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Comparison of Protein Transduction Domains in Mediating Cell Delivery of a Secreted CRE Protein[†]

Paul A. Shaw,[‡] Ian R. Catchpole,[§] Catharine A. Goddard,[‡] and William H. Colledge*[,‡]

Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, CB2 3EG, U.K., and BioPharm CEDD Biology, GlaxoSmithKline R&D Ltd, Medicines Research Centre, Stevenage, SG1 2NY, U.K.

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ABSTRACT: Protein transduction domains (PTDs) are versatile peptide sequences that facilitate cell delivery of several cargo molecules including proteins. PTDs usually consist of short stretches of basic amino acids that can cross the plasma membrane and gain entry into cells. Traditionally, to assess PTD mediated protein delivery, PTD-fusion proteins have been used as purified proteins. To overcome the requirement for a protein purification step, we used a secretory signal peptide to allow PTD-CRE fusion proteins to be exported from transfected mammalian cells. PTD induced protein transduction into cells was assessed by a CRE-mediated recombination event that resulted in β-galactosidase expression. Several PTDs were tested including the prototypic TAT, different TAT variants, Antp, MTS and polyarginine. A negative correlation was observed between the cationic charge on the PTD and the extent of secretion. Poor secretion was found when the PTD charge was greater than +5. One TAT-CRE protein variant had a 14-fold enhancement above CRE alone when added to cells in the presence of chloroquine. This PTD domain also enhanced gene expression after plasmid delivery. These data illustrate that some secreted PTD proteins may be useful reagents to improve protein delivery in mammalian systems and a novel approach to enhancing the response to DNA transfections.

Most protein transduction domains (PTDs[1]) consist of short basic peptide sequences (<20 amino acids) that can cross the cell membrane of eukaryotic cells. The first description of a protein with this activity was for the full-length transactivator (TAT) protein (86 amino acids) from the HIV-1 retrovirus. TAT demonstrated translocation across the lipid bilayer and transcriptional activation of the viral genome (*1*, *2*). A highly basic region located between amino acids 47 and 57 was subsequently identified as the PTD (*3*). PTDs have been identified in other proteins including a 16 amino acid sequence corresponding to the third helix of the Antennapedia homeodomain (Antp) and a short peptide (12 amino acids) derived from the hydrophobic region of Kaposi fibroblast growth factor termed membrane translocating sequence (MTS) (*4*−*6*).

PTDs offer the potential to deliver a range of substances into cells. They have been used to deliver cargo proteins into cells both *in vitro* and *in vivo* (*7*). Intraperitoneal injection of a purified TAT-β-galactosidase fusion protein into mice gave delivery of the 120 kDa protein to all tissues, including the brain (*8*). A recombinant CRE-MTS fusion protein injected into the intraperitoneal cavity of ROSA26R mice (a transgenic mouse line in which a CRE mediated recombination event activates expression of β-galactosidase protein) showed widespread delivery (*9*). TAT has been used in anticancer research to deliver a range of proteins, including p53 (*10*). Antp has been used in a number of studies including enhancing transduction of adenovirus-GFP and adenovirus-β-galactosidase *in vivo* (*5*).

The mechanism by which cationic PTD sequences facilitate entry into cells probably involves an electrostatic interaction with negatively charged proteoglycans on the cell surface followed by a mode of endocytosis. Reagents that disrupt the endosomal compartment like chloroquine have been shown to enhance PTD-mediated protein delivery (*10*−*14*).

Current approaches using PTDs to facilitate protein delivery often rely on producing large quantities of fusion proteins in bacteria. Subsequent purification steps are costly, time-consuming and at risk from contaminating bacterial proteins. We have tested a novel approach involving secretion of PTD-fusion proteins from transfected cells to enhance protein delivery. This approach uses plasmid DNA as the initial delivery reagent, which is considerably easier to purify than protein. We identify a TAT PTD sequence that is efficiently secreted from mammalian cells via a natural secretory pathway while retaining potent transduction activity, enabling spread and providing proof of principle for a controlled eukaryotic based protein spread model for gene

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* To whom correspondence and reprint requests should be addressed. Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, CB2 3EG, U.K. Tel: +44 (0)1223 333881. Fax +44 (0)1223 333840. E-mail: whc23@cam.ac.uk.

[‡] University of Cambridge.

[§] GlaxoSmithKline R&D Ltd.

[1] Abbreviations: PTD, protein transduction domain; TAT, transactivator of transcription; Antp, Antennapedia; MAntp, mutant Antennapedia; MTS, membrane translocating sequence; BFA, brefeldin A; NLS, nuclear localization signal; RLU, relative light units; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; pCRE, pVacssCRE; p(PTD)CRE, pVacss-(PTD)CRE; 3T3PZ, NIH 3T3.loxP.lacZ.

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therapy applications. In addition, PTDs have been assessed at physiological concentrations by virtue of cellular production of PTD-CRE fusion protein as apposed to high concentrations of purified proteins routinely reported (*10*).

MATERIALS AND METHODS

Materials. Brefeldin A was obtained from Fluka. Furin Inhibitor I was obtained from Calbiochem. Oligonucleotides were synthesized by MWG-Biotech (Nottingham, U.K.).

Vector Design. The CRE recombinase DNA sequence was amplified by PCR from the expression plasmid, pPGKCre (*15*). pPGKCre contains the SV40 NLS sequence at the N-terminus of CRE. Primers were designed to incorporate restriction enzyme recognition sites at either end of the PCR product to assist subcloning. The forward primer contained an *Xho*I site and the reverse an *Asc*I site (both underlined).

Forward primer (start at position −9 with respect to the CRE start codon):

5′-CCG CTC GAG ATG CCC AAG AAG AAG AGG AAG GT-3′

Reverse primer (start at position 1029 with respect to the CRE start codon):

5′-TTG GCG CGC CCA TTT ACG CGT TAA TGG CTA ATC G-3′

CRE DNA was cloned directly downstream of the IgH secretory signal sequence (*16*) in *Xho*I/*Asc*I-digested pVacss2 (vector provided by GSK, Stevenage, U.K.). The resulting plasmid, pVacssCRE (abbreviated to pCRE) retained the *Xho*I site directly downstream of the signal sequence to allow in frame fusion of PTD sequences at the N-terminus of CRE. The signal sequence was MGWSCIILFLVATATGVH-**SQ**VQQRSPALER with the signal sequence cleavage site highlighted in bold and the *Xho*I site underlined. PTD sequences were introduced using double-stranded oligonucleotides that when annealed produced overhangs allowing direct cloning into the *Xho*I site. Oligonucleotide sequences were as shown in Table 1.Constructs were named according to the pVacss(PTD)CRE nomenclature (abbreviated to p(PTD)CRE). Plasmids were quantitated using a nanodrop NP1000 spectrophotometer (Wilmington DE), and 260/280 nm ratios were between 1.85 and 1.9. The correct orientation of the PTD was confirmed by DNA sequencing. The luciferase plasmid pVR1223 (generously provided by Vical Inc. San Diego, CA) was also used.

Cell Culture and Transfection. HEK 293T cells (ECACC: 85120602) were cultured in Minimal Essential Medium (MEM) + GlutaMAX I (Invitrogen, Paisley, U.K.) and NIH 3T3.loxP.lacZ (abbreviated to 3T3PZ) cells (*17*) in Dulbecco's MEM + GlutaMAX I (Invitrogen, Paisley, U.K.). All media were supplemented with 10% fetal calf serum (FCS) (TCS Biologicals, Buckingham, U.K.) and 100 U/mL penicillin and streptomycin (Invitrogen, Paisley, U.K.). HEK 293T and 3T3PZ cells were grown to 80% confluency (1×10^6) for transfection of the PTD-CRE expression vectors using Lipofectamine 2000 (Invitrogen, Paisley, U.K.) according to the manufacturer's instructions. Transfected cells were left for 24 h in complete medium before analysis.

Table 1: Oligonucleotides Encoding PTD Sequences

TAT8	l5′-TCG AGT ACG GAC GAA AAA AAC GAC GAC AAC GAC GAC GAC-3′
	5′-TCG AGT CGT CGT CGT TGT CGT CGT TTT TTT CGT CCG TAC-3′
ARG8	5′-TCG AGG GAC GAC GAC GAC GAC GAC GAC GAC GAG GAC-3′
	5′-TCG AGT CCT CGT CGT CGT CGT CGT CGT CGT CGT CCC-3′
Antp7	5′-TCG AGG GAC GAC AAA TAA AAA TAT GGT TCC AAA ACC GAC GAA TGA AAT GGA AAA AAG GAC-3′
	5′-TCG AGT CCT TTT TTC CAT TTC ATT CGT CGG TTT TGG AAC CAT ATT TTT ATT TGT CGT CCC-3′
TAT7	5′-TCG AGT ACG GAC GAA AAA AAC GAC GAC AAC GAC GAG CAG GAC-3′
	5′-TCG AGT CCT GCT CGT CGT TGT CGT CGT TTT TTT CGT CCG TAC-3′
TAT6	5′-TCG AGT ACG GAC GAA AAA AAC GAC GAC AAG CAC GAG CAG GAC-3′
	5′-TCG AGT CCT GCT CGT GCT TGT CGT CGT TTT TTT CGT CCG TAC-3′
TAT6A	5′-TCG AGT ACG GAC GAA AAA AAG CAC GAC AAC GAC GAG CAG GAC-3′
	5′-TCG AGT CCT GCT CGT CGT TGT CGT GCT TTT TTT CGT CCG TAC-3
TAT6B	5′-TCG AGT ACG GAC GAA AAA AAG CAC GAC AAG CAC GAC GAG GAC-3
	5′-TCG AGT CCT CGT CGT GCT TGT CGT GCT TTT TTT CGT CCG TAC-3
TAT6C	5′-TCG AGT ACG GAC GAA AAA AAG CAC GAC GAG CAC GAG CAG GAC-3
	5′-TCG AGT CCT GCT CGT GCT CGT CGT GCT TTT TTT CGT CCG TAC-3
TAT5	5′-TCG AGT ACG GAC GAA AAA AAG CAC GAC AAG CAC GAG CAG GAC-3′
	5′-TCG AGT CCT GCT CGT GCT TGT CGT GCT TTT TTT CG CCG TAC-3′
TAT5A	5′-TCG AGT ACG GAG CAC GAG CAC AGC GAG CAA AAA AAC GAG GAC-3′
	5′-TCG AGT CCT CGT TTT TTT GCT CGC TGT GCT CGT GCT CCG TAC-3
TAT4	5′-TCG AGT ACG GAC GAA AAG CAG CAC GAC AAG CAC GAG CAG GAC-3′
	5′-TCG AGT CCT GCT CGT GCT TGT CGT GCT GCT TTT CGT CCG TAC-3′
TAT4a	5′-TCG AGT ACG CTC GTG CTG CTG CTC GTC GTC GTG CTC GTG CTC-3′
	5′-TCG AGA GCA CGA GCA CGA CGA GCA GCA GCA CGA GCG TAC-3′
MAntp4	5′-TCG AGG GAC GAC AAA TAG CAA TAT GGT TCC AAA ACC GAC GAA TGA AAT GGG CAG CAG GAC-3′
	5′-TCG AGT CCT GCT GCC CAT TTC ATT CGT CGG TTT TGG AAC CAT ATT GCT ATT TGT CGT CCC-3′
TAT3	5′-TCG AGG GTT ACG CTC GTG CTG CTG CTC GTC AAG CTC GTG CTG GTC-3′
	5′-TCG AGA CCA GCA CGA GCT TGA CGA GCA GCA GCA CGA GCG TAA CCC-3′
MTS	5′-TCG AGG AGC AGC CGT TGC CCT TCT CCC TGC CGT TCT TCT CGC TCT TCT CGC CC CAC-3′
	5′-TCG AGT GGG GCG AGA AGA GCG AGA AGA ACG GCA GGG AGA AGG GCA ACG GCT GCT CCC-3′

Western Blotting. Opti-MEM I + GlutaMAX I (Invitrogen, Paisley, U.K.) was placed upon transfected cells for a period of 4 h. At this time point cells were healthy and no cell death

was observed. The PTD-CRE medium was removed and passed through a MILLEX-GV 0.22 μm PVDF filter unit (Millipore, U.K.) and concentrated by an Amicon Ultrafilter Device (10,000 MWCO). Transfected cells were washed in phosphate buffered saline (PBS) and lysates prepared using RIPA buffer (25 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS). Samples were passed through a fine gauge (27G) needle and spun to remove debris prior to electrophoresis. Proteins were separated on a reducing 12% SDS PAGE gel and transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences) via electroblotting (Trans-Blot SD transfer cell, BIO-RAD, Hemel Hempstead, U.K.). CRE protein was visualized using a rabbit polyclonal anti-CRE primary antibody (1:10,000 dilution) (Novagen, 69050-3) and a monoclonal anti-rabbit horse-radish peroxidase conjugated secondary antibody (1:160,000 dilution) (Sigma, A1949). For blocking the membrane and antibody dilution, 5% BSA fraction V (Sigma, Gillingham, U.K.) in PBS-Tween (5%) was used.

CRE Recombinase ELISA. To measure the amount of secreted CRE protein in the medium a CRE sandwich ELISA was developed. Rabbit anti-CRE polyclonal capture antibody (Novagen, 69050-3) was bound (0.4 μL/well) to wells (Maxisorp ELISA plate, nunc) overnight at 20 °C in coating buffer (50 mM carbonate buffer, pH 9.6, 150 mM Na_2CO_3, 350 mM $NaHCO_3$). Wells were washed with 0.05% Tween-20 in PBS, pH 7.2, 3 × 2 min with gentle agitation and then blocked in 3% BSA/PBS at 37 °C for 4 h. Wells were washed prior to adding samples (blocking buffer was used for dilutions). Samples were incubated in the wells at 37 °C for 2 h followed by a wash and addition of a biotinylated detection antibody (1:100 dilution) (Covance, BIOT-106L) to wells for 2 h in a humid environment with gentle agitation. The wells were washed with PBS and incubated with a streptavidin−horse radish peroxidase (HRP) conjugate (1:15,000 dilution) (Abcam, ab7403) for 30 min at 20 °C. A further wash preceded the addition of the HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, T0440) (50 μL/well). Color development took place over 10 min. The reaction was stopped with 0.5 M sulfuric acid (50 μL/well) and absorbance measured at 450 nm.

In Vitro Delivery of PTD-CRE Protein. 3T3PZ cells were pretreated for 2 h with chloroquine (100 μM). PTD-CRE protein was quantitated in medium using the CRE ELISA and equal amounts placed upon reporter cells in medium containing chloroquine (100 μM). After incubation, the PTD-CRE medium was replaced with complete medium for 36 h prior to detection of the β-galactosidase protein as an indicator of cell transduction by the PTD-CRE protein. To monitor the kinetics of uptake, PTD-CRE medium was left in contact with 3T3PZ cells for different amounts of time. To ensure complete removal of PTD-CRE from the cell surface, cells were washed in PBS, trypsinized and plated in complete medium prior to detection of β-galactosidase.

β-Galactosidase Detection and Quantitation. For detection of β-galactosidase, cells were washed in PBS twice, fixed in 4% paraformaldehyde/PBS at 4 °C for 5 min, washed in PBS and stained in X-Gal solution overnight at 37 °C. X-Gal solution was prepared by adding 1 mg/mL X-gal (prepared as 25 mg/mL stock in dimethyl formamide) into the staining solution (PBS with 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, pH adjusted to 7.5−8). For quantitation of β-galactosidase activity the Galacto-Light Plus System (Applied Biosystems, Warrington, U.K.) was used according to manufacturer's instructions.

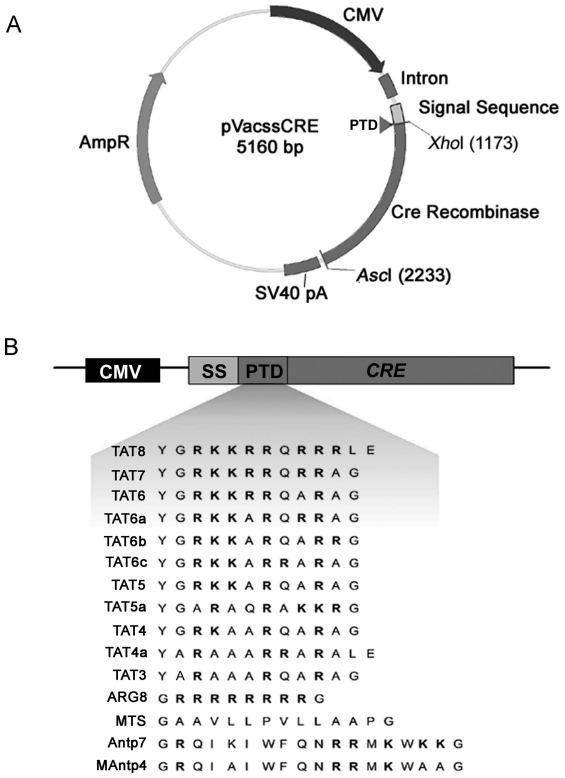

FIGURE 1: PTD expression vector and PTD sequences. (A) PTD-CRE fusion protein expression vector. PTD sequences were cloned into the *Xho*I restriction site between the secretory signal sequence and the CRE recombinase sequence. (B) The amino acid sequences of each PTD used in this study. Cationic amino acids are highlighted in bold. The nomenclature used is the PTD name followed by a number corresponding to the cationic charge, e.g., Antp7 originates from *Drosophila Antennapedia* and has 7 cationic amino acids. TAT = HIV1 transcriptional transactivating factor peptide sequence, MTS = membrane translocating sequence from acid fibroblast factor.

Luciferase Detection and Quantitation. Luciferase protein was detected using the Luciferase assay system (Promega, Madison, WI) according to manufacturer's instructions.

Statistical Analysis. A two-tailed Student's *t* test was used to calculate *P* values and significance taken as $P < 0.05$.

RESULTS

Construction and Validation of p(PTD)CRE Expression Plasmids. To determine whether secreted PTD recombinant proteins represent a viable approach to enhance gene delivery via protein spread, a series of mammalian expression plasmids were constructed, each containing unique PTD sequences fused to the full-length CRE recombinase protein. To ensure efficient secretion of the PTD-CRE recombinant protein from mammalian cells, PTD sequences were cloned in frame between the C-terminus of the immunoglobulin-H secretory signal sequence and the N-terminus of the CRE sequence (Figure 1A). It has been postulated that PTD uptake relies on initial electrostatic interactions between cationic PTD sequences and negatively charged phospholipid bilayers. A range of PTD sequences were tested, each varying in cationic amino acid content, with the aim of determining

how charge might influence function (Figure 1B). The prototypic TAT PTD sequence contains 8 cationic amino acids and is therefore referred to as TAT8. To investigate a charge effect, cationic amino acids were sequentially replaced with the uncharged amino acid alanine in the TAT sequence (Figure 1B) to produce a repertoire of PTDs with decreasing charge (TAT7−TAT3). TAT3 is based on an optimized TAT sequence previously described and MAntp4 is a mutant form of Antp (*18*), previously described to have no PTD activity (*5*).

To confirm expression of a functional PTD-CRE fusion protein, all constructs were transfected into the 3T3PZ reporter cell line (*17*) (generously provided by Dr. M. Bessler, Washington University). CRE protein expression in these cells results in removal of an intervening sequence that inhibits expression of the *lacZ* gene (Figure 2A). This provides an assay by which PTD-CRE fusion proteins can be functionally validated. All of the plasmids were found to produce functional CRE protein as judged by induction of β-galactosidase expression in the 3T3PZ reporter line (Figure 2B). β-Galactosidase expression was never observed following transfection with a vector without CRE (pVacss2). Immunocytochemistry using an anti-CRE antibody showed that PTD-CRE was expressed by 24 h post-transfection but not detectable at an earlier time point of 9 h (Figure 2C). Therefore, to investigate secretion, medium samples were collected 24 h post-transfection.

Secretion of PTD-CRE from Mammalian Cells Is Influenced by the Cationic Charge of the PTD Sequence. To assess the expression levels and extent of secretion of PTD-CRE proteins, Western blotting was used to compare CRE protein levels in the cell lysates and the medium of transfected cells. Medium samples were collected for a period of 1 h after the 24 h transfection period. This was to eliminate any possibility that release of PTD-CRE protein was due to cell death (cells remain healthy after 1 h in Optimem (results not shown). Medium samples were also filtered to remove any cells that may have become detached from the surface of the tissue culture plate.

PTD-CRE expression plasmids were transfected into HEK 293T cells and 3T3PZ cells. All plasmids expressed CRE protein in the cell lysate fraction at approximately similar levels (assuming similar transfection efficiencies for each plasmid) (Figure 3A). The 41 kDa and 39 kDa bands probably represent different glycosylation states of the protein. The pPGKCRE expression plasmid was included as a nonsecreted CRE control. This was demonstrated by absence of CRE in the medium from these transfected cells. CRE protein encoded from the pCRE plasmid (containing the IgH secretory signal) was present in both the cell lysate and the medium, indicating secretion from the transfected cells (Figure 3A). Analysis of each PTD-CRE expression plasmid showed that only a subset of the PTD-CRE recombinant proteins were secreted efficiently from 293T cells. From HEK 293T cells, MTSCRE, TAT3CRE, MAntp4CRE, TAT4CRE, TAT4aCRE, TAT5CRE and TAT5aCRE were secreted at comparatively high levels compared to the other PTD-CRE fusion proteins (Figure 3A). A similar pattern was not as noticeable in the 3T3PZ cells. In the 3T3PZ cells, the MTSCRE, TAT3CRE and TAT4CRE demonstrated the best secretion with the remaining PTD-CRE proteins demonstrating lower secretion, which reduced

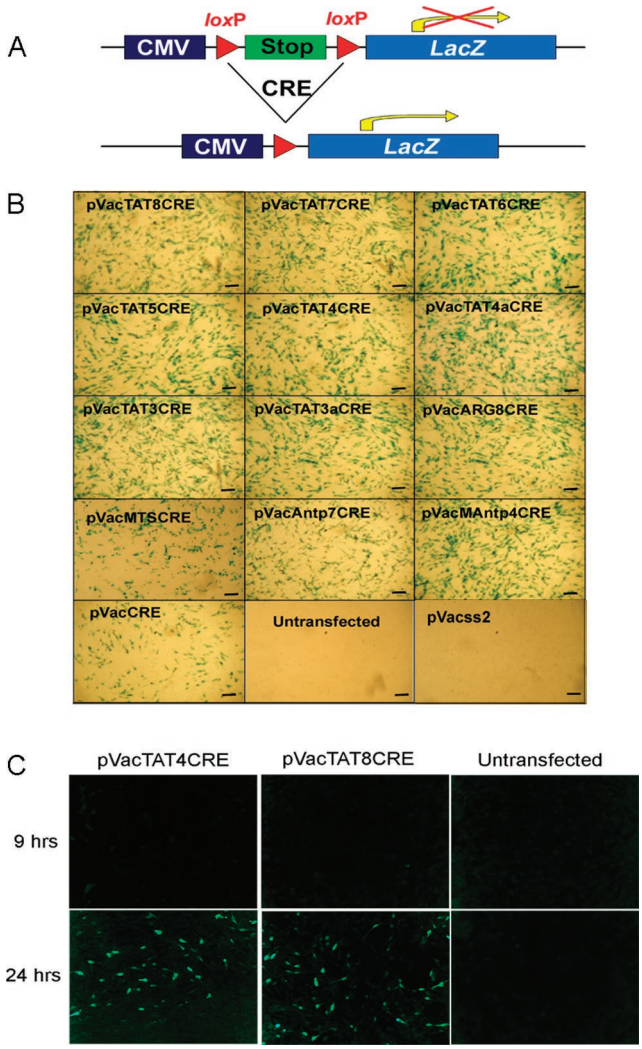

FIGURE 2: PTD-CRE fusion proteins are expressed by 24 h and retain functional CRE activity. (A) Structure of the *LacZ* transgene in the 3T3PZ reporter cell line. CRE-mediated recombination catalyzes removal of the stop cassette, allowing expression of β-galactosidase protein from a CMV enhancer modified chicken β-actin promoter. The stop cassette is the chloramphenicol acetyltransferase gene followed by SV40-t-intron poly-A signal. (B) Validation of functional CRE activity in PTD-CRE fusion proteins. Plasmids were transfected into the 3T3PZ reporter cell line and CRE mediated recombination monitored by staining the cells for β-galactosidase activity. Scale = 10 μm. (C) Time course of CRE expression. 3T3PZ reporter cells were transfected with PTD-CRE expression plasmids. After 9 and 24 h, cells were fixed and probed with FITC labeled anti-CRE antibody to detect CRE recombinase. Untransfected cells were included as a negative control.

as cationic charge increased (Figure 3A). A marked difference was found between secretion of TAT5CRE and TAT6CRE protein particularly in HEK 293T cells suggesting an effect of PTD charge on secretion (Figure 3A). To investigate whether the positional effect of the cationic charge within the PTD influenced secretion, sequences from both TAT5 and TAT6 PTDs were rearranged. The TAT5 sequence was reversed producing TAT5a and the arginine residues in TAT6 were rearranged to form TAT6a, TAT6b and TAT6c. In HEK 293T cells, TAT5aCRE was secreted similarly to TAT5CRE (Figure 3A) and TAT6aCRE, TAT6bCRE and TAT6cCRE were secreted more than TAT6CRE. In the 3T3PZ cells, TAT5CRE and TAT5aCRE showed similar secretion profiles as did TAT6bCRE and TAT6cCRE, which

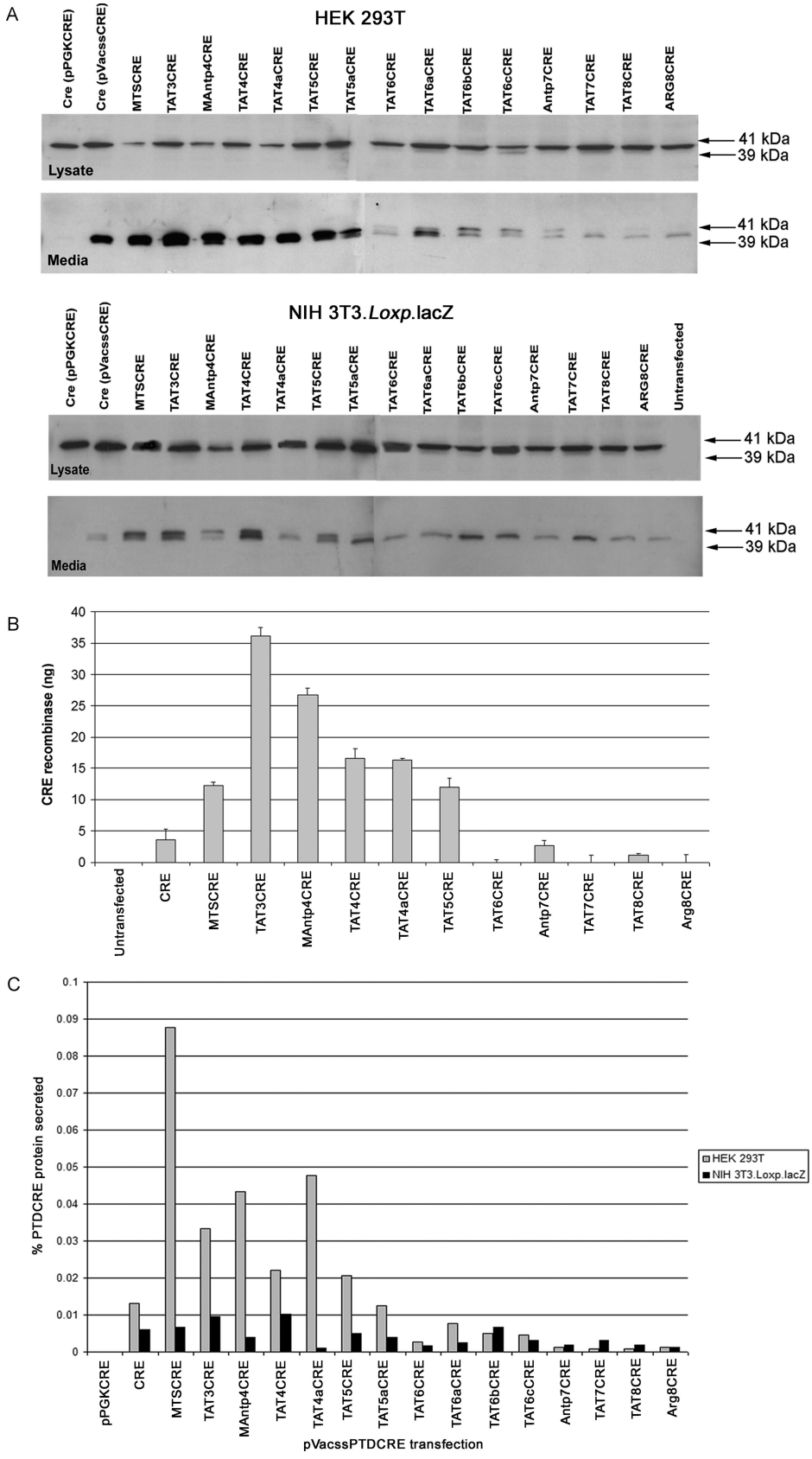

FIGURE 3: PTD-CRE fusion protein expression and secretion from mammalian cells. (A) Expression plasmids were transfected into both HEK 293T (1×10^6) and NIH 3T3.loxP.lacZ (1×10^6) cells, and CRE protein was detected by Western blotting of cell lysates and the media using a rabbit anti-CRE polyclonal antibody. The pPGKCRE plasmid expresses a nonsecreted CRE protein and the pVacssCRE plasmid produces a secreted CRE. (B) Amount of secreted CRE in media samples. Media samples were collected from HEK 293T transfected plates, filter concentrated and the amount of CRE recombinase in each sample determined by ELISA. Transfections were performed in triplicate, and error bars represent standard deviations. (C) Efficiency of PTD-CRE secretion. Efficiency was calculated as % of PTD-CRE in the medium compared to total PTD-CRE (lysate + medium). Band intensities were taken from the Western blots in (A) and calculated using ImageJ software (http://rsb.info.nih.gov/ij/). HEK 293T cells in gray, NIH 3T3.loxP.lacZ cells in black.

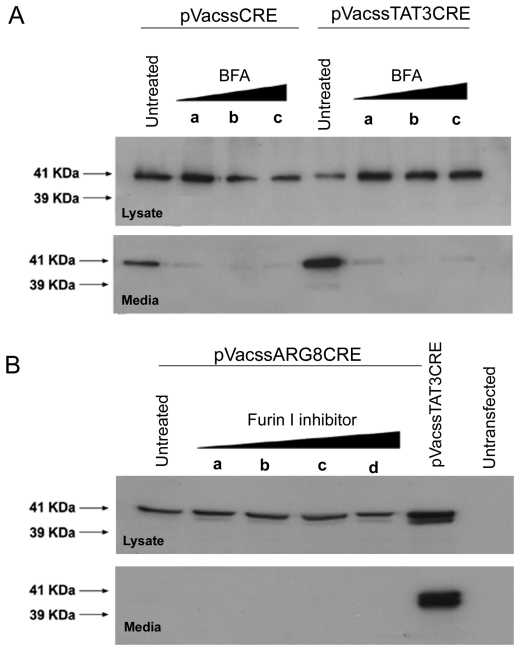

FIGURE 4: Effects of brefeldin A and furin I inhibitor. (A) HEK 293T cells were transfected with expression plasmids and 22 h later pretreated with BFA for 1 h at increasing concentrations of (a) 1 μg/mL, (b) 5 μg/mL and (c) 10 μg/mL. Transfection medium was replaced with serum free medium (including BFA) for a further 1 h prior to analysis via Western blot using rabbit anti-CRE polyclonal antibody. (B) HEK 293T cells were transfected with pARG8CRE expression plasmid and 22 h later pretreated with furin I inhibitor for 2 h at increasing concentrations of (a) 25 mM, (b) 50 mM, (c) 75 mM and (d) 100 mM. Transfection medium was replaced with serum free medium for 4 h prior to Western blot analysis. pTAT3CRE was transfected into HEK 293T cells and left untreated as a positive control for secretion.

were secreted more than TAT6CRE, and TAT6aCRE (Figure 3A).

The amount of PTD-CRE protein secreted was quantitated using an ELISA developed in house as no commercially available CRE ELISA kit exists. This ELISA showed a linear response for purified CRE protein over a range of 0−100 ng (data not shown) and was used to measure the amount of PTD-CRE in the medium for a subset of the expression plasmids (Figure 3B). Reflecting the HEK 293T medium Western blot (Figure 3A) more TAT3CRE was secreted (36 ng) than the other PTD-CRE proteins (Figure 3B). The efficiency of PTD-CRE secretion from both HEK 293T and 3T3PZ cells was assessed by comparing the amount of protein secreted relative to the total amount of protein produced (Figure 3C). MTSCRE demonstrated the highest secretion efficiency followed by TAT4aCRE, MAntp4CRE and TAT3CRE.

Brefeldin A Inhibits Secretion of TAT3CRE Protein. Brefeldin A (BFA) is a fungal metabolite that is known to block protein secretion in eukaryotic cells (*19*). BFA mediates its effects through the disassembly of the Golgi apparatus and redistribution of resident Golgi proteins into the ER (*20*). pTAT3CRE and pCRE were transfected into HEK 293T cells treated with increasing concentrations of BFA. In this study, BFA had no major effect on protein expression as judged by the total amount of CRE protein in the cell lysate (Figure 4A). In contrast, at 1 μg/mL BFA, secretion was reduced with complete inhibition of secretion at 5 μg/mL BFA treatment. Thus, secretion seems to be via the Golgi pathway.

A Furin Endoprotease Inhibitor Does Not Affect the Secretion Profile of ARG8CRE Protein. The Ca^{2+} dependent, membrane bound serine endoprotease furin can cleave a minimal recognition sequence of R*XX*R and is located in the Golgi apparatus (*21*). It was therefore possible that furin could be responsible for inhibition of secretion of arginine rich PTD sequences. To test this we used a furin I inhibitor, which is a peptidyl chloromethylketone reversible inhibitor that blocks furin activity via direct binding to the active site (*22*). HEK 293T cells were transfected with pARG8CRE, which produces a nonsecreted CRE. The transfected cells were subsequently treated with furin I inhibitor over a concentration range of 25−100 mM. pTAT3CRE was transfected into HEK 293T cells as a positive control for secretion (Figure 4A). Furin I had no detrimental effects on intracellular expression of the ARG8CRE protein as judged by the amount of protein in the cell lysate (Figure 4B). At the highest concentration of furin I, ARG8CRE remained unsecreted from the HEK 293T cells suggesting that the furin endoprotease is not responsible for prevention of ARG8CRE secretion.

PTD-CRE Cell Transduction Is Enhanced by Chloroquine. Initial experiments did not detect any protein transduction activity of the secreted PTD-CRE proteins when added to the 3T3PZ reporter cell line. It has been reported that uptake of PTD-fusion proteins occurs via a mode of endocytosis and this can be enhanced by addition of lysomotrophic agents such as chloroquine or the pH-sensitive fusogenic TAT-HA2 peptide (*11*, *13*, *23*−*25*). These compounds were tested to see whether they could enhance PTD-CRE mediated β-galactosidase gene expression in the 3T3PZ reporter cell line (Figure 5A). Pretreatment of the 3T3PZ cells with TAT-HA2 did not seem to enhance PTD-CRE mediated β-galactosidase expression above that found in untreated cells. In contrast, chloroquine enhanced PTD-CRE mediated β-galactosidase expression with the highest signal using TAT3CRE. No chloroquine-mediated cytotoxicity was observed (assessed by a LDH release cytotoxicity assay, data not shown). In the absence of chloroquine, TAT3CRE had a comparable level of transduction to CRE protein without a PTD (Figure 5B). In the presence of chloroquine, the TAT3CRE fusion protein had a 14-fold enhancement compared to CRE (Figure 5B).

TAT3CRE Demonstrates the Highest Cell Transduction Activity. A direct comparison of each of the secreted PTD-CRE proteins highlighted that TAT3CRE had the highest PTD activity. Equal amounts of protein were added to the 3T3PZ reporter cells to monitor transduction efficiency in the presence of chloroquine (Figure 6A). Surprisingly, the remaining secreted PTD-CRE fusion proteins did not demonstrate significant enhancement of transduction above that of secreted CRE alone.

TAT3CRE Demonstrates Dose and Time Dependent Responses. Increasing amounts of TAT3CRE were placed on 3T3PZ reporter cells in the presence of chloroquine (Figure 6B). Increasing the quantity of TAT3CRE placed upon the 3T3PZ reporter cells corresponded with an increased β-galactosidase expression. TAT3CRE (4800 ng) was placed upon cells for different periods of time to assess how increased length of contact might influence uptake and response (Figure 6C). The longer the TAT3CRE protein was

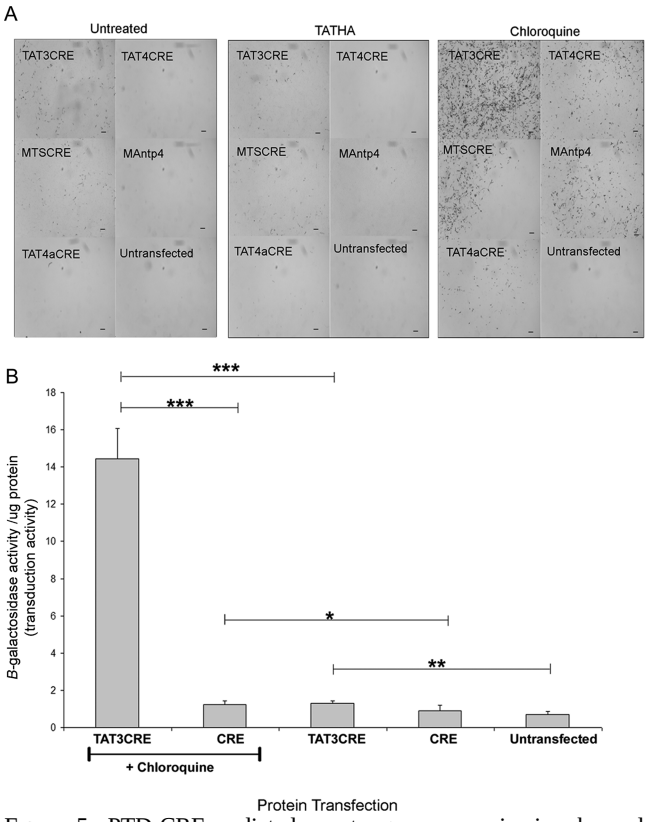

FIGURE 5: PTD-CRE mediated reporter gene expression is enhanced in the presence of chloroquine. (A) HEK 293T cells were transfected with different PTD-CRE expression plasmids, and 24 h later the medium was collected, filtered and placed on pretreated NIH 3T3.loxP.lacZ reporter cells for 4 h. Cells were stained for β-galactosidase activity 24 h later. 3T3PZ reporter cells were either pretreated with TATHA fusogenic peptide (5 μM, overnight) or chloroquine (100 μM, 2 h). Scale bar = 20 μm. (B) Secreted TAT3CRE and CRE were quantitated by ELISA and equal amounts (400 ng) added to the medium surrounding confluent 3T3PZ reporter cells for 4 h. Some reporter cells were pretreated with chloroquine (100 μM) for 2 h. β-Galactosidase activity was measured by luminescent assay and normalized to total protein in each sample. Experiments were performed in triplicate, and error bars represent the standard deviation; (***) <0.0005, (**) <0.005 and (*) <0.05 (Student's two-tailed *t* test).

in contact with the 3T3PZ reporter cells, the greater the response. This CRE mediated recombination response increased over a period of 4 h.

PTD Can Enhance Protein Spread after Plasmid Transfection. To determine whether protein transduction could occur following plasmid transfection into a single cell type, 3T3PZ reporter cells were transfected with either pTAT3CRE or pCRE expression plasmids to assess TAT3CRE spread. Each plasmid was cotransfected with the luciferase reporter plasmid pVR1223 (1:10 w/w) to assess transfection efficiency. β-Galactosidase expression was normalized to transfection efficiency. Therefore increased β-galactosidase expression resulting from transfection of either plasmid would relate to increased spread of CRE protein. pTAT3CRE mediated β-galactosidase expression was significantly higher ($p < 0.01$) than pCRE suggesting that TAT3CRE spread to a larger number of cells after initial plasmid transfection compared to secreted CRE protein (Figure 6D).

DISCUSSION

We have identified a functional PTD sequence that is capable of passage through the secretory pathway of mammalian cells and retains PTD activity. A secretory signal sequence was included to allow initially transfected cells to act as a source of PTD-CRE for transduction into adjacent cells.

PTD sequences have been well documented for their ability to traverse the cell membrane and enter cells from the extracellular environment. However, less attention has been paid to the reverse process, i.e., PTD export from the cell. A number of proteins have been identified that exit the cell via a leaderless secretory pathway, i.e., not requiring a secretory signal sequence and hence bypassing the classical ER secretory pathway. These include the acidic fibroblast growth factor (aFGF) (*26*, *27*), basic fibroblast growth factor and the full-length HIV-1 TAT protein (*28*, *29*). A possible reason for alternative secretory pathways could be to bypass the oxidizing environment of the ER lumen for proteins that rely on cysteine residues for their bioactivity (*28*). However, the mechanism by which this export takes place remains unclear. There is some suggestion that the TAT8 PTD sequence itself may act as an export signal for secretion of fusion proteins via a leaderless pathway. The TAT8 PTD peptide has been shown to aid secretion of the herpes simplex virus thymidine kinase (TK) protein from stable HEK 293 cell lines (*30*). However, it was unclear if the secreted TAT8-TK protein retained biological activity. In addition, the TAT8-TK protein was collected in serum free medium after 3 days of contact with the HEK 293 cell lines. We found nonspecific protein release from transfected cells caused by cell death if the serum free medium was left in contact for greater than 14 h. We collected secreted protein from transiently transfected cells for a period of 1−4 h where no adverse cytotoxic effects were observed. It is not possible to directly compare this study with ours as the amount of TAT8-TK present in the medium was not measured. TAT8 PTD has also been shown to enhance the biodistribution of β-glucuronidase protein following injection into the mouse brain (*31*).

TAT-EGFP protein secreting cell lines have been reported previously (*32*). These authors collected medium over a 14 h period and confirmed secretion by Western blotting. Protein released into the medium over a shorter time point (3 h) was also capable of transducing reporter cells. Interestingly, the amount of protein secreted as estimated from the Western blot was around 14 ng/mL/h, which is similar to the amount of TAT3CRE secreted (10 ng/mL/h) from the HEK 293 cells measured by ELISA.

It is still not clear whether PTD sequences can act as protein export signals in their own right. Identification of this type of activity in our experiments is confounded by the presence of the secretory signal sequence which would tend to mask this property. It is noteworthy, however, that more TAT3CRE protein was apparently secreted than CRE, even though both proteins contain the same signal sequence. TAT sequences might enhance protein export by increasing movement through the ER by more productive interactions with chaperone proteins. Alternatively, the apparent enhancement in TAT3CRE secretion could simply reflect higher protein degradation of CRE in the culture medium.

Brefeldin A (BFA) treatment was used to confirm that the PTD-CRE fusion proteins investigated here were secreted through the Golgi pathway, with secretion of TAT3CRE inhibited in a dose-dependent manner. Brefeldin A has been

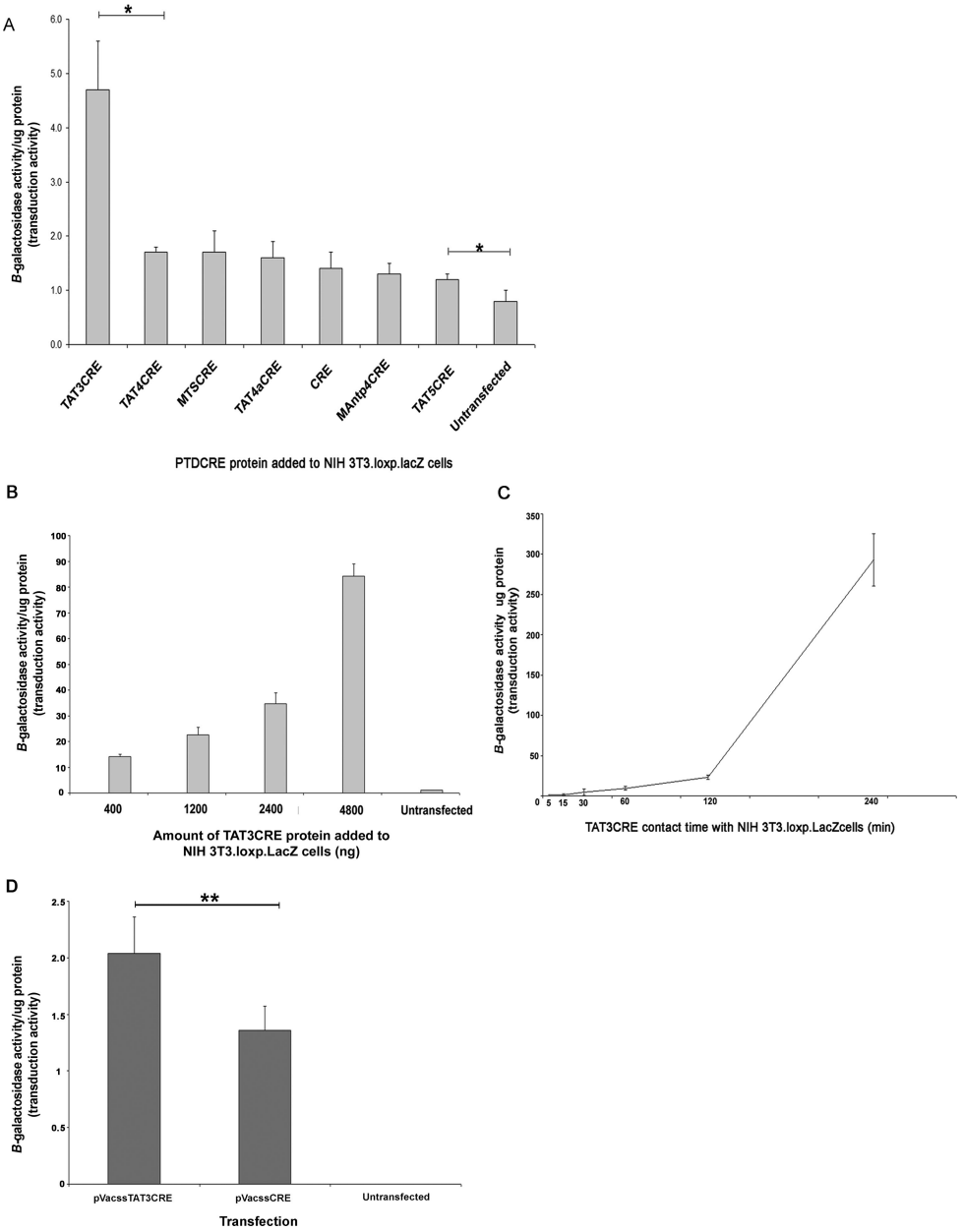

FIGURE 6: PTD-CRE transduction. (A) Comparison of different PTD-CRE protein transduction abilities. Secreted PTD-CRE fusion proteins were quantitated by ELISA and equal amounts (260 ng) added to NIH 3T3.loxP.lacZ reporter cells for 4 h in the presence of chloroquine (100 μM). The transduction activity of each PTD-CRE was measured as β-galactosidase activity normalized to total protein in each sample. Experiments were performed in triplicate, and error bars represent standard deviation. (*) <0.05 (Student's two-tailed *t* test). (B) Evaluation of transduction related to the amount of TAT3CRE protein present. Secreted TAT3CRE was quantitated by ELISA and increasing amounts (400−4800 ng) placed in the medium surrounding confluent NIH 3T3.loxP.lacZ reporter cells for 4 h. The reporter cells were pretreated with chloroquine (100 μM) for 2 h. β-Galactosidase activity was measured by luminescent assay and normalized to total protein in each sample. Experiments were performed in triplicate, and error bars represent the standard deviation. (C) Evaluation of contact time of TAT3CRE with cells. TAT3CRE (4800 ng) was placed on NIH 3T3.loxP.lacZ reporter cells for different lengths of time, and associated β-galactosidase activity was measured by luminescent assay and normalized to total protein in each sample. Experiments were performed in triplicate, and error bars represent the standard deviation. (D) TAT3CRE demonstrates spread after plasmid delivery. NIH 3T3.loxP.lacZ cells were transfected with pTAT3CRE or pCRE with a pVR1223 luciferase expression plasmid spike. 24 h post transfection cells were treated with chloroquine (100 μM, 2 h). Fresh medium was placed on the cells and left for a further 24 h prior to β-galactosidase activity detection. β-Galactosidase activity was measured by luminescent assay and normalized to total protein in each sample. In addition, responses were normalized to initial transfection using the pVR1223. (**) $P < 0.01$ (Student's *t* test).

reported to inhibit protein synthesis, but we found no detrimental affects on protein production at the concentration of BFA used to inhibit protein secretion (*33*). In this context, there was no indication that the TAT3-PTD domain caused secretion through a nonclassical pathway.

PTD-CRE fusion proteins with a PTD sequence charge greater than +5 were not secreted efficiently in this study. We investigated the possibility that some of the PTDs might be acting as internal targeting signals to reroute the protein from the secretory pathway. To explore this possibility we analyzed the sequences using the subcelluar N-terminal sorting signal program iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/). TAT7, TAT4a, MAntp4 and TAT3 PTD sequences all contained potential mitochondrial targeting signals. Of these, only TAT7CRE was not secreted, making it unlikely that a mitochondrial import sequence inhibited

secretion. There are a number of possible reasons why some PTD-CRE fusion proteins were not secreted efficiently from the cell. The cationic charge on PTDs could bind to internal structures such as RNA or become associated with negatively charged phospholipids or proteins. The PTD sequence could interfere with the signal recognition particle, required to guide growing polypeptides and their associated ribosomes to the translocon.

A marked difference between the secretory potential of HEK 293T and 3T3PZ cells was found. Fibroblasts such as NIH 3T3 are noted for secretion of growth factors, and HEK 293 cells are an epithelial cell line and thus might be expected to show good secretory activity. This variation in the efficiency of secretion between different cell types means that predicting how well a fusion protein might behave in a particular tissue should be done with caution.

The mechanism of PTD uptake is still under scrutiny, but it is generally accepted to be a mode of endocytosis rather than direct translocation across the membrane. Consistent with these observations, we showed that chloroquine treatment, which causes buffering of the endosome and release of contents, was essential for efficient CRE transduction. In our experiments, TAT-HA did not act as lysomotrophic agent as previously reported (*13*). It is possible that there was competitive inhibition between the TAT-HA and the PTD groups. The TAT3 peptide, which is based on an optimized TAT sequence (*18*), demonstrated the best PTD activity compared to the other secreted PTD sequences in this study. This peptide sequence has been optimized for α-helical structure and when chemically conjugated to FITC has been shown to enhance *in vivo* delivery into whole blood cells compared to normal TAT (*18*).

CRE recombinase has been reported to have an intrinsic ability to enter cells when applied extracellularly, possibly due to its overall cationic charge. We have confirmed this observation, with CRE being able to enter our reporter cell line and catalyze recombination between LoxP sites. A CRE-MTS fusion protein has been used *in vivo* and showed extensive delivery to a wide range of tissues including crossing the blood−brain barrier (*9*). The authors, however, did not provide a CRE only control which makes it difficult to assess the contribution of the MTS to the overall transduction. In our study, TAT3-CRE delivery is significantly higher than CRE alone.

We have also shown protein spread following plasmid delivery and expression in a single cell type. Using the 3T3PZ reporter line for these experiments meant that the pCRE plasmid gave a background β-galactosidase activity. Nevertheless, the β-galactosidase activity after pTAT3CRE transfection was significantly higher ($P < 0.01$) than with pCRE indicating PTD-mediated protein spread. More dramatic differences between pCRE and pTAT3CRE could have been obtained by using a different cell line for the initial plasmid transfection and then adding the 3T3PZ reporter analogous to the experiments in Figure 5B, but we wished to show protein spread in a single cell population similar to what might be required after delivery *in vivo*.

PTDs offer a potentially powerful approach for drug delivery by allowing transduction of large hydrophobic molecules into cells. However, for enhancing the response to transfection strategies and ultimately gene therapy applications, more research should be focused on the protein spread model proposed here. Ultimately, this will require PTDs that are eligible for secretion from mammalian cells and also retain transduction potential for their cargo. This report presents a PTD sequence that has been evaluated to fulfill these criteria *in vitro*.

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REFERENCES

1. Frankel, A. D., and Pabo, C. O. (1988) Cellular uptake of the tat protein from human immunodeficiency virus, *Cell 55*, 1189−1193.
2. Green, M, L. P. (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein, *Cell 55*, 1179−1188.
3. Vives, E., Brodin, P., and Lebleu, B. A. (1997) Truncated HIV-1 Tat Protein Basic Domain Rapidly Translocates through the Plasma Membrane and Accumulates in the Cell Nucleus, *J. Biol. Chem. 272*, 16010−16017.
4. Derossi, D., Joliot, A., Chassaing, G., and Prochiantz, A. (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes, *J. Biol. Chem. 269*, 10444−10450.
5. Gratton, J.-P., Yu, J., Griffith, J. W., Babbitt, R. W., Scotland, R. S., Hickey, R., Giordano, F. J., and Sessa, W. C. (2003) Cell-permeable peptides improve cellular uptake and therapeutic gene delivery of replication-deficient viruses in cells and *in vivo*, *Nat. Med. 9*, 357−362.
6. Rojas, M., Donahue, J. P., Tan, Z., and Lin, Y.-Z. (1998) Genetic engineering of proteins with cell membrane permeability, *Nat. Biotechnol. 16*, 370−375.
7. Dietz, G. P. H., and Bahr, M. (2004) Delivery of bioactive molecules into the cell: the Trojan horse approach, *Mol. Cell. Neurosci. 27*, 85−131.
8. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) *In vivo* protein transduction: delivery of a biologically active protein into the mouse, *Science 285*, 1569−1572.
9. Jo, D., Nashabi, A., Doxsee, C., Lin, Q., Unutmaz, D., Chen, J., and Ruley, H. E. (2001) Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase, *Nat. Biotechnol. 19*, 929−933.
10. Wadia, J. S., and Dowdy, S. F. (2005) Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer, *Adv. Drug Delivery Rev. 57*, 579−596.
11. Kaplan, I. M., Wadia, J. S., and Dowdy, S. F. (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis, *J. Controlled Release 102*, 247−253.
12. Kaplan, I. M., Wadia, J. S., and Dowdy, S. F. (2005) Corrigendum to "Cationic TAT peptide transduction domain enters cells by macropinocytosis" [(2005) *J. Controlled Release 102*, 247−253], *J. Controlled Release 107*, 571−572.
13. Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, *Nat. Med. 10*, 310−315.
14. Hyndman, L., Lemoine, J. L., Huang, L., Porteous, D. J., Boyd, C., and Nan, X. (2004) HIV-1 Tat protein transduction domain peptide facilitates gene transfer in combination with cationic liposomes, *J. Controlled Release 99*, 435−444.
15. Lallemand, Y., Luria, V., Haffner-Krausz, R., and Lonai, P. (1998) Maternally expressed PGK-Cre transgene as a tool for early and uniform activation of the Cre site-specific recombinase, *Transgenic Res. 7*, 105−112.
16. Ellis, J., Burden, M., Vinogradov, D., Linge, C., and Crowe, J. (1996) Interactions of CD80 and CD86 with CD28 and CTLA4, *J. Immunol. 156*, 2700−2709.
17. Araki, K., Araki, M., Miyazaki, J., and Vassalli, P. (1995) Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase, *Proc. Natl. Acad. Sci. U.S.A. 92*, 160−164.
18. Ho, A., Schwarze, S. R., Mermelstein, S. J., Waksman, G., and Dowdy, S. F. (2001) Synthetic protein pransduction domains: enhanced transduction potential *in vitro* and *in vivo*, *Cancer Res. 61*, 474-477.

19. Misumi, Y., Miki, K., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes, *J. Biol. Chem. 261*, 11398−11403.
20. Klausner, R., Donaldson, J., and Lippincott-Schwartz, J. (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure, *J. Cell Biol. 116*, 1071−1080.
21. Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins, *Biochem. J. 327*, 625−635.
22. Garten, W., Hallenberger, S., Ortmann, D., Schafer, W., Vey, M., Angliker, H., Shaw, E., and Klenk, H. D. (1994) Processing of viral glycoproteins by the subtilisin-like endoprotease furin and its inhibition by specific peptidylchloroalkylketones, *Biochimie 76*, 217−225.
23. Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F., and Giacca, M. (2003) Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins, *J. Biol. Chem. 278*, 34141−34149.
24. Ferrari, A., Pellegrini, V., Arcangeli, C., Fittipaldi, A., Giacca, M., and Beltram, F. (2003) Caveolae-Mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time, *Mol. Ther. 8*, 284−294.
25. Caron, N. J., Quenneville, S. P., and Tremblay, J. P. (2004) Endosome disruption enhances the functional nuclear delivery of Tat-fusion proteins, *Biochem. Biophys. Res. Commun. 319*, 12−20.
26. Jackson, A., Tarantini, F., Gamble, S., Friedman, S., and Maciag, T. (1995) The release of fibroblast growth factor-1 from NIH 3T3 cells in response to temperature involves the function of cysteine residues, *J. Biol. Chem. 270*, 33−36.
27. Jackson, A., Friedman, S., Zhan, X., Engleka, K. A., Forough, R., and Maciag, T. (1992) Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells, *Proc. Natl. Acad. Sci. U.S.A. 89*, 10691−10695.
28. Chang, H. C., Samaniego, F., Nair, B. C., Buonaguro, L., and Ensoli, B. (1997) HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region, *AIDS 11*, 1421−1431.
29. Mignatti, P., Morimoto, T., and Rifkin, D. B. (1992) Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex, *J. Cell Physiol. 151*, 81-93.
30. Tasciotti, E., Zoppe, M., and Giacca, M. (2003) Transcellular transfer of active HSV-1 thymidine kinase mediated by an 11-amino-acid peptide from HIV-1 Tat, *Cancer Gene Ther. 10*, 64−74.
31. Xia, H., Mao, Q., and Davidson, B. L. (2001) The HIV Tat protein transduction domain improves the biodistribution of beta-glucuronidase expressed from recombinant viral vectors, *Nat. Biotechnol. 19*, 640−644.
32. Barka, T., Gresik, E. S., and Henderson, S. C. (2004) Production of cell lines secreting TAT fusion proteins, *J. Histochem. Cytochem. 52*, 469−477.
33. Fishman, P., and Curran, P. (1992) Brefeldin A inhibits protein synthesis in cultured cells, *FEBS Lett. 314*, 371-374.

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