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Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis

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The TAT protein transduction domain (PTD) has been used to deliver a wide variety of biologically active cargo for the treatment of multiple preclinical disease models, including cancer and stroke. However, the mechanism of transduction remains unknown. Because of the TAT PTD's strong cell-surface binding, early assumptions regarding cellular uptake suggested a direct penetration mechanism across the lipid bilayer by a temperature- and energy-independent process. Here we show, using a transducible TAT–Cre recombinase reporter assay on live cells, that after an initial ionic cell-surface interaction, TAT-fusion proteins are rapidly internalized by lipid raft–dependent macropinocytosis. Transduction was independent of interleukin-2 receptor/raft-, caveolar- and clathrin-mediated endocytosis and phagocytosis. Using this information, we developed a transducible, pH-sensitive, fusogenic dTAT-HA2 peptide that markedly enhanced TAT-Cre escape from macropinosomes. Taken together, these observations provide a scientific basis for the development of new, biologically active, transducible therapeutic molecules.

In 1988, Green and Frankel independently observed the ability of HIV-1 TAT protein to penetrate cells in a receptor-independent, concentration-dependent fashion and activate HIV-1-specific target genes[1–3]. Using the TAT PTD, a short basic region comprising residues 48–57, subsequent studies have generated heterologous TAT fusions to deliver a wide, size-independent variety of molecules into cells, including peptides, proteins, antisense oligonucleotides, large iron beads and liposomes[4–7]. Multiple groups have recently used PTDs to successfully treat preclinical models of human disease, including cancer, psoriasis and stroke[5–7]. However, although recent research using the TAT PTD has shown the usefulness of delivering large macromolecules to treat disease, the mechanism of protein transduction has remained elusive.

Early mechanistic studies showed that TAT-mediated transduction occurs through a rapid, temperature- and energy-independent process, suggesting direct penetration across the lipid bilayer[2,8]. Because of the strong binding of the PTD to the cell surface, however, measurements of protein internalization by flow cytometry or after fixation led to some incorrect assumptions regarding cellular uptake[9–12]. In contrast, biologically studies have shown that TAT-fusion proteins or peptides can elicit phenotypic changes in live cells in culture and *in vivo*[5–7]. Therefore, to understand the mechanism of transduction while avoiding difficulties in interpretation caused by fixation, we used a TAT–Cre recombination reporter assay on live cells. We found that the interaction of TAT-PTD with the cell surface leads to the internalization of TAT-fusion proteins by lipid raft–dependent macropinocytosis. Using this information, we generated a transducible fusogenic influenza dTAT-HA2 peptide that markedly and specifically enhanced macropinosome escape.

RESULTS

TAT-Cre enters cells and recombines DNA

To avoid potential difficulties associated with fixation, we used TAT-Cre–mediated recombination of a *lox*P-STOP-*lox*P enhanced green fluorescent protein (EGFP) reporter gene in live mouse reporter T cells (tex.loxP.EG) as a measure of cellular uptake (**Fig. 1a**). This system requires that exogenous TAT-Cre protein enter the cell, translocate to the nucleus and excise the transcriptional STOP DNA segment in live cells in a nontoxic fashion before scoring positive for EGFP expression. Treatment of reporter T cells with TAT-Cre resulted in site-specific recombination and subsequent induction of EGFP expression (**Fig. 1b** and **Supplementary Fig. 1** online) that was inhibited at 4 °C (data not shown) and negatively affected by the presence of serum (**Fig. 1c**), although no degradation was observed. In contrast, cells treated with control Cre (TAT-minus) protein, expressed and purified under identical conditions, did not undergo recombination or express EGFP (**Fig. 1b** and **Supplementary Fig. 1** online). Incubation of cells with TAT-Cre for as little as 5 min was sufficient to induce recombination, confirming that cellular uptake of TAT-Cre is a rapid process (**Fig. 1c**). Thus, expression of EGFP is dependent on TAT-Cre transduction into cells, followed by nuclear import, recombination and continued cell viability.

Full-length TAT protein has previously been shown to bind strongly to cell-surface heparan sulfate proteoglycans[10,13]. We also

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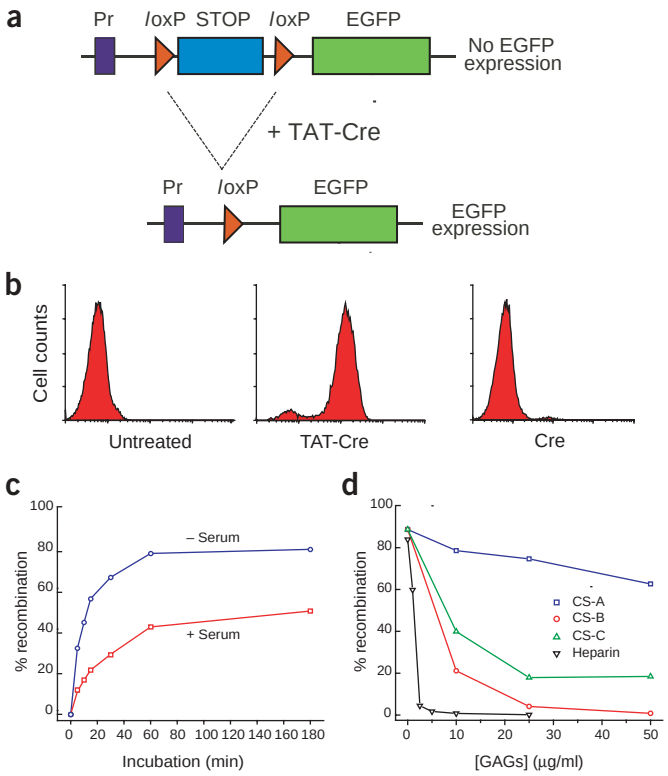

Figure 1 Expression of EGFP requires transduction of TAT-Cre. (**a**) TAT-Cre–mediated excision of transcriptional STOP region flanked by *lox*P sites induces constitutive EGFP expression. Pr, promoter. (**b**) EGFP expression in untreated cells or reporter T cells treated with 2 μM TAT-Cre or 2 μM control Cre (TAT-minus). (**c**) Time course of recombination in reporter T cells treated with 0.5 μM TAT-Cre, with or without serum. (**d**) Incubation of reporter T cells with extracellular glycosaminoglyans (GAGs) of varying doses (0–50 μg/ml chondroitin sulfate (CS) A, B or C, or 0–25 μg/ml heparin) prevents TAT-Cre recombination.

found that incubation of reporter T cells with TAT-Cre fluorescently labeled with Alexa 488 (TAT-Cre-488) resulted in substantial trypsin-sensitive surface binding at 4 °C (**Supplementary Fig. 1** online). To determine whether cell-surface binding is a prerequisite for internalization, we incubated cells with TAT-Cre and increasing concentrations of free glycosaminoglyans for 1 h in serum-free medium, then trypsinized, washed and replated the cells in complete medium and measured EGFP expression after 18 h. Chondroitin sulfates B and C and heparin prevented surface binding of TAT-Cre and strongly inhibited recombination (**Fig. 1d** and **Supplementary Fig. 1** online). These observations indicate that cell-surface binding of TAT-Cre, probably by electrostatic interaction between the basic TAT-PTD and negatively charged cell-surface constituents, is a necessary event before internalization.

TAT-Cre enters cells by lipid raft macropinocytosis

Endocytosis, an essential cellular process for the internalization of a wide variety of extracellular factors, occurs through functionally distinct mechanisms[14]. Several recent studies have suggested that uptake of full-length TAT protein and recombinant TAT-fusion proteins occurs by endocytosis[10–12,15]. Similarly, we found that fluorescently labeled TAT-Cre-488 colocalized in live cells to intracellular endocytic vesicles with FM4-64, a general fluorescent marker of endocytosis (**Fig. 2a**).

We next determined whether cellular uptake of TAT-Cre occurs through a specific endocytic pathway. Removal of cholesterol from the plasma membrane disrupts several lipid raft–mediated endocytic pathways, including caveolae and macropinocytosis[16–18]. We pre-

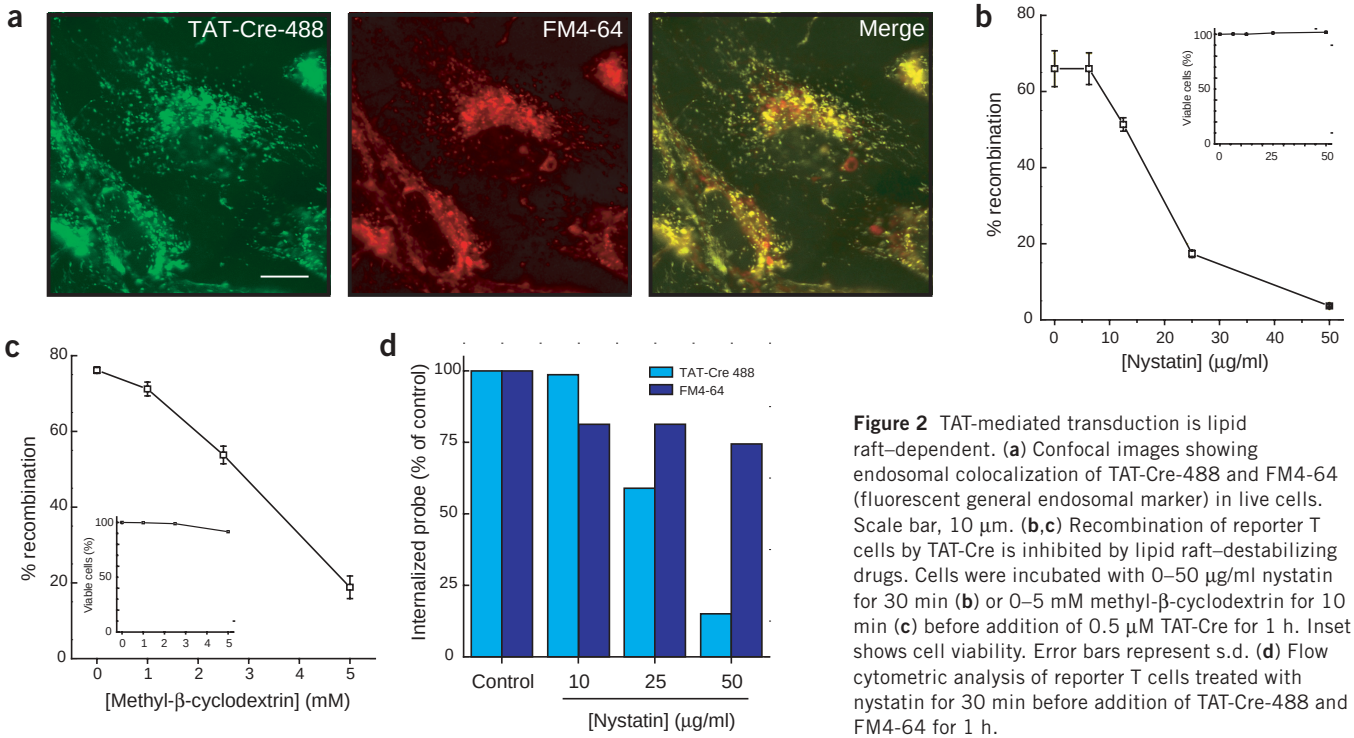

Figure 2 TAT-mediated transduction is lipid raft–dependent. (**a**) Confocal images showing endosomal colocalization of TAT-Cre-488 and FM4-64 (fluorescent general endosomal marker) in live cells. Scale bar, 10 μm. (**b**,**c**) Recombination of reporter T cells by TAT-Cre is inhibited by lipid raft–destabilizing drugs. Cells were incubated with 0–50 μg/ml nystatin for 30 min (**b**) or 0–5 mM methyl-β-cyclodextrin for 10 min (**c**) before addition of 0.5 μM TAT-Cre for 1 h. Inset shows cell viability. Error bars represent s.d. (**d**) Flow cytometric analysis of reporter T cells treated with nystatin for 30 min before addition of TAT-Cre-488 and FM4-64 for 1 h.

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treated reporter T cells with β-cyclodextrin or nystatin to deplete or sequester cholesterol, respectively, then added TAT-Cre for an additional 1 h. This was followed by trypsinization, washing and replating in complete media for 18 h. Surprisingly, both β-cyclodextrin and nystatin disruption of lipid rafts resulted in a dose-dependent inhibition of recombination (**Fig. 2b,c**) that was independent of cytotoxicity. Cotreatment of cells with nystatin, followed by incubation with TAT-Cre-488 and FM4-64, caused a near-complete loss of TAT-Cre-488 internalization, and only a minor (~20%) decrease in FM4-64 uptake (**Fig. 2d**). Taken together, these observations indicate that transduction of TAT-Cre into cells specifically requires lipid raft–mediated endocytosis.

One mechanism of lipid raft–mediated endocytosis is through caveolae involved in slow transcellular vesicle trafficking[17]. It was recently suggested that transduction of TAT-EGFP occurs through caveolar uptake[15]. But consistent with previous reports showing the absence of caveolae in lymphoid cells[19], we did not detect caveolin expression in our T-cell reporter line (**Fig. 3a**). Moreover, cells transfected with red fluorescent protein-labeled caveolin-1 (caveolin-1-RFP) and treated with TAT-Cre-488 did not show colocalization at any time points between 2 and 120 min (**Fig. 3b** and data not shown).

Multiple forms of endocytosis, including phagocytosis and clathrin- and caveolar-mediated endocytosis, require dynamin GTPase activity for vesicle formation at the cell surface. Expression of a dominant-negative mutant of dynamin, Dyn^{K44A}, effectively blocks these endocytic pathways[14]. We cotransfected cells with Dyn^{K44A} and Z/EG *lox*P-STOP GFP reporter plasmids (10:1 ratio, respectively), then treated the cells with TAT-Cre 24 h after transfection and analyzed EGFP expression at 48 h. Expression of Dyn^{K44A} did not block TAT-Cre uptake and recombination (**Fig. 3c**) or decrease the recombination frequency compared with cells transfected with control vector (**Fig. 3d**). In contrast, Dyn^{K44A} expression inhibited uptake of control Alexa 546–conjugated transferrin (**Fig. 3d**). Taken together, these observations show that TAT-Cre internalization occurs through a lipid raft–dependent process that is exclusive of caveolar- and clathrin-mediated endocytosis.

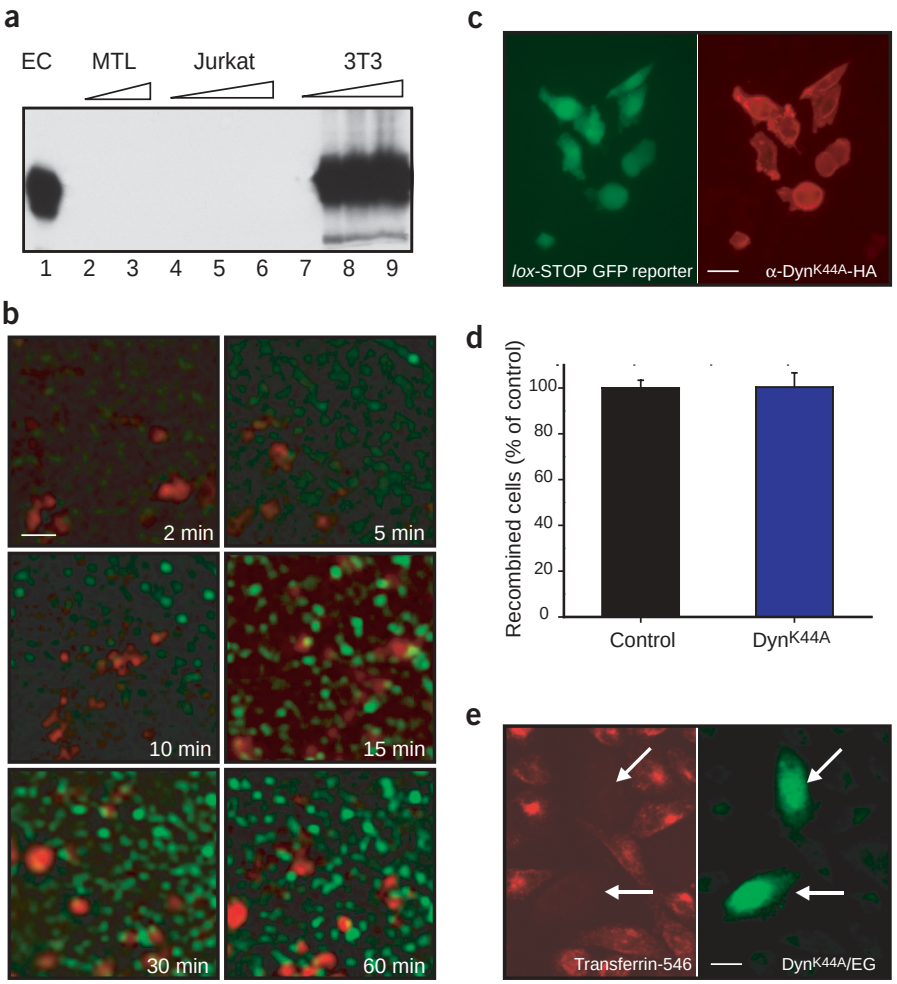

Figure 3 TAT-mediated transduction does not occur by caveolar- or clathrin-mediated endocytosis. (**a**) Caveolin-1 immunoblot analysis of endothelial cells (EC), reporter T cells (MTL), Jurkat T cells and mouse NIH 3T3 fibroblasts. (**b**) Live-cell confocal images of caveolin-1-RFP–transfected cells incubated with fluorescent TAT-Cre-488, taken at indicated time points. Scale bar, 1 μm. (**c**) Left, fluorescent image of cells transfected with Dyn^{K44A}-HA dominant negative and pZ/EG reporter plasmids (10:1 ratio) and treated with TAT-Cre after 24 h. Right, immunohistochemistry using antibodies to Dyn^{K44A}-HA. Scale bar, 20 μm. (**d**) Recombination in TAT-Cre–treated cells transfected with control empty vector or pDynK44A plasmid and pZ/EG reporter plasmid (10:1 ratio). Error bars indicate s.d. (**e**) Transferrin–Alexa 546 uptake in cells transfected with pDynK44A and pEGFP constitutive expression plasmids (10:1 ratio). White arrows indicate Dyn^{K44A}-expressing cells that were inhibited for transferrin uptake. Scale bar, 20 μm.

Macropinocytosis is a rapid, lipid raft–dependent and receptor-independent form of endocytosis. It requires actin membrane protrusions that envelope into vesicles, termed macropinosomes[14,16,18]. To examine the involvement of macropinocytosis in transduction, we pretreated cells with amiloride, a specific inhibitor of the Na^+/H^+ exchange required for macropinocytosis[20], or cytochalasin D, an F-actin elongation inhibitor[21]. Pretreated cells were then incubated with TAT-Cre for 1 h, trypsinized, washed and replated for 18 h. Treatment of cells with both macropinosome inhibitors resulted in a dose-dependent reduction in TAT-Cre transduction and recombination (**Fig. 4a,b**), with no cytotoxicity. Amiloride and cytochalasin D also prevented uptake of TAT-Cre-488 into vesicles, but did not block cell-surface association (**Fig. 4c**). In contrast, the inhibitors caused only a minor reduction in FM4-64 uptake (**Supplementary Fig. 1** online and data not shown). Taken together, these observations showing rapid transduction into cells, dynamin-1-independence, inhibition of uptake by amiloride and cytochalasin D, and variable cargo sizes (peptides to iron beads[22,23]) indicate that TAT PTD–mediated cellular entry occurs by lipid raft–mediated macropinocytosis.

Enhanced endosomal escape by dTAT-HA2 peptide

The majority of the TAT-Cre peptide remained trapped in macropinosomes, even after 24 h (data not shown), indicating that escape from macropinosomes is an inefficient process. To enhance

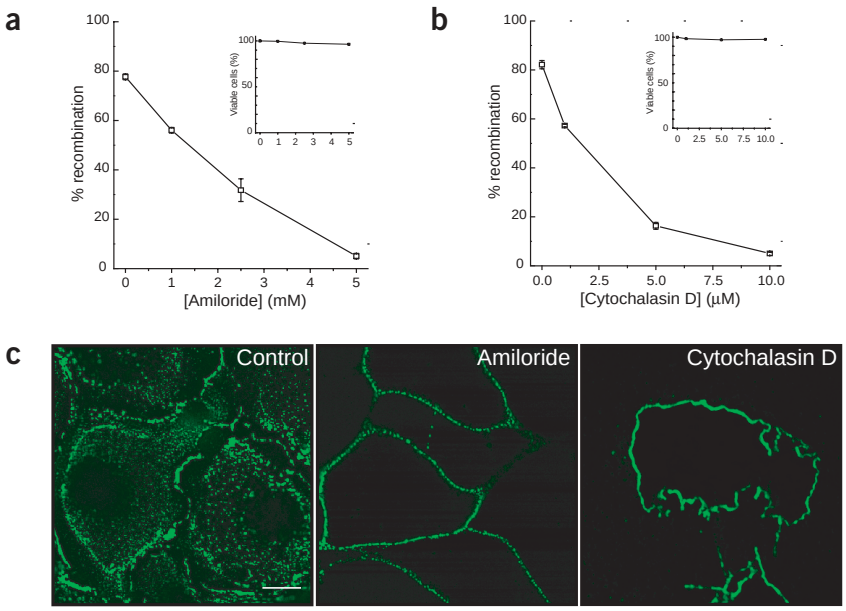

Figure 4 Inhibition of macropinocytosis prevents TAT-Cre–mediated recombination. (**a**,**b**) Flow cytometric analysis of EGFP expression (main panel) and viability (inset) of reporter T cells preincubated with 0–5 mM amiloride (**a**) or 0–10 μM cytochalasin D (**b**) for 1 h before addition of 0.5 μM TAT-Cre for 1 h. Error bars indicate s.d. (**c**) Confocal images of cells treated with amiloride or cytochalasin D for 30 min, then with TAT-Cre-488 for 30 min. Scale bar, 10 μm.

TAT-Cre release from macropinosomes, we treated *lox*P.LacZ reporter 3T3 cells with a subthreshold dose of TAT-Cre in combination with increasing concentrations of chloroquine, an ion-transporting ATPase inhibitor that disrupts endosomes by preventing their acidification[24] (**Fig. 5a**). The combination of TAT-Cre with 100 μM or 200 μM chloroquine resulted in a marked increase in TAT-Cre recombination (**Fig. 5a**). However, the effective dose of chloroquine (≥100 μM) was associated with extremely high cytotoxicity (<95%) in multiple cell lines.

Several viruses have evolved endosomal escape mechanisms that take advantage of the pH drop in mature endosomes[25]. The N-terminal 20 amino acids of the influenza virus hemagglutinin protein, termed HA2, is a well characterized, pH-sensitive fusogenic peptide that destabilizes lipid membranes at low pH[25,26]. Cotreatment of reporter cells with a transducible dTAT-HA2 peptide (5.0 μM; **Fig. 5b**) and increasing concentrations of TAT-Cre resulted in a marked dose-dependent increase in recombination, with no cytotoxicity, compared with cells treated with TAT-Cre only (**Fig. 5c**). In contrast, treatment with control dTAT peptide or control dHA2 peptide had little to no effect (**Fig. 5c**). In addition, enhanced transduction by TAT-HA2 peptide was inhibited by nystatin treatment (**Supplementary Fig. 1** online). These observations indicate that TAT-HA2 markedly enhances the biological activity of TAT-mediated transduction and release into cells.

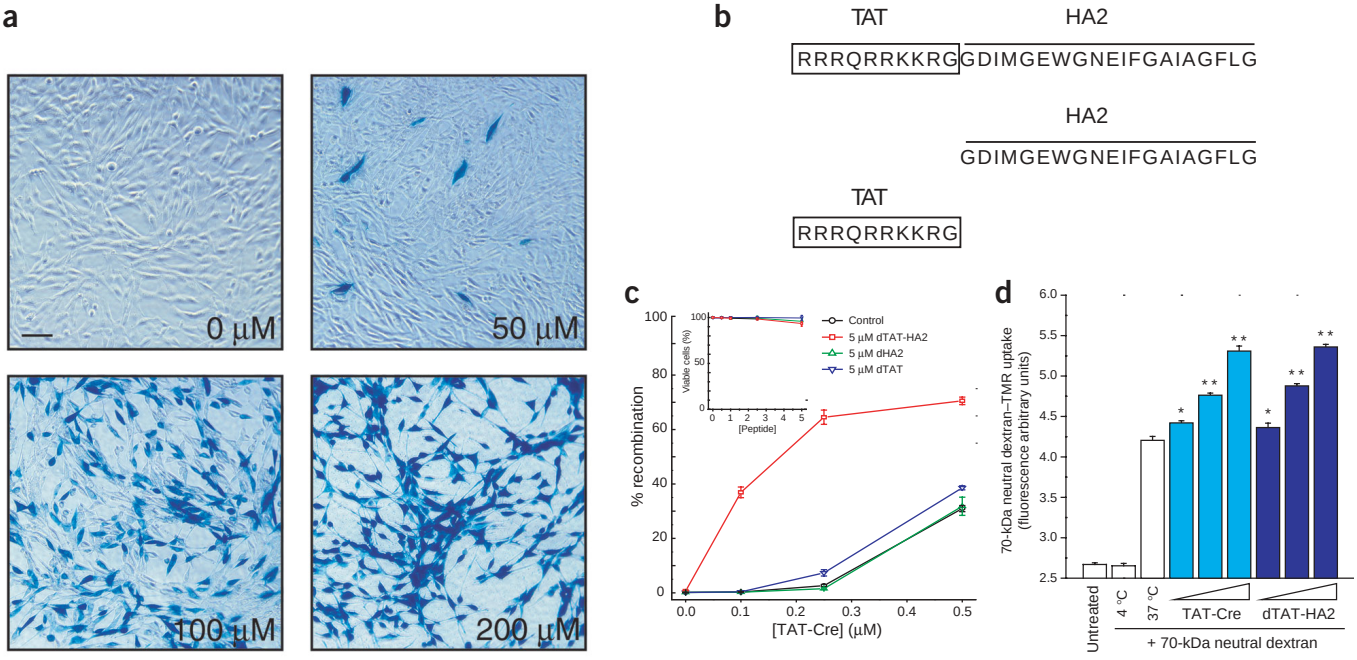

Figure 5 Enhancement of transduction by endosomal disruption. (**a**) Chloroquine increases TAT-Cre recombination in NIH 3T3 *lox*P.LacZ reporter cells. Light micrographs showing X-gal staining (blue cells). Scale bar, 50 μm. (**b**) Amino-acid sequences of dTAT-HA2, dHA2 and dTAT peptides. (**c**) EGFP expression (main panel) and viability (inset) of reporter T cells treated with TAT-Cre alone or with 5.0 μM dTAT-HA2 peptide, 5.0 μM control dHA2 peptide or 5.0 μM control dTAT peptide for 18 h. Error bars indicate s.d. (**d**) Incubation of cells with TAT-Cre or dTAT-HA2 peptide (0.5, 1.0 or 2.0 μM) treatment significantly increased macropinocytosis of 70-kDa neutral dextran–Texas red fluid-phase marker. *, $P < 0.05$; **, $P < 0.001$ compared with 37 °C control. Error bars indicate s.d.

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Finally, to determine the effects of TAT binding on induction of macropinocytosis, we incubated cells with a fluorescent fluid-phase macropinocytosis marker, 70-kDa neutral dextran-tetramethylrhodamine[27,28], in combination with TAT-Cre or dTAT-HA2 (**Fig. 5d**). Consistent with the observations of others[27,28], 70-kDa neutral dextran was primarily taken up by amiloride-sensitive macropinocytosis (**Supplementary Fig. 1** online). Both TAT-Cre and dTAT-HA2 induced a similar significant ($P < 0.05$ to 0.001), dose-dependent increase in 70-kDa neutral dextran fluid-phase uptake over steady-state control levels, independent of the HA2 fusogenic domain (**Fig. 5d**). These observations suggest that cell-surface binding by the TAT PTD stimulates macropinocytotic uptake.

DISCUSSION

Although initially observed in 1988, it is only within the last several years that delivery of macromolecules by PTDs has been refined sufficiently to transport biologically active cargo for the treatment of preclinical animal models of disease[7]. Recent studies on cell-surface binding properties of PTDs[9–12,15], as well as the data reported here, show that the initial binding of PTD to the cell surface involves an ionic interaction. However, our observations strongly support a multistep mechanism whereby the PTD first interacts with cell membrane lipid rafts in a receptor-independent manner, stimulating a rapid internalization by macropinocytosis, followed by a pH drop and destabilization of integrity of the macropinosome vesicle lipid bilayer, and ultimately resulting in the release of TAT-cargo release into the cytosol and nuclear transport. Notably, both TAT-fusion proteins and TAT peptides (J.S.W. & S.F.D., unpublished observations) transduce into cells by macropinocytosis.

Negatively charged sulfated proteoglycans and glycoproteins containing sialic acids are present on all cells. The dependency and specificity of TAT PTD for lipid rafts is therefore somewhat surprising. However, glycophosphatidylinositol-anchored proteoglycans and glycoproteins are present in lipid rafts, suggesting that the TAT PTD may have an increased avidity for certain types of proteoglycans, or perhaps for direct binding to the cholesterol membrane constituents that trigger macropinocytosis. Although the exact nature of TAT-mediated release from macropinosomes into the cytosol remains unclear, macropinosomes are thought to be inherently leaky vesicles compared with other types of endosomes[29,30]. Notably, although the pH of macropinosomes decreases, macropinosomes do not fuse into lysosomes to degrade their contents[14].

Finally, the data presented here suggest that the rate-limiting step is macropinosome escape. Although this principle has been demonstrated in general endosomal disruptors, such as chloroquine and polyethylenimine, their associated toxicity at effective doses prevents their therapeutic use. In contrast, we found that a transducible, pH-sensitive fusogenic dTAT-HA2 peptide delivered *in trans* markedly enhanced release of TAT-Cre from macropinosomes. Trafficking the HA2 fusogenic domain specifically to macropinosomes containing the TAT-Cre cargo, by linking the HA2 domain to the TAT PTD, resulted in both an absence of cytotoxicity and a marked reduction in the effective concentration of HA2 peptide required. This approach has the beneficial aspect of only disrupting macropinosomes and not other types of vesicles. Thus, our discovery that protein transduction occurs by macropinocytosis, and that its efficiency can be enhanced using transducible fusogenic peptides to deliver biologically active, macromolecular cargo, could open new avenues for the treatment and experimental investigation of disease.

METHODS

Purification of TAT-Cre. We cloned the *Cre* cDNA into the pTAT v2.2 vector and expressed it in BL21 pLysS *Escherichia coli* (Novagen). Overnight LB cultures were induced using 500 μM IPTG for 3 h. We purified TAT-Cre by IMAC chromatography and ion exchange on a HiPrep Source 30S 5/5 column (Pharmacia). Aliquots were stored at –80 °C in 10% glycerol. We fluorescently labeled TAT-Cre by coupling it to Alexa 488 or Alexa 546 dyes (Molecular Probes).

Peptide synthesis. All peptides were synthesized as D-amino acid, *retro-inverso* forms using solid-phase FMOC chemistry on an Applied Biosystems 433A synthesizer. The sequences were *R*QRRKKRGGDIMGEWGNEIFGAIAGFLG for dTAT-HA2, *R*QRRKKRG for dTAT and GDIMGEWGNEIFGAIAGFLG for dHA2. We purified the peptides on a C18 HPLC column and analyzed their mass by electrospray mass spectrography. We lyophilized the peptides and stored them at –80 °C. Concentration was determined by absorbance at 215 and 225 nm.

Recombination experiments. To measure the rate of TAT-Cre internalization, we plated tex.loxP.EG cells (E. Ruley, Vanderbilt University) at 5×10^5 cells/well and treated them with 0–0.5 μM TAT-Cre in RPMI, with or without 10% FBS. After each time period, we trypsinized the cells to remove extracellularly bound protein, then washed and replated them into complete medium for 18 h. Cells were then pretreated for 30 min (except amiloride, 10 min) in serum-free medium with the following drugs: chondroitin sulfate A, B or C, 0–50 μg/ml (Sigma); heparin, 0–25 μg/ml (Sigma); nystatin, 0–50 μg/ml (Fluka); methyl-β-cyclodextrin, 0–5 mM (Sigma); amiloride, 0–5 mM (Sigma); or cytochalasin D, 0–10 μM (Sigma). After addition of Tat-Cre, cells were maintained for 1 h in the presence of inhibitors (except cyclodextrin, 10 min; and nystatin, 30 min), washed twice and replated for 18 h in complete medium. To measure the effect of nystatin on TAT-Cre internalization, we pretreated tex.loxP.EG T cells as described with nystatin for 30 min, before adding 2 μM TAT-Cre-488 and 4 μM FM4-64. After 1 h, the cells were trypsinized and washed, and fluorescence was measured by flow cytometry (50,000 cells; gated on live cells by forward/side scatter and propidium iodide exclusion). To determine the effect of endosomal release by chloroquine, 3T3 *lox*P.LacZ cells were treated overnight with 0.25 μM TAT-Cre and 0–200 μM chloroquine (Sigma) in DMEM with 10% FBS. LacZ expression was visualized by *in situ* β-galactosidase staining (Stratagene). For peptide treatments, tex.loxP.EG cells maintained in RPMI and 10% FBS were incubated with 0–0.5 μM TAT-Cre and either 5 μM dTAT-HA2, 5 μM dTAT or 5 μM dHA2 for 16–20 h. EGFP expression was analyzed by flow cytometry.

Microscopy. To visualize TAT-Cre internalization, we incubated 3T3 cells with 2 μM fluorescent TAT-Cre-488 and 4 μM FM4-64. After 8 h, cells were washed and images were acquired using a BioRad MRC1024 confocal microscope. For colocalization studies, COS7 cells were transiently transfected with 0.2 μg caveolin-1-RFP expression vector using Fugene-6. After 24 h, cells were washed and TAT-Cre-488 was added. Live-cell confocal images were taken at time points from 2 to 120 min as indicated. To measure the effect of macropinosome inhibitors on TAT-Cre vesicle formation, we treated COS7 cells with either 10 μM cytochalasin D or 100 μM amiloride (EIPA) for 30 min, before adding 2 μM TAT-Cre-488 and 4 μM FM4-64 for 30 min.

Dynamin-1 (K44A). We transfected CHO-K cells at a ratio of 10:1 with Dyn-1^{K44A}-HA (pDyn^{K44A}-HA) expression plasmid (S. Schmid, Scripps Research Institute) and a pZ/EG (A. Nagy, University of Toronto) reporter vector that expresses GFP after *lox*P-STOP-*lox*P excision. After 24 h, cells were washed and treated with 1.0 μM TAT-Cre in complete medium. After overnight incubation, recombination was scored by GFP expression. Immunohistochemistry using antibody to pDyn^{K44A}-HA was used to verify that GFP-positive cells expressed Dyn^{K44A}. We also transfected control cells at a 10:1 ratio with Dyn^{K44A} and pEGFP (Stratagene) as a marker. After 24 h, cells were washed and incubated in serum-free medium for 4 h before the addition of 25 μg/ml Alexa-546–conjugated transferrin (Molecular Probes) for 15 min. As a control for transferrin endocytosis, cells were transfected 10:1 with an empty vector and pEGFP before treatment with transferrin–Alexa 546. Transfection did not affect the uptake of transferrin in these cells (data not shown).

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Measurement of 70-kDa dextran uptake. To measure the effects of TAT-Cre and dTAT-HA2 on macropinocytosis, we incubated cells with 100 μg/ml of 70-kDa neutral dextran-tetramethylrhodamine or neutral dextran–Texas red (Molecular Probes), along with 0–0.5 μM TAT-Cre or dTAT-HA2 peptide, for 1 h at 37 °C. Cells were then trypsinized and washed in PBS before flow cytometric analysis. To confirm that amiloride inhibited macropinocytosis, we treated cells with 0, 1, 2.5 and 5 mM amiloride and either 100 μg/ml of neutral 70-kDa dextran–Texas red or 4 μM FM4-64 for 1 h at 37 °C.

Immunoblot analysis. We solubilized equal numbers of cells in SDS-PAGE sample buffer and resolved them on a 12% gel. Proteins were blotted onto PVDF and probed with polyclonal antibody to caveolin-1 (1:4,000; Becton Dickinson). Bound antibody was detected using horseradish peroxidase–conjugated antibody to rabbit IgG (1:5,000), followed by enhanced chemiluminescence (Pierce).

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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