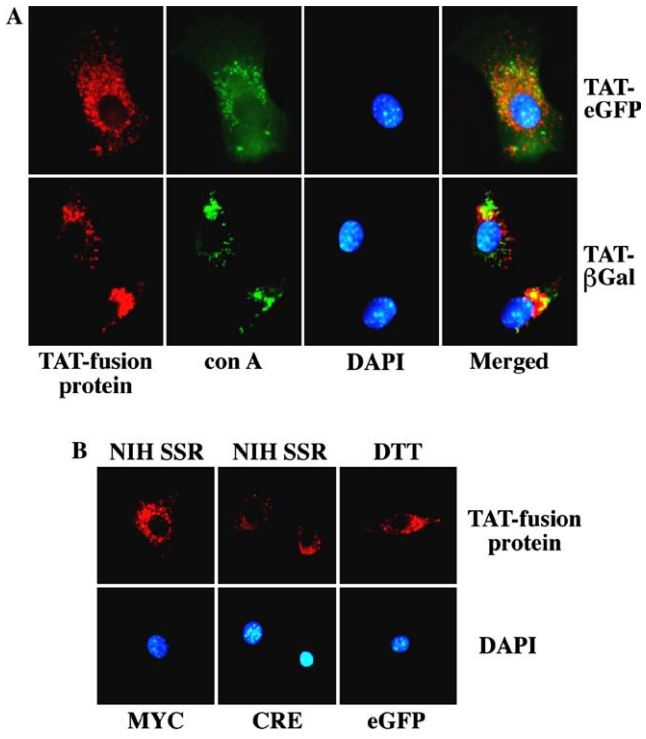

Fig. 2. Subcellular localization of Tat-fusion proteins. (A) Fluorescence microscopy analysis of NIH SSR fibroblasts pulse treated with rhodamine-labeled proteins Tat-β-Gal or Tat-eGFP (50 μg/ml) for 2 h. After a 4 h incubation in complete medium, they were fixed, labeled with concanavalin A-FITC (50 μg/ml), and stained with DAPI. The lectin con A recognizes glycoproteins and core oligosaccharides commonly found in the endoplasmic reticulum and Golgi. (B) Perinuclear accumulation of Tat-Myc and TAT-Cre in fibroblasts and Tat-eGFP in myoblasts. Same experiment as in A, using rhodamine-labeled Tat-Myc, Tat-Cre in NIH SSR fibroblasts, and rhodamine-labeled Tat-eGFP in DTT myoblasts.

Functional nuclear Cre recombinase assay

If TFPs proceed through the endosomal pathway to be later directed to a compartment where they are degraded or sequestered, promoting endosomal release and access to the cytosol should theoretically increase functional nuclear delivery. To exclude potential artifacts from our study, we have designed an assay based on the detection of the biological activity of the Cre recombinase in the nucleus. When added exogenously to cultured cells, the native Cre protein from bacteriophage P1 is internalized and performs site-specific recombination of DNA between LoxP sites [21]. Fusion of Cre to PTDs such as the leader sequence from Kaposi FGF-4 [22] or the Tat peptide [16] can greatly enhance its cellular uptake and subsequent recombination. A target cell line (NIH SSR) was generated by infection with SSR 69 [15], a retroviral vector yielding G418 resistance following LoxP recombination in the presence of Cre recombinase (Fig. 3A). When treated with a Tat-Cre fusion protein (HTNC), NIH SSR cells undergo LoxP specific recombination. After selection with G418, resistant colonies are generated and can

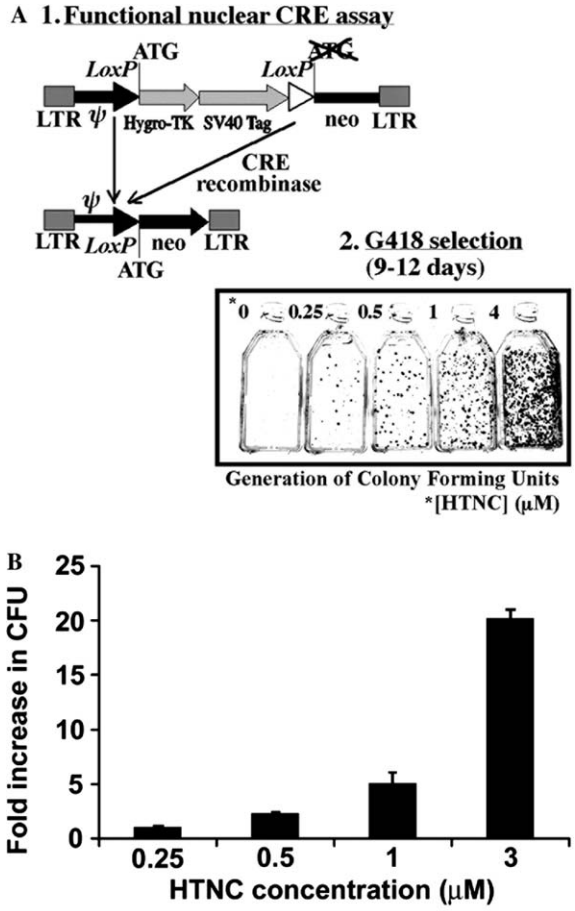

Fig. 3. Functional nuclear Cre assay. (A) Schematic representation of functional nuclear Cre assay and dose-dependent increase in G418 resistant CFU generated by exogenously added HTNC to NIH SSR cells. (B) Concentration-dependent HTNC functional delivery in NIH SSR fibroblasts. Cells were treated with increasing concentrations of HTNC for 1 h in serum-free conditions and then subjected to the functional nuclear Cre recombinase assay. Resistant colonies were stained and then counted. Fold increase was measured using the mean CFU of each group against the control (0.25 μM HTNC). Each column represents the mean ± SD of duplicates.

be counted to evaluate the efficacy of the treatment (Fig. 3A). NIH SSR cells were exposed to increasing concentrations of HTNC and subjected to functional nuclear Cre assay. The quantification of neomycin resistant colonies shows concentration-dependent increase relative to the HTNC added in the culture medium (Fig. 3B). This shows that our method can detect relatively small variations in the amounts of biologically active HTNC delivered into the nucleus of the targeted cells.

Endosome-disrupting compounds dramatically increase functional nuclear delivery

We tested compounds known to increase the delivery of macromolecules, like DNA, that enter cells via the endosomes. Chloroquine is a lysosomotropic agent thought to have a buffering capacity preventing

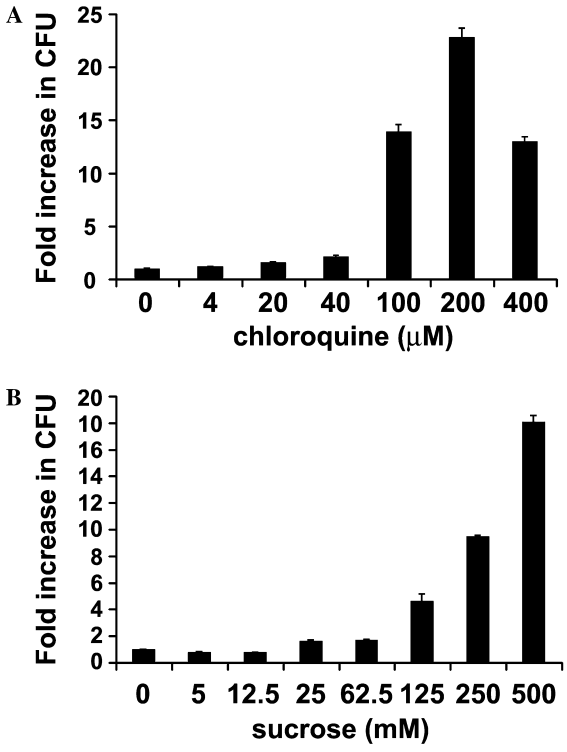

Fig. 4. Effects of lysosomotropic agents known to promote endosomal release on the functional nuclear delivery of Tat-Cre fusion protein (HTNC). Dose dependence of the increase of genomic DNA recombination mediated by exogenously added HTNC in cells by lysosomotropic agents: chloroquine (A) or sucrose (B). NIH SSR fibroblasts were rinsed in HBSS once, then co-treated with 0.5 μM HTNC and one of the lysosomotropic agents, chloroquine (0–400 μM) or sucrose (0–500 mM), in serum-free culture medium for 45 min, and subjected to the functional nuclear Cre recombinase assay. Fold increase was measured using the mean CFU of each group against the control (concentration = 0). Each column represents the mean ± SD of duplicates.

endosomal acidification. In addition, it can lead to swelling and bursting of the endosomes and was shown to enhance DNA transfection [23–25]. When target cells were co-incubated with 500 nM HTNC and 200 μM chloroquine, genomic recombination was increased up to 23-fold in NIH SSR fibroblasts (Fig. 4A). Sucrose was also tested because mammalian cells, in general, lack the intracellular disaccharidase enzyme for metabolizing sucrose. Adding sucrose to the cell culture medium has thus been noted to cause intracellular cytoplasmic swelling within endosomes [26] and was used previously to enhance plasmid DNA transfection efficiency in fibroblasts [23]. Indeed, sucrose co-incubation with HTNC resulted in up to 18-fold increased recombination efficiency in NIH SSR (Fig. 4B) submitted to functional nuclear Cre recombinase assay.

Increased functional nuclear delivery induced by lysosomotropic agents is not the result of increased uptake

Membrane permeation or glycoprotein over-expression resulting in increased uptake could have been responsible for the increased LoxP recombination measured when co-incubating HTNC, respectively, with chloroquine or sucrose. To demonstrate that the increased functional nuclear delivery of HTNC was caused by endosome disruption and subsequent intracellular trafficking modifications and not by an increased cell uptake, we examined the internalization of HTNC in cells co-incubated with a lysosomotropic agent. We quantified the effects of chloroquine and sucrose on cell uptake, using an antibody directed against the poly-His Tag, part of the HTNC fusion protein. Chloroquine treatment did not significantly increase HTNC internalization as measured in total proteins recovered from treated cells (Figs. 5A and C). Far from increasing HTNC uptake, sucrose co-treatment resulted in significant decreases in HTNC immunodetection (Figs. 5B and D). Most strikingly, co-treatment with 500 mM sucrose that increased 18-fold the functional nuclear delivery of HTNC decreased by 9-fold the levels of HTNC uptake. These results clearly show that treatment with chloroquine or sucrose led to strong increases in LoxP recombination due to a modification in intracellular trafficking and not increased cell uptake.

Once internalized, the HTNC fusion protein is not rapidly degraded in lysosomes

The perinuclear accumulation observed in fluorescence microscopy 4 h post-incubation could possibly have resulted from the recycling of labeled amino acids in the ER for neosynthesis following the rapid degradation by lysosomal enzymes or the proteasome complex. The effects of chloroquine are not restricted to its buffering capacity and thus interfere with intracellular transport beyond lysosomal enzyme degradation. To evaluate more specifically the influence of lysosomal enzymes on internalized proteins, we tested the effects of inhibitors of vacuolar H+-ATPases, bafilomycin A1 (BFLA), and concanamycin A (CCM) [27] on the functional delivery of HTNC to the nucleus in NIH SSR cells. These inhibitors of lysosomal acidification, which do not result in transport vesicle swelling, did not increase the functional HTNC delivery to the nucleus (Figs. 6A and B).

While lysosomal enzymes did not seem to be significantly involved in HTNC degradation, we wanted to confirm that internalized proteins persisted inside cells long enough to be shipped to a proper compartment. We evaluated the apparent degradation of the internalized HTNC using immunodetection. Immunoblotting against an epitope of the Cre protein shows a slow decrease in intracellular Cre (Fig. 7). Nonetheless, more than 50% of the internalized HTNC still remains intact after 8 h.

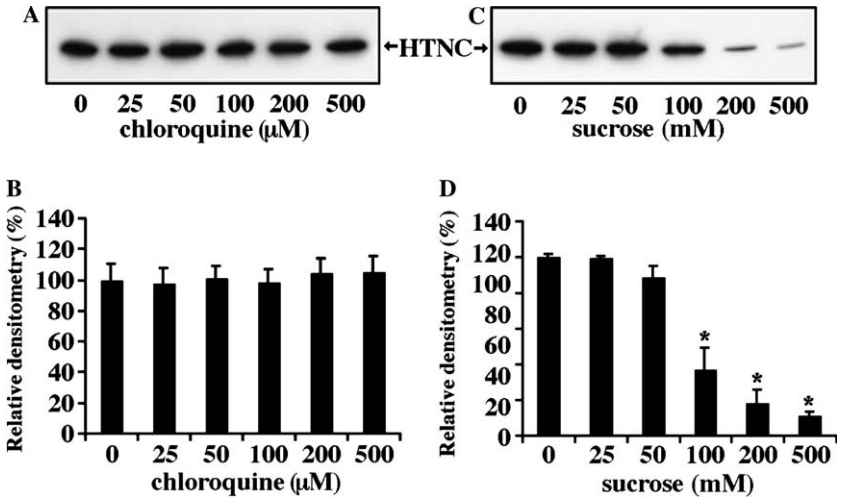

Fig. 5. Effects of lysosomotropic agents on the Tat-Cre fusion protein (HTNC) uptake and binding to NIH SSR. NIH SSR fibroblasts were co-incubated for 1 h with 0.5 μM HTNC and concentrations of 0–500 μM chloroquine (A,B) or 0–500 mM sucrose (C,D) in serum-free DMEM. Cells were proliferated for 1 h and then rinsed three times in HBSS before recuperation in protein sample buffer. In (A,C) immunoblot analysis cell lysates using an anti-polyhistidine antibody. Results of band densitometry are illustrated in (B,D). Each measurement is expressed as a percentage of relative densitometry compared to the untreated control (defined as 100%). Each column represents the mean ± SD of two readings per sample from duplicates. Asterisks indicate significance level (P value <0.05) attained in comparison to the control group (concentration = 0).

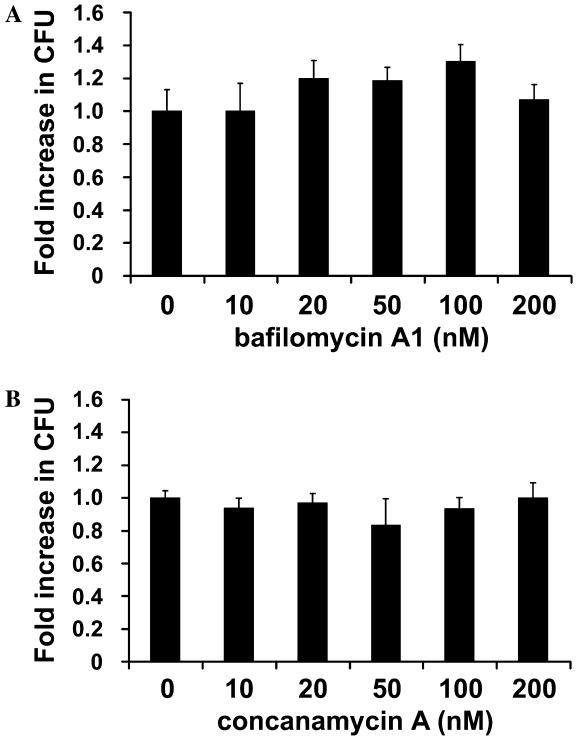

Fig. 6. Effects of vacuolar H+-ATPase inhibitors, BFLA and CCM, on the functional nuclear delivery of Tat-Cre fusion protein (HTNC) in NIH SSR. Cells were pre-treated for 2 h with increasing concentrations of BFLA or CCM (0–200 nM) in complete DMEM. Cells were then co-incubated in 1 μM HTNC with BFLA or with CCM (0–200 nM) in serum-free medium and then subjected to the functional nuclear Cre recombinase assay. Fold increase was measured using the mean CFU of each group against the control (concentration = 0). Each column represents the mean ± SD of duplicates.

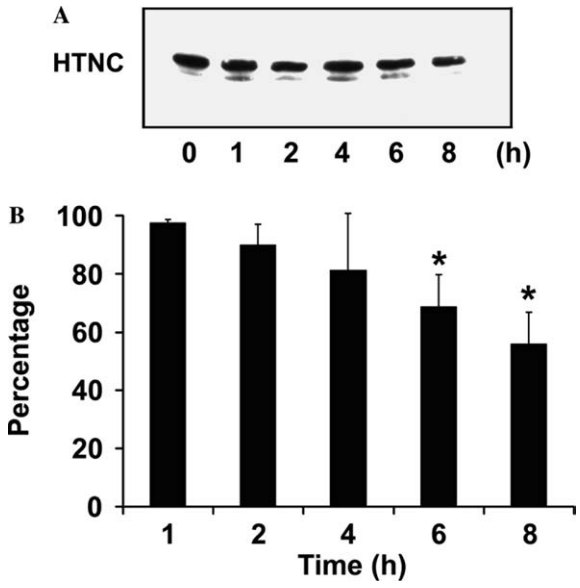

Fig. 7. Tat-Cre fusion protein (HTNC) stability following internalization. (A) Immunoblot analysis of the intracellular stability of the HTNC internalized by NIH SSR cells. Cells were pulse-treated with 1 μM HTNC in serum-free DMEM for 1 h before cells were rinsed and proliferated in HTNC-free culture medium for different amounts of time. After proliferation, cells were directly harvested in protein sample buffer. Cell lysates were analyzed by immunoblotting using an anti-Cre antibody. (B) Results of band densitometry are expressed as percentage of the relative densitometry compared to the time 0 control (defined as 100%). Each column represents the mean ± SD of duplicates from three separate experiments. Asterisks indicate significance level (P value <0.05) attained in comparison to the control group (time = 0).

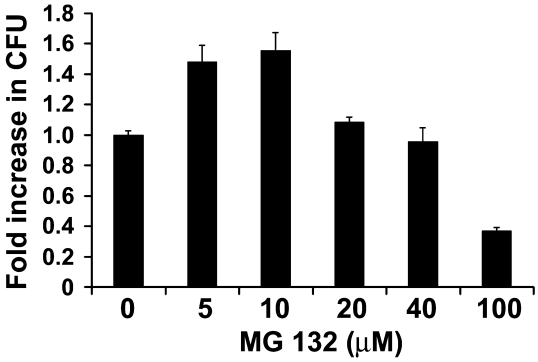

Fig. 8. Effects of the proteasome inhibitor MG 132 on the functional nuclear delivery of HTNC. NIH SSR cells were submitted to functional nuclear Cre recombinase assay in the presence of MG 132. Cells were pulse treated with 1 μM Tat-Cre (HTNC) and increasing amounts of MG 132 (0–100 μM) in serum-free DMEM HG for 1 h. Cells were then washed in HBSS, submitted to an additional treatment of MG 132 (0–100 μM) in complete medium for 3 h, and then submitted to functional nuclear Cre recombinase assay. Fold increase was measured using the mean CFU of each group against the control (concentration = 0). Each column represents the mean ± SD of triplicates.

Proteasome inhibitor MG 132 increases HTNC functional nuclear delivery

The degradation of internalized TFPs by the proteasome complex could be responsible for limited access to the nucleus and the decrease in the intracellular content of HTNC and Tat-β-GAL after the uptake. To examine whether TFPs were degraded by the proteasome, NIH SSR cells were co-treated with HTNC and increasing amounts of MG 132 and then subjected to functional Cre nuclear delivery. MG 132 is a potent, reversible, and cell-permeable proteasome inhibitor targeting the chemotrypsin-like activity of the proteasome [28]. As shown in Fig. 8, HTNC functional nuclear delivery was increased, respectively, by 48% and 56% using MG 132 concentrations of 5 and 10 μM. At a higher dose, MG 132 showed some toxicity resulting in decreased recombination efficiency.

Discussion

In this study, we have investigated the events implicated in the intracellular transport of Tat-fusion proteins (TFPs) using a novel reporter system based on the detection of the functional nuclear delivery of a Tat-Cre fusion protein. Our results showed that the endosomal pathway plays a major role in the process of TFP internalization. TFPs demonstrated a punctuate and temperature sensitive internalization. TFPs slowly accumulated around the nucleus, not inside. Mild fixation procedure (0.25% glutaraldehyde) imposed by β-Gal enzymatic detection led to a higher background in β-Gal controls and to an apparently faint nuclear localization, which was never detected using fluorescent detection. We attribute this discrepancy to the mild fixation procedure used in this experiment that can lead to artifactual nuclear uptake of highly charged peptides [10,11]. Co-treatment with molecules known to disrupt endosomal integrity (chloroquine or sucrose) caused large increases in functional delivery of HTNC to the nucleus. Chloroquine treatment was previously reported to inhibit endosomal acidification leading to a reduction in endosome delivery to lysosomes [25] and to cause membrane disruption [24]. However, the co-treatment with inhibitors of endosome/lysosome acidification did not increase LoxP recombination efficiency, suggesting that vesicles containing TFPs did not fuse with lysosomes or that the neutralization of endocytic vesicles is not sufficient. When cells were co-incubated with rhodamine-labeled TFPs and lysosomotropic agents, there was no apparent increase in the fluorescent labeling of the nuclei (results not shown). This indicates that only a small proportion of delivered proteins can escape endosomes, even in cells treated with lysosomotropic agents. This small amount of proteins is nevertheless sufficient to produce a detectable recombinase activity. This capacity to evade endocytic vesicles could be the result of the amphipathic helix structure found in the basic domain of Tat.

These observations bring us to reconsider the main factors limiting direct protein delivery using cationic peptides such as the basic domain from Tat. While most groups have been developing means to increase the intracellular uptake [6,29], our data suggest that an inadequate intracellular trafficking of TFPs, resulting in perinuclear sequestration, is the major factor limiting functional delivery. We cannot exclude the possibility that proteolysis played a role in the limited access of Tat-fusion proteins to the nucleus, since inhibitors of the proteasome did produce small increases in LoxP recombination efficiency. As shown in Fig. 8 using anti-Cre immunoblotting, internalized HTNC is degraded slowly by the cell, more than 50% remaining after 8 h. Therefore, perinuclear accumulation could not result exclusively from the recycling of labeled amino acids through neosynthesis in the ER. Inhibitors of vacuolar H+-ATPases produced no effect on functional nuclear delivery of HTNC, while proteasome inhibitor MG 132 resulted in limited increases compared to endosome-disrupting agents. We cannot, however, exclude the possibility that the perinuclear accumulation is also responsible for the absence of a rapid degradation. The small proportion of TFPs that escape endocytic vesicles, with or without help from disrupting molecules, could be more accessible to the proteolytic machinery. In consequence, it would be interesting to test the synergistic effects of chloroquine in combination with MG 132.

TFPs can bind to many cell types by associating non-specifically to sulfated-membrane-bound glycoproteins

[30]. Our results confirm that TFPs purified from bacteria, unlike small cationic peptides, enter cells via the endosomal pathway. Endocytic vesicles containing TFPs mainly follow the caveolar endocytosis pathway [14] or the lipid raft macropinocytosis pathway [31]. They are then mainly segregated in the surroundings of the nucleus, where they are non-functional. Only a very small proportion of internalized TFPs can reach the nucleus. While the general localization of internalized TFPs is consistent with the ER or Golgi, and would be a logical outcome for proteins entering using the caveolar endocytic pathway [20] the exact compartmentalization is still uncertain. Indeed in accordance with recent observations [14], and although TFPs generally accumulate in structures resembling the ER/Golgi, we cannot rigorously co-localize TFPs with markers of those compartments. Interestingly, the cholera toxin (CTX) and ricin localize to the ER/Golgi and are internalized via the clathrin-independent/caveolae-mediated endocytic pathway [20]. In fact, CTX which possesses the ability to be internalized in cells is directed to the Golgi through caveolae/raft-dependent endocytosis. It is still unclear whether this perinuclear sequestration of TFPs is due to their internalization pathway or if this phenomenon is restricted to proteins produced in bacteria being recognized as misfolded. Internalized TFPs might be sorted back to follow a path that is analogous to the retrotranslocation process imposed by the quality control mechanism to aggregated or misfolded proteins directed to the ER or TGN for degradation or refolding [32]. Perinuclear accumulation is not uncommon with fusion proteins. It is often observed in the case of over-expressed or misfolded recombinant proteins [33].

The punctuate fluorescence observed following the application of TFPs and the stimulating effects of lysosomotropic agents on their efficient nuclear delivery unquestionably point toward endocytosis. It was previously demonstrated that the basic domain of the Tat protein could not bind to negatively charged membrane glycoproteins as efficiently as polylysines or polyarginines [6]. The properties exhibited by the basic domain of Tat could extend beyond binding to sulfated proteoglycans. While TFPs cannot translocate across the cell membrane like small peptides, the Tat peptide may confer some endosomolysis capacities resulting from the charge distribution found in its helix [29]. We therefore postulate that the unexpected efficiency reported in the literature in recent years, with which the basic domain from Tat can internalize large active cargos inside cells, does not result from a new membrane-translocating mechanism. It is most probably the result of the multitasking nature of the Tat basic domain (strongly cationic, amphipathic helix and nuclear localization signal). Our hypothesis, far from disqualifying the basic domain of Tat as an instrument to perform the internalization of active cargos in cells, suggests that its properties could be enhanced to optimize active intracellular delivery.

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References

[1] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, In vivo protein transduction: delivery of a biologically active protein into the mouse, Science 285 (1999) 1569–1572.
[2] S. Fawell, J. Seery, Y. Daikh, C. Moore, L.L. Chen, B. Pepinsky, J. Barsoum, Tat-mediated delivery of heterologous proteins into cells, Proc. Natl. Acad. Sci. USA 91 (1994) 664–668.
[3] G. Elliott, P. O'Hare, Intercellular trafficking and protein delivery by a herpesvirus structural protein, Cell 88 (1997) 223–233.
[4] A. Joliot, C. Pernelle, H. Deagostini-Bazin, A. Prochiantz, Antennapedia homeobox peptide regulates neural morphogenesis, Proc. Natl. Acad. Sci. USA 88 (1991) 1864–1868.
[5] J. Hawiger, Noninvasive intracellular delivery of functional peptides and proteins, Curr. Opin. Chem. Biol. 3 (1999) 89–94.
[6] J.C. Mai, H. Shen, S.C. Watkins, T. Cheng, P.D. Robbins, Efficiency of protein transduction is cell type-dependent and is enhanced by dextran sulfate, J. Biol. Chem. 277 (2002) 30208–30218.
[7] M. Silhol, M. Tyagi, M. Giacca, B. Lebleu, E. Vives, Different mechanisms for cellular internalization of the HIV-1 Tat-derived cell penetrating peptide and recombinant proteins fused to Tat, Eur. J. Biochem. 269 (2002) 494–501.
[8] D.A. Mann, A.D. Frankel, Endocytosis and targeting of exogenous HIV-1 Tat protein, Embo J. 10 (1991) 1733–1739.
[9] S.R. Schwarze, K.A. Hruska, S.F. Dowdy, Protein transduction: unrestricted delivery into all cells?, Trends Cell Biol. 10 (2000) 290–295.
[10] J.P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M.J. Gait, L.V. Chernomordik, B. Lebleu, Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake, J. Biol. Chem. 278 (2003) 585–590.
[11] M. Lundberg, M. Johansson, Is VP22 nuclear homing an artifact?, Nat. Biotechnol. 19 (2001) 713–714.
[12] Y. Yang, J. Ma, Z. Song, M. Wu, HIV-1 TAT-mediated protein transduction and subcellular localization using novel expression vectors, FEBS Lett. 532 (2002) 36–44.
[13] N.J. Caron, Y. Torrente, G. Camirand, M. Bujold, P. Chapdelaine, K. Leriche, N. Bresolin, J.P. Tremblay, Intracellular delivery of a Tat-eGFP fusion protein into muscle cells, Mol. Ther. 3 (2001) 310–318.
[14] A. Fittipaldi, A. Ferrari, M. Zoppe, C. Arcangeli, V. Pellegrini, F. Beltram, M. Giacca, Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins, J. Biol. Chem. 278 (2003) 34141–34149.
[15] K.A. Westerman, P. Leboulch, Reversible immortalization of mammalian cells mediated by retroviral transfer and site-specific recombination, Proc. Natl. Acad. Sci. USA 93 (1996) 8971–8976.
[16] M. Peitz, K. Pfannkuche, K. Rajewsky, F. Edenhofer, Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient

genetic engineering of mammalian genomes, Proc. Natl. Acad. Sci. USA 99 (2002) 4489–4494.
[17] S. Seigneurin-Venin, V. Bernard, J.P. Tremblay, Telomerase allows the immortalization of T antigen-positive DMD myoblasts: a new source of cells for gene transfer application, Gene Ther. 7 (2000) 619–623.
[18] P. Chapdelaine, K. Vignola, M.A. Fortier, Protein estimation directly from SDS–PAGE loading buffer for standardization of samples from cell lysates or tissue homogenates before Western blot analysis, Biotechniques 31 (2001) 478, pp. 480, 482.
[19] E. Vives, P. Brodin, B. Lebleu, A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus, J. Biol. Chem. 272 (1997) 16010–16017.
[20] P.U. Le, I.R. Nabi, Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum, J. Cell Sci. 116 (2003) 1059–1071.
[21] E. Will, H. Klump, N. Heffner, M. Schwieger, B. Schiedlmeier, W. Ostertag, C. Baum, C. Stocking, Unmodified Cre recombinase crosses the membrane, Nucleic Acids Res. 30 (2002) e59.
[22] D. Jo, A. Nashabi, C. Doxsee, Q. Lin, D. Unutmaz, J. Chen, H.E. Ruley, Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase, Nat. Biotechnol. 19 (2001) 929–933.
[23] K. Ciftci, R.J. Levy, Enhanced plasmid DNA transfection with lysosomotropic agents in cultured fibroblasts, Int. J. Pharm. 218 (2001) 81–92.
[24] P. Erbacher, A.C. Roche, M. Monsigny, P. Midoux, Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes, Exp. Cell Res. 225 (1996) 186–194.
[25] I. Mellman, R. Fuchs, A. Helenius, Acidification of the endocytic and exocytic pathways, Annu. Rev. Biochem. 55 (1986) 663–700.
[26] T. Kato, S. Okada, T. Yutaka, H. Yabuuchi, The effects of sucrose loading on lysosomal hydrolases, Mol. Cell. Biochem. 60 (1984) 83–98.
[27] Z. Ouar, M. Bens, C. Vignes, M. Paulais, C. Pringel, J. Fleury, F. Cluzeaud, R. Lacave, A. Vandewalle, Inhibitors of vacuolar H+-ATPase impair the preferential accumulation of daunomycin in lysosomes and reverse the resistance to anthracyclines in drug-resistant renal epithelial cells, Biochem. J. 370 (2003) 185–193.
[28] S. Tsubuki, Y. Saito, M. Tomioka, H. Ito, S. Kawashima, Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine, J. Biochem. Tokyo 119 (1996) 572–576.
[29] A. Ho, S.R. Schwarze, S.J. Mermelstein, G. Waksman, S.F. Dowdy, Synthetic protein transduction domains: enhanced transduction potential in vitro and in vivo, Cancer Res. 61 (2001) 474–477.
[30] M. Tyagi, M. Rusnati, M. Presta, M. Giacca, Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans, J. Biol. Chem. 276 (2001) 3254–3261.
[31] J.S. Wadia, R.V. Stan, S.F. Dowdy, Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, Nat. Med. 10 (2004) 310–315.
[32] R.E. Chapman, S. Munro, Retrieval of TGN proteins from the cell surface requires endosomal acidification, Embo J. 13 (1994) 2305–2312.
[33] R.E. Cudna, A.J. Dickson, Endoplasmic reticulum signaling as a determinant of recombinant protein expression, Biotechnol. Bioeng. 81 (2003) 56–65.