Efficiency of Protein Transduction Is Cell Type-dependent and Is Enhanced by Dextran Sulfate

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Protein transduction domains (PTDs), both naturally occurring and synthetic, have been increasingly utilized to deliver biologically active agents to a variety of cell types in vitro and in vivo. We report that in addition to previously characterized arginine-rich PTDs, including TAT, lysine homopolymers were able to mediate transduction of a wide variety of cell types, as measured by flow cytometric and enzymatic assays. The efficiency of PTD-mediated transduction was influenced by the cell type tested, although polylysine homopolymers demonstrate levels of internalization that consistently exceeded those of TAT and arginine homopolymers. Transduction of arginine/lysine-rich PTDs occurred at 4 °C and following depletion of cellular ATP pools, albeit generally at reduced levels. Although transduction was reduced in Chinese hamster ovary mutant lines deficient in either heparan sulfate or glycosaminoglycan synthesis, uptake was restored to wild-type levels by incubating target cells with dextran sulfate. The enhancement of transduction by dextran sulfate suggests that electrostatic interactions play an important first step in the process by which PTDs and their cargoes traverse the plasma membrane.

Since the simultaneously published reports by Green and Loewenstein (2) and Frankel and Pabo (3) that the human immunodeficiency virus type 1 TAR protein is able to cross the plasma membrane, with subsequent mapping of the activity to the 11-amino acid TAR protein transduction domain (PTD),1 the protein transduction field has rapidly gained prominence as an efficient method to modify cellular function in the absence of ectopic gene expression (1–3). Protein transduction is rapid and titratable, with a broad range of transducible cell types. PTDs have been employed to deliver oligonucleotides, peptides, full-length proteins, 40-nm iron nanoparticles, bacteriophages, and even 200-nm liposomes (4–9). Recently published reports suggest that the guanidinium head group of arginine plays a critical role in enhancing the uptake of arginine homopolymers, with levels of transduction increased severalfold over the TAR PTD (19, 21). These findings have led to increased focus on arginine homopolymers and synthetic peptide analogues of arginine incorporating the guanidinium moiety as transduction domains (19, 21–23, 25).

The precise mechanism of transduction mediated by PTDs has been the subject of some debate. It has been suggested that transduction of arginine homopolymers is dependent on the presence of active phosphate stores (21). However, more recent work has shown that treatment with sodium azide does not block internalization of PTDs (22, 23). The fact that internalization of PTDs occurs at 4 °C supports the notion that cell-mediated processes do not participate in the transduction process (6). The search for specific cellular components required for transduction has identified few essential mediators. Internalization of Antennapedia, TAR, and arginine homopolymers has been shown to be nonsaturable and achiral, and it is not reliant on particular secondary structural elements within the PTD, as evidenced by mutagenesis experiments, use of retro-inverso peptides, β-peptides, peptoids, and substitution with d-enantiomers of PTD residues (19, 21, 26–30). The broad range of transducible cell targets points toward the involvement of ubiquitously shared cellular structures, such as plasma membrane phospholipids (18, 29, 31–37). Data have supported the role of heparan sulfate proteoglycans as a surface binding target for the TAR protein (38–40), although more recent work has suggested that they are not required for internalization of the TAR PTD (23, 41, 42). Furthermore, it is unclear whether the presence of glycosaminoglycans (GAGs) serves as a prerequisite for transduction by PTDs other than TAR (23).

Due to the rising importance of PTDs in delivering bioactive cargoes for the manipulation of cells in vitro and in vivo, we undertook a systematic, quantitative comparison of various cationic PTDs delivering cargoes ranging from 60 kDa to >500 kDa in size with the objective of determining key parameters governing efficiency of protein transduction. Previous studies have focused on measuring the efficiency of transduction of...
PTDs covalently linked to small fluorophore cargoes (19–23). Surprisingly, lysine homopolymers yielded the highest levels of internalization of all PTDs tested, across nearly all tested cell types. Additionally, we investigated the contribution of heparan sulfate proteoglycans and GAGs on uptake using CHO K1 cell line mutants. Preincubation of GAG-deficient cells with dextran sulfate significantly enhanced transduction of short PTDs, providing a method for enhancing in vitro and ex vivo manipulation of cells by protein transduction. This result also suggests that the initial step for internalization is mediated via electrostatic interactions.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Quantitation—PTDs were synthesized by Fmoc (N-9-fluorenylmethoxycarbonyl) solid phase synthesis, N-terminally biotinylated, purified by reversed-phase high performance liquid chromatography to >90% purity on an octonitrile/H_2O-trifluoroacetic acid gradient, and confirmed by electrospray ionization mass spectrometry (Peptide Synthesis Facility, University of Pittsburgh). Lysylphosphopeptides were reconstituted in distilled water to 2 mM stock concentrations. Peptide solutions containing primary amines (PTD-5, TAT, and lysine homopolymers; Table I) were quantitated by ninhydrin chemistries. Peptide solutions containing primary amines (PTDs, providing a method for enhancing in vitro and ex vivo manipulation of cells by protein transduction. This result also suggests that the initial step for internalization is mediated via electrostatic interactions.

Cell Culture—CHO K1 Chinese hamster ovary cells and their HS-deficient (pgs D-677) and GAG-deficient (pgs A-745) derivatives and the HIG-82 rabbit synovial cell line were cultured in Ham’s F-12 medium at 37 °C in 5% CO_2. HeLa cells (human epithelial cervical carcinoma line) were grown in Dulbecco’s modified Eagle’s medium, and the human Jurkat T cell and A549 (human epithelial lung carcinoma) lines were cultured in RPMI 1640. All medium was supplemented with 10% fetal calf serum, 10 mM HEPES, and 2 mM L-glutamine.

Enzymatic Quantitation of Protein Transduction—PTD-β-galactosidase complexes were formed by preincubating streptavidin-β-galactosidase (SA-β-Gal; Rockland Chemical Co.) with a 30-fold excess of biotinylated peptides for 30 min at room temperature. Uncoupled biotinylated peptides were left in solution, since competition effects from excess peptides have been previously observed only at significantly higher concentrations (24). Complexes were added to serum-containing medium (10% fetal calf serum) in each well of 24-well plates with confluent cell monolayers at 20 nm streptavidin concentrations and incubated for 1.5 h at 37 °C. Cells were washed twice in serum-containing media and once in PBS, and soluble extracts were obtained by incubating cells in 0.2% Triton X-100 lysis buffer, followed by pelleting insoluble debris at 14,000 rpm. β-Galactosidase recovered from the cellular extracts was quantitated by 1,2-dioxetane-based light emission (Tropix, Inc.). Fluorescence was measured in a Berthold Autolumat luminometer, and relative light units were normalized to protein content as measured by Bradford protein assay (Bio-Rad) using bovine serum albumin as a standard. Each condition tested was performed in triplicate, with means and S.D. values calculated.

For complementation experiments using anionic polymers (salmon protamine sulfate (salmine, Grade II), porcine intestinal mucosal heparin sodium salt, bovine kidney heparan sulfate sodium salt, and dextran sulfate, M_r 500,000; Sigma), cells were preincubated with various concentrations of polymers in serum-containing media and washed twice in serum-containing media prior to the addition of complexes. Steps following incubation with complexes were performed as described above.

Confocal Microscopy for Internalized Peptide Complexes—Cells analyzed by flow cytometry were fixed in 2% paraformaldehyde and mounted in gelvatol on slides using number 1 coverslips. Internalized PTD-streptavidin-Alexa Fluor 488 was visualized using a ×100 oil-1.3 NA immersion objective on an inverted Leica TCS NT laser-scanning confocal microscope. The argon 488-nm laser line was used to detect Alexa-488, and corresponding differential interference contrast images were taken of each section. Successive 0.4-μm optical sections were taken, and maximum intensity projections were generated using Leica TCS-NT software. Photomultiplier tube and laser power settings were identical for each data set.

RESULTS

Measuring PTD-mediated Transduction—To assess the transductional efficiency mediated by a variety of PTDs, including arginine and lysine homopolymers (see Table I), the internalization of cargoes was examined by two different methods. The first method involved quantitating internalization efficiency of SA-β-Gal complexes (≥500 kDa) coupled to the biotinylated PTDs by measuring enzymatic activity recovered from cellular extracts. Confluent cell monolayers were incubated with the complexes for 1.5 h at 37 °C, as peak transduction was measured to be reached in that time frame (data not shown). As shown in Fig. 1A, cell line-specific differences in
transduction are observed, with the HIG-82 synovial cell line demonstrating a 3.7–7.3-fold greater transduction than the other cell lines. The shorter PTDs (4R, 4K, and 5RQ) fail to mediate efficient transduction in the cell lines tested. Notably, no single PTD appears to be optimal in mediating transduction of the SA-β/galactosidase complexes, although in all cases, polylysine homopolymers, particularly 8K and 10K, yield the highest levels of transduction from the PTD panel across all of the screened cell lines (see Table II). Furthermore, the internalization efficiency of the polylysines is not a simple function of length, since peak transduction occurs between 6 and 10 mers in size. Complexes with the 6R PTD yield the highest levels of transduction of the polyarginine PTDs. Interestingly, the 8RQ and 11RQ peptides, which were screened on the basis of the shared RRQR motif between PTD-5 and TAT, show no enhancement of transduction, except in the case of HIG-82 cells.

To determine whether results from the quantitation of β-galactosidase complexes were extendable to smaller cargo sizes (60 kDa), we examined the ability of biotinylated PTDs to facilitate internalization of SA-488 (60 kDa) in the same cell lines, as measured by flow cytometry (Fig. 1B). Of concern was the fact that despite extensive washing, nonspecific electrostatic interactions may have led to quantitation of both surface-bound and internalized complexes in the SA-β/Gal transduction assays. By trypsinizing the cells for 20 min following incubation with the PTD-SA-488 complexes, noninternalized, surface-bound SA-488 was eliminated, a finding confirmed by confocal microscopy (data not shown). Trypsin was chosen because of its preference for cleavage following lysine and arginine residues, which are present in the PTDs, as well as its limited impairment of cell viability in comparison with other proteases. The addition of 7-AAD prior to flow cytometric analysis enabled elimination of nonviable cells from the quantitation. Results obtained by incubating the cell lines with PTD-SA-488 (Fig. 1B) yield similar patterns of uptake to the PTD-SA-β-Gal data (Fig. 1A), with the lysine homopolymers, particularly 8K and 10K, demonstrating the highest mean levels of uptake. These data are summarized in Tables II and III. An example of fluorescence distributions derived following incubation with the PTD-SA-488 complexes is shown in Fig. 1C for CHO K1 cells. For the PTDs shown, greater than 99.7% of live CHO K1 cells exhibit fluorescence greater than 10^4, which is adjusted for background fluorescence in untreated cells. Distributions of fluorescence are generally narrow (>95% of events within 1 log fluorescence intensity), although 6K reproducibly resulted in a broader distribution in CHO K1 cells. Results from flow cytometry indicate that there are reproducible cell line-specific, as well as PTD-specific, patterns of overall uptake.
levels and distribution of uptake (data not shown). In this assay, CHO K1 cells demonstrate 4.4–9.7-fold greater transduction of the 60-kDa PTD-SA-488 complexes in comparison with the other cell lines. PTD-mediated internalization of Alexa Fluor 488 marker was confirmed by laser-scanning confocal microscopy (Fig. 1D), with observable levels of internalized fluorescence that correlate with the flow cytometry results. A punctate distribution of internalized marker, against a weaker diffuse signal, was present for the PTDs, suggesting that larger aggregates of SA-488 may have been internalized. No signal was detectable in the SA-488 control. Nonspecific, surface-bound peptide complexes were absent from all of the samples as a result of trypsin treatment. Interestingly, unlike in the SA-β-Gal quantitative assays, 8RQ and 11RQ peptides mediate efficient transduction of CHO K1 cells as shown by flow cytometry and confocal microscopy.

Role of Surface GAGs in Mediating Protein Transduction—To assess the role of surface-bound GAGs in protein transduction, two mutants of the CHO K1 parental line were used. The CHO K1 mutant, pgs D-677, fails to express both GlcNAc transferase and GlcA transferase, resulting in defective HS synthesis with a concomitant 3° up-regulation of chondroitin sulfate (44). Another CHO K1 mutant, pgs A-745, is deficient in xylosyltransferase, the key enzyme required for attachment of GAGs to the core protein. This defect leads to nearly complete elimination (1% of wild-type levels) of GAG expression (45, 46). Both SA-β-Gal and SA-488 assays were used to quantitate transduction mediated by the PTDs in the pgs D-677 and pgs A-745 lines (Fig. 2, A and B). Overall, internalization of PTD-SA-β-Gal complexes is reduced in HS- and GAG-deficient cells. Transduction of SA-β-Gal coupled to the 6K PTD, the highest transducer of the CHO K1 parental line, is diminished 90-fold in the pgs D-677 line and 42.8-fold in the pgs A-745 line (Fig. 2A). The most efficient PTD of the arginine homopolymers, 6R, transduces 12.7 and 10.8-fold less efficiently in the pgs D-677 and pgs A-745 line, respectively. The TAT PTD, which has been shown to directly bind heparin sulfate, shows a 1.66-fold reduction in transduction in the HS- pgs D-677 line but a greater reduction of 4.8-fold in the GAG-pgs A-745 line. Interestingly, a trend emerges in which increasing lengths of arginine and lysine homopolymers show increasing levels of internalization of SA-β-Gal in the GAG mutant cells. In fact, 12R, the most efficient PTD for GAG mutant cells, mediates higher levels of transduction in the pgs D-677 and pgs

| PTD | CHO K1 | HIG-82 | HELa | A549 | Mean |
|-----|--------|--------|------|------|------|
| 5R  | 50     | 378    | 54   | 96   | 147  |
| 4R  | 41     | 1      | 1    | 1    | 2    |
| 6R  | 110    | 581    | 62   | 185  | 234  |
| 8R  | 94     | 574    | 51   | 137  | 214  |
| 10R | 59     | 191    | 15   | 50   | 79   |
| 12R | 34     | 85     | 20   | 45   | 46   |
| 4K  | 0      | 0      | 0    | 1    | 0    |
| 6K  | 267    | 379    | 70   | 98   | 202  |
| 8K  | 173    | 560    | 179  | 209  | 280  |
| 10K | 111    | 753    | 142  | 286  | 323  |
| 12K | 68     | 518    | 58   | 146  | 198  |
| 5RQ | 0      | 0      | 0    | 1    | 1    |
| 8RQ | 0      | 0      | 0    | 1    | 1    |
| 11RQ| 1      | 243    | 0    | 61   |      |

Table II

Summary of transductional efficiency of PTD-SA-β-Gal complexes

- Fold increase in transduction is shown, compared with control, of PTD-SA-β-Gal complexes in CHO K1, HIG-82, HELa, and A549 cell lines. Highest level of transduction for each cell line is shown in boldface type. Mean increase in transduction across all four cell lines is shown in the right-hand column, with the two highest transducing PTDs, 8K and 10K, depicted with gray shading.

| PTD | CHO K1 | HIG-82 | HELa | A549 | Jurkat | Mean |
|-----|--------|--------|------|------|--------|------|
| 5R  | 71     | 21     | 6    | 13   | 4      | 23   |
| 4R  | 81     | 38     | 19   | 24   | 3      | 64   |
| 6R  | 40     | 19     | 9    | 12   | 2      | 62   |
| 8R  | 6R     | 415    | 33   | 44   | 92    | 52   |
| 10R | 10R    | 6R     | 225  | 55   | 33    | 57   |
| 12R | 12R    | 17B    | 46   | 34   | 51    | 17   |
| 4K  | 4K     | 25     | 9    | 3    | 4     | 2     |
| 6K  | 6K     | 232    | 57   | 22   | 47    | 41   |
| 8K  | 8K     | 759    | 133  | 75   | 177   | 50   |
| 10K | 10K    | 518    | 105  | 81   | 103   | 64   |
| 12K | 12K    | 430    | 90   | 46   | 99    | 52   |
| 5RQ | 5RQ    | 35     | 10   | 4    | 7     | 3     |
| 8RQ | 8RQ    | 388    | 60   | 29   | 75    | 20   |
| 11RQ| 11RQ   | 297    | 70   | 22   | 46    | 14   |

Table III

Summary of transductional efficiency of PTD-SA-488 complexes

- Fold increase in transduction is shown, compared with control, of PTD-SA-488 complexes in CHO K1, HIG-82, HELa, A549, and Jurkat cell lines, as measured by flow cytometry. Highest level of transduction for each cell line is shown in boldface type. Mean increase in transduction across all four cell lines is shown in the right-hand column, with the two highest transducing PTDs, 8K and 10K, depicted with gray shading.

Influence of Anionic Polymer Preincubation on PTD-mediated Internalization—The effect of preincubation of GAG-deficient cells with various anionic polymers was measured by β-galactosidase quantitation. Dextran sulfate (DS), a polysulfonated polymer of α-1,6-linked glucose units, protamine sulfate, a sulfated 33-mer peptide, and heparin and HS polysaccharides (HS is less highly sulfated than heparin) were reconstituted in serum-containing Ham’s F-12 medium and incubated with the CHO K1 and GAG mutant derivative lines under varying conditions, prior to the addition of 6K-SA-β-Gal complexes. The 6K PTD was chosen, since it was the most efficient PTD for delivering SA-488 to both GAG mutants. Surprisingly, TAT shows almost no reduction in SA-488 delivery to pgs D-677 and pgs A-745 lines (48-fold increase for both lines) than in the CHO K1 parental line (35-fold increase over control).

Similar results arise from comparison of internalization levels of SA-488 in the GAG mutants, as measured by flow cytometry (Fig. 2B). The most efficient transducer of CHO K1 wild type cells, 8K, shows a 2.8- and 10.3-fold reduction in internalization in the pgs A-677 and pgs D-745 lines, respectively. Likewise, 6R, the highest transducer of the polyarginines in CHO K1 cells, shows a 14-fold reduction in delivering SA-488 to both GAG mutants. Surprisingly, TAT shows almost no reduction in SA-488 delivery to pgs D-677 cells and only a 21% reduction in transduction of pgs A-745 cells. As in the SA-β-Gal assay (Fig. 2A), PTD-mediated transduction of SA-488 complexes in GAG-mutant cell lines demonstrates increased internalization as the PTD length increases. The most efficient PTD complex in these mutant lines, 12K-SA-488, is able to mediate a 172-fold increase in uptake, even in the absence of GAGs in the pgs A-745s. Unlike the 12R-SA-β-Gal complexes, the 12R-SA-488 complexes are unable to transduce the GAG mutants at levels equivalent to those observed in the CHO K1 cells. Additionally, in this assay, total loss of GAGs more strongly impairs transduction than loss of only HS, particularly in the lysine homopolymers. Distributions of Alexa Fluor 488 fluorescence from representative PTD-SA-488 complexes are shown in Fig. 2, C and D, for the pgs D-677 and pgs A-745 lines, respectively. Analysis by laser-scanning confocal microscopy shows definitive evidence of PTD-mediated internalization of SA-488 in the absence of HS and GAG synthesis (Fig. 2E).
to washing and the subsequent addition of 6K-SA-β-Gal complexes. As shown in Fig. 3A, of the polymers, only DS significantly increases transduction in the GAG deficient line, with transduction peaking (up to a 17-fold increase) between 32 and 320 μg/ml dextran sulfate. Preincubation with heparin, HS, and protamine sulfate increases uptake of the complexes up to 2.7-fold at 1 mg/ml, 1.6-fold at 100 μg/ml, and 1.4-fold at 1 mg/ml, respectively. No noticeable impairment of cell viability on the monolayers was observed at the concentrations tested. The direct addition of 6K-SA-β-Gal complexes following a 30-min preincubation of pgs A-745 or pgs D-677 cells at 37 °C with DS, without washing, still results in an enhancement of transduction. However, enhancement occurs at lower concentrations of DS, and overall increases are lower than when washing is used prior to the addition of SA-β-Gal complexes. For pgs A-745 cells, an 8.8-fold increase in transduction is observed at 10 μg/ml DS, and a 6.6-fold increase in transduction is observed at 100 μg/ml DS when complexes are directly added to media containing DS (data not shown).

A time course was carried out in order to determine the optimal length of preincubation of cells with DS prior to washing and the addition of 6K-SA-β-Gal complexes (Fig. 3B). Enhancement of uptake plateaus at ~180 min of preincubation of pgs A-745 cells with media containing 32 μg/ml DS. The effect
of DS preincubation on uptake of 6K-SA-β-Gal in HS-deficient cells and the CHO K1 parental line was also tested (Fig. 3C). pgs D-677 cells, which lack HS but have a compensatory 3-fold up-regulation of chondroitin sulfate expression, show a benefit from preincubation with DS, although the levels of increase are lower (maximal enhancement of 8.4-fold at 100 μg/ml DS) than observed for pgs A-745 cells. In CHO K1 cells, which have normal GAG synthesis, moderate impairment of transduction was observed by use of DS (40% reduction at 1 mg/ml DS).

The effects of DS preincubation were also examined across
the entire PTD panel in the context of the CHO K1 wild type and GAG-defective genetic backgrounds. PTD-mediated transduction of SA-488 complexes was measured by flow cytometry, and internalization was confirmed by confocal microscopy (Fig. 4). Cells were preincubated for 2 h at 37 °C in 100 μg/ml DS and washed twice prior to incubation with the PTD-SA-488 complexes. Internalization of Alexa Fluor 488 marker was quantitated by flow cytometry, gating on the 7-AAD-negative cell population. Mean -fold increases in transduction over SA-488 controls are shown. 

Preincubation of cells with dextran sulfate enhances transduction mediated by short PTDs. A, effect of dextran sulfate preincubation on transduction of CHO K1, pgs D-677, and pgs A-745 cell lines. Cells were preincubated with Ham’s F-12 with 10% fetal calf serum and 100 μg/ml DS for 2 h at 37 °C and washed twice prior to incubation with the PTD-SA-488 complexes. Internalization of Alexa Fluor 488 marker was quantitated by flow cytometry, gating on the 7-AAD-negative cell population. Mean -fold increases in transduction over SA-488 controls are shown. B, -fold enhancement of transduction by dextran sulfate preincubation in CHO K1, pgs D-677, and pgs A-745 cell lines. -Fold increase in PTD-SA-488 transduction is shown, as measured by flow cytometry, of cells preincubated with serum-containing media plus 100 μg/ml DS for 2 h at 37 °C compared with cells incubated under the same conditions, without added DS. C, confocal microscopy of 4R-SA-488 and 6K-SA-488 transduction, with or without DS preincubation, in pgs D-677 and pgs A-745 cells. Cells were preincubated with Ham’s F-12 plus 10% fetal calf serum with or without 100 μg/ml DS for 2 h at 37 °C prior to washing and the addition of PTD-SA-488 complexes. Internalized Alexa Fluor 488 marker (green) is shown composited against a corresponding DIC image (red).

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medium for both the pgs D-677 and pgs A-745 lines in Fig. 4C. Measuring the Influence of Low Temperature and ATP Depletion on Transduction—The effect of both low temperature (4°C incubation) and ATP depletion on internalization of PTD-SA-488 complexes was investigated in the GAG-deficient CHO line, pgs A-745. Parallel incubations of single cell suspensions with PTD-SA-488 complexes were carried out at both 37 and 4°C, and uptake was measured by flow cytometry. As shown in Fig. 5A, transduction is not completely blocked in any of the tested PTDs by shifting to lower temperatures, although levels of internalization are lower overall. Mean levels of transduction at 4°C compared with 37°C are reduced anywhere from 12% for 4K to 79% for 11RQ. To determine whether transduction is mediated by ATP-dependent processes, cells were pre-incubated in ATP-depletion medium (43) for 1 h at 37°C and maintained in the presence of 6 mM 2-deoxyglucose and 10 mM sodium azide throughout incubation with the PTD-SA-488 complexes. Although impairment of transduction is more acute than observed when shifting to 4°C, depletion of intracellular pools of ATP fails to completely abolish transduction. Transduction is reduced by up to 90%, as seen for the 11RQ-SA-488 complex, in ATP-depleted cells. Curiously, 10R complexes show a 20% increase in transduction following depletion of ATP compared with cells incubated in normal media. Internalization of PTD-SA-488 complexes under either 4°C or ATP depletion conditions in pgs A-745 cells was corroborated by confocal microscopy analysis (data not shown). Similar results were obtained in CHO wild type and human Jurkat T cell lines; specifically, neither 4°C incubation nor ATP depletion was able to completely block PTD-mediated internalization (data not shown).

The ability of dextran sulfate preincubation to enhance PTD-mediated internalization of SA-488 complexes in pgs A-745 incubated at 4°C was also examined (Fig. 5B). Again, internalization levels were generally reduced by the shift to 4°C. Nevertheless, a substantial enhancement in transduction by DS preincubation was still observed. At 4°C, 6K-SA-488 complexes show a 364-fold increase in transduction over the control, and 8K-SA-488 complexes mediate an even higher 438-fold increase. The reductions in transductional efficiencies by incubating at 4°C versus 37°C for the two PTDs are 35.7 and 20.1%, respectively. The most efficient transducer of the arginine homopolymer PTDs at both 37°C (381-fold increase in uptake) and 4°C (169-fold increase in uptake) is 4R, which experiences a net 56% reduction in transduction by shifting to the lower temperature. Confocal microscopy analysis, confirming DS-mediated enhancement of transduction at 4°C, is shown in Fig. 5C for the 4R and 6K complexes.

DISCUSSION

Previous comparative studies of PTDs have measured transductional efficiency by monitoring net peptide uptake in cells incubated with fluoresceinated PTDs but have not followed this in the context of delivery of large molecules or molecular complexes (19–21). Furthermore, detailed studies pertaining to the kinetics and optimization of uptake have been generally limited to observations made in a single cell line, such as Jurkat T cells or HeLa cells (19–23). To address these issues, we employed a screening system to quantitatively assess efficiency of delivery of large cargos (60 kDa for streptavidin-fluorophore to greater than 500 kDa for streptavidin-β-galactosidase) to a variety of cell types. As shown in Fig. 1, A and B, marked differences in transducibility in delivering PTD-SA-β-Gal and PTD-SA-488 complexes are observed from one cell line to another. The removal of nonspecific cell surface binding by trypsinization in the flow cytometric analysis ensured that only internalized Alexa Fluor 488 marker was quantitated. In these experiments, only cell line-specific patterns of transduction were observed by using flow cytometry, but also differential patterns of distribution of uptake were noted from one PTD to another. We and others have previously shown that the degree of intracellular delivery is a function of the extracellular concentration of the PTDs (21, 23, 24, 42). Some of the PTDs studied here are able to mediate homogenous uptake in a very narrow range (>95% within 1 log fluorescence), which suggests that in particular cell lines, they may be more useful for titrating delivery of bioactive cargoes to a desired concentration.

Current attention has been focused on arginine-rich peptides, due to data suggesting that they mediate transduction far more efficiently than other cationic homopolymers, such as histidine, ornithine, and lysine (21). The efficacy of transduction mediated by arginine homopolymers and their peptoid analogues has been attributed to the presence of the guanidine head group. This moiety has been postulated to form a bidentate hydrogen bond with phosphate or sulfate groups on the cell surface, conferring a unique and critical feature required for effective transduction.

Large poly(L-lysine) molecules were shown over 3 decades ago to mediate internalization of coupled methotrexate, serum albumin, and horseradish peroxidase (47–50). Enhancement by covalent linkage of polylysine molecules to various cargoes yielded increases in cellular uptake by as much as 1000-fold, and no difference was observed in uptake using poly(Lys) ranging from 3.1 to 130 kDa (49, 51, 52). However, the mechanism of entry was shown to occur by adsorptive endocytosis with proteins accumulating in coated pits, pinocytotic vesicles, lysosomes, and vacuoles (49–51, 53). In addition, Blanke et al. (54) were able to show that 6-mer lysine homopolymers fused to diphtheria toxin were 100-fold more active than 6-mers of arginine or histidine recombinant fusions. Nevertheless, the presence of another factor, protective antigen, was absolutely required for diphtheria toxin activity in their study. Thus, it is unclear whether the internalization was occurring through a protein transduction mechanism or by receptor-mediated endocytic delivery via electrostatic binding to protective antigen.

The patterns established in both the SA-488 and SA-β-Gal assays presented here unambiguously establish the ability of short lysine homopolymers to deliver large cargoes in a manner consistent with protein transduction (Figs. 1, 2, and 5). Unexpectedly, the efficiency of delivery by polylysine PTDs consistently exceeds that of previously identified TAT and l-polyarginine PTDs (Tables II and III). Although we cannot rule out the ability of lysine homopolymers longer than those we have tested (>12 mers) to mediate internalization via protein transduction, in addition to the previously described adsorptive endocytic pathway, we have observed that when lysine length progresses beyond 10 mers, a drop-off in transductional efficiency occurs (Fig. 1). Since lysine residues lack the specific guanidium moiety present in polyarginine, the absolute requirement for a bidentate hydrogen bond interaction for protein transduction can be ruled out. More likely is the explanation that at physiological pH values, both lysine (pK_a < 10.5) and arginine (pK_a > 12) are fully protonated, enabling them to interact with charged moieties present on the cell surface. Unlike the acute cellular toxicity elicited by long polylysine molecules, the short lysine homopolymers (<12 mers) have no demonstrable cytotoxic effects, even at the highest concentrations we have tested (100 μg; data not shown) (55).

Apparent from the transduction data, summarized in Tables II and III, is that there is no single optimal PTD for delivery across all cell lines. Nevertheless, polylysine PTDs (particularly 8–10 mers in size) appear to mediate the highest levels of internalization in all of the cell lines tested here, with the
exception of the GAG-deficient CHO K1 derivative lines. As a comparison of results from the SA-β-Gal and SA-488 assays suggests (Fig. 1, Tables II and III), the efficiency of transduction may be dependent on the cargo to be delivered. This observation is consistent with the findings of others that the protein transduction efficiency of the TAT PTD varies, depending on the cargo carried (1, 10, 56). Such differences may be explained by the accessibility of the PTD as well as the overall steric, conformational, charge, and hydrophobicity/hydrophilicity characteristics of the cargo itself. Cell type-specific interac-

**Fig. 5.** Transduction of lysine and arginine-rich PTDs occurs at 4 °C and following depletion of cellular ATP pools. A, effect of incubation at 4 °C and incubation with ATP-depleting medium on transduction of PTD-SA-488 complexes in pgs A-745 cells. Conditions were performed as described under “Experimental Procedures.” Internalization was quantitated by flow cytometry, and the mean fluorescence intensity was calculated. Fold increase in transduction over an SA-488 control is shown. B, transduction of PTD-SA-488 complexes in pgs A-745 cell lines preincubated with DS at 37 and 4 °C. Cells were preincubated with 100 μg/ml DS for 2 h at 37 °C, washed twice, and equilibrated to the appropriate temperatures prior to the addition of PTD-SA-488 complexes. C, confocal microscopy of transduction at 37 and 4 °C mediated by 4R-SA-488 and 6K-SA-488 complexes in pgs A-745 cells preincubated with DS. Cells were treated as in B, fixed, and analyzed by confocal microscopy.
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Proteins also contribute to the internalization. Whereas PTD-SA-β-Gal more avidly transduces the HIG-82 cell line, the smaller PTD-SA-488 complexes transduce the CHO K1 cell lines more efficiently in comparison with the other cell lines tested. These phenomena may underlie why polyarginine was previously reported to be the optimal transduction domain in Jurkat cells, since polyarginine may well be optimal for delivering small molecule cargoes, such as the fluorescein isothiocyanate labels (21). Flow cytometry studies using PTD-SA-488 complexes show that 10K is the most efficient PTD of the peptide panel when tested in Jurkat cells (64-fold increase), not the polystyrenes (Table III). It is also important to note that the experiments here describe efficient PTD-mediated delivery of large cargoes in their native conformations. Denaturation has been previously shown to increase transductional efficiency of TAT chimeric in-line fusions (10, 57–59).

To more closely examine the role that GAGs play in the transduction of arginine- and lysine-rich PTDs, we have examined patterns of transduction in the HS-deficient pgs D-677 and GAG-deficient pgs A-745 CHO cell lines (Fig. 2). Both mutants demonstrate a clear reduction in internalization efficiency, compared with the wild type parental line. Nevertheless, the relatively high levels of internalization mediated by 12K-SA-488 (172-fold increase in uptake; 2.5-fold reduction compared with the CHO K1 line), even in the pgs A-745 line, which expresses 1% of wild type GAGs, indicate that their presence is not absolutely required. Furthermore, the transduction of longer PTDs (10- and 12-mers of lysine and arginine) is less strongly influenced by the loss of HS or GAG expression.

The reductions observed in transduction in the GAG-deficient cell lines suggested that electrostatic interactions on the cell surface, separate from PTD-lipid interactions, contribute to protein transduction (60). To address this question, we investigated the role of anionic polymers in PTD-mediated delivery of SA-β-Gal and SA-488 cargoes in GAG-deficient cells. Preincubation with dextran sulfate dramatically enhances uptake of 6K-SA-β-Gal (Fig. 3A). All other polymers tested mediate weak enhancement, including protamine sulfate, which has been reported to increase Antennapedia-mediated transduction 8-fold (37). Preincubation with DS is even able to restore levels of transduction at the highest levels of transduction observed in the wild type context (Fig. 4A). Notably, short PTDs show dramatic increases in transductional efficiency following treatment with DS, with nearly 2 log increases in enhancement for 4R, 4K, and 5RQ PTDs (Fig. 4B).

All of the tested PTD classes, including lysine homopolymers, are able to mediate transduction at 4 °C and following depletion of ATP (Fig. 5). At 37 °C, the uptake of PTDs may reflect a combination of delivery to both endocytic compartments and direct cytosolic “protein transduction” type internalization. Since the internalization of TAT, 6R, and 6K SA-β-Gal complexes was unaffected by chloroquine or monensin treatment (data not shown). The great reduction in molecular motion at 4 °C, rigidifying the plasma membrane in the process, may solely explain the reduced internalization levels. In addition, the fact that internalization still occurs to varying degrees following depletion of cellular ATP pools implies that either transduction mediated by all tested PTDs operates by pathways that can occur independently of active ATP-dependent processes or transduction requires only small amounts of ATP (Fig. 5A).

Furthermore, these experiments do not rule out participation by other high energy stores (i.e. GTP) in the internalization process. Enhancement of PTD uptake by DS preincubation also occurs at 4 °C (Fig. 5, B and C), indicating that the internalization observed occurs by a true protein transduction pathway and not simply through adsorption of dextran sulfate with electrostatically bound PTD-SA-488 complexes (61, 62).

These data collectively imply that entry of PTDs into cells generally occurs by two steps. The first is the electrostatic interaction of PTDs with anionic elements, such as GAGs, on the cell surface. These contacts draw the PTDs in close to the plasma membrane, where, by an unknown mechanism, a rapid nonendocytic process occurs, which delivers the PTDs and their cargoes into the cell. This second step may be mediated by interactions of charged heads of phospholipids with the cationic residues of the PTDs, as suggested by studies from the Antennapedia PTD (18, 29, 31–37). These data are supported by the fact that longer PTDs (10- and 12-mers) are better able to mediate transduction in GAG-deficient lines than short PTDs (Fig. 2), and short PTDs (4- and 6-mers) still possess an intrinsic capacity for protein transduction, provided they can bind to the cell surface via interactions with charged dextran polymers. The use of DS preincubation presents a technique to potently enhance PTD delivery of biologically relevant cargoes to poorly transducible, GAG-low cell lines and primary cells for their manipulation in vitro and ex vivo. Since primary tissues, such as hematopoietic stem cells and differentiated muscle tissue, are known to have down-regulated GAG surface expression, DS might be used to enhance ex vivo protein transduction of these cell types (63, 64). Furthermore, the widespread presence of short, arginine/lysine-rich stretches of residues within proteins opens up the possibility that, with or without DS treatment, such proteins may be capable of receptorless entry into cells without the need for modification of their primary sequences.

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