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Revised Role of Glycosaminoglycans in TAT Protein Transduction Domain-mediated Cellular Transduction[S]

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Cellular uptake of the human immunodeficiency virus TAT protein transduction domain (PTD), or cell-penetrating peptide, has previously been surmised to occur in a manner dependent on the presence of heparan sulfate proteoglycans that are expressed ubiquitously on the cell surface. These acidic polysaccharides form a large pool of negative charge on the cell surface that TAT PTD binds avidly. Additionally, sulfated glycans have been proposed to aid in the interaction of TAT PTD and other arginine-rich PTDs with the cell membrane, perhaps aiding their translocation across the membrane. Surprisingly, however, TAT PTD-mediated induction of macropinocytosis and cellular transduction occurs in the absence of heparan sulfate and sialic acid. Using labeled TAT PTD peptides and fusion proteins, in addition to TAT PTD-Cre recombination-based phenotypic assays, we show that transduction occurs efficiently in mutant Chinese hamster ovary cell lines deficient in glycosaminoglycans and sialic acids. Similar results were obtained in cells where glycans were enzymatically removed. In contrast, enzymatic removal of proteins from the cell surface completely ablated TAT PTD-mediated transduction. Our findings support the hypothesis that acidic glycans form a pool of charge that TAT PTD binds on the cell surface, but this binding is independent of the PTD-mediated transduction mechanism and the induction of macropinocytotic uptake by TAT PTD.

Cationic peptide-mediated cellular transduction represents a cell entry modality with enormous potential for the delivery of macromolecular therapeutic agents. The human immunodeficiency virus TAT protein basic domain (RKKRRQRRR) and other protein/peptide transduction domains (PTDs)[2] have been used to deliver a wide variety of bioactive cargo into cells in culture and preclinical models *in vivo* (1–3). Efficient delivery of peptide, nucleic acid, and full-length protein cargoes by cationic PTDs has been demonstrated by many groups, and several clinical trials are currently under way using PTD-mediated delivery. Despite a number of studies on the mechanism used by PTDs to enter cells, many questions about their uptake remain unanswered (4–7). We and others (8–10) have previously shown that TAT PTD and other cationic PTDs induce a ubiquitous form of fluid-phase endocytosis termed macropinocytosis and enter cells in this manner. However, many mechanistic aspects of TAT PTD transduction remain poorly defined. For instance, we have yet to identify the molecules that TAT PTD binds on the cell surface to induce macropinocytosis, and we do not know how escape across the endosomal lipid bilayer into the cytoplasm is accomplished.

Using fluorescently labeled peptides, it has previously been reported that TAT PTD avidly binds to sulfated glycans and that the presence of acidic polysaccharides like heparin and chondroitin sulfate in solution can compete with TAT PTD for binding to the cell surface and inhibit transduction (8, 11, 12). Based on these observations, several studies argue for a significant role for glycans in the transduction of cationic PTDs, with some researchers concluding that transduction is dependent on heparan sulfate (HS) proteoglycans (13–20). However, these studies relies predominantly on both flow cytometry and microscopic visual observations of fluorescently labeled PTD peptides and are therefore did not necessarily assay for the amount of PTD cargo that has escaped from endocytic vesicles into the cytoplasm. We refer to these observations as "cell association" because they do not sufficiently distinguish between cells that have taken up fluorescently labeled PTDs in endocytic vesicles (or bound to the cell surface) and PTD peptide and cargo that have actually escaped endosomes into the cytoplasm. This distinction is critical because the inside of the endosome is essentially the outside of the cell; no biological response will be elicited simply by uptake into vesicles. Indeed, endosomal escape is requisite for transduction of cargo into the cytoplasm to induce phenotypic responses, and the majority of labeled TAT PTD taken up by cells is present in endosomes, with only a small fraction actually escaping into the cytoplasm.

Here, we sought to explore the role of glycans in TAT PTD-mediated transduction using Chinese hamster ovary (CHO) glycan-deficient cell lines that are genetically deficient for cell-surface expression of heparan and chondroitin sulfate glycosaminoglycans or sialic acids (SAs) (21–24). Although previous studies using fluorescently labeled PTD peptides indicated a requisite role for HS in transduction (13, 16, 17), surprisingly, we found, via a phenotypic assay, that cells lacking HS are in fact fully functional for TAT PTD-mediated transduction. Moreover, transduction occurs efficiently in cells completely lacking

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[2] The abbreviations used are: PTD, protein transduction domain; HS, heparan sulfate; CHO, Chinese hamster ovary; SA, sialic acid; EGFP, enhanced green fluorescent protein; FITC, fluorescein isothiocyanate; TMR, tetramethylrhodamine; LSL, LoxP-STOP-LoxP; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting.

all glycosaminoglycans, including HS, in cells lacking SAs, and in cells depleted of both together. Our findings support the hypothesis that acidic glycans form a pool of negative charge that TAT PTD binds on the cell surface but are dispensable for cationic PTD-mediated transduction into cells.

EXPERIMENTAL PROCEDURES

Generation of Stable Cell Lines—Parental CHO-K1 cells and glycosaminoglycan mutant pgsA (745) cells lacking xylosyltransferase necessary for glycosaminoglycan chain initiation were a gift from Dr. Jeffrey Esko (University of California, San Diego). Lec2 cells, which lack activity of the SA Golgi transporter SLC35A1 and are extremely deficient in expression of cell-surface SA, were a gift from Dr. Ajit Varki (University of California, San Diego). Cells were maintained in Ham's F-12 medium (Invitrogen) containing 10% fetal bovine serum (Sigma). Stable clones were made using a construct (pZ/EG) that contains a Lox-STOP-Lox-EGFP motif, resulting in strong EGFP expression only after Cre-mediated recombination. Cells were transfected with pZ/EG and selected with G418. Multiple subclones were analyzed by flow cytometry for Cre-inducible EGFP expression following transfection with a Cre expression construct and subsequently by treatment with TAT PTD-Cre. The clones chosen for use in this study had low background EGFP expression and showed comparable induction of EGFP expression after expression of Cre.

Peptide Synthesis and Purification of Recombinant Proteins—Peptides were synthesized by SPPS on a Symphony Quartet peptide synthesizer (Rainin) using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-protected L-amino acids on an amidated support (EMD). Fluorescein isothiocyanate (FITC) or tetramethylrhodamine (TMR) labeling was performed by reaction of the peptide with carboxyfluorescein (Sigma) while still on solid-phase support. Crude peptides were purified by reverse-phase high pressure liquid chromatography and confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy.

Recombinant TAT-Cre and Cre proteins were expressed in BL21-CodonPlus(DE3)-RIPL cells (Stratagene) using pET28.2 protein expression constructs (Novagen). Both the TAT PTD-Cre and Cre constructs contain a His_6 motif at the C terminus, with TAT at the N terminus in the TAT-Cre expression construct. Cells were transformed and grown overnight at 37 °C under kanamycin and chloramphenicol selection. Expression was induced with 500 μM isopropyl β-D-thiogalactopyranoside for 4 h at 37 °C. Bacteria were lysed by sonication in the presence of protease inhibitors, lysozyme, RNase A, and DNase I. Recombinant Cre was purified from cleared lysate first by nickel-nitrilotriacetic acid (Qiagen) chromatography followed by ion exchange chromatography using a Mono S column on an AKTA FPLC system (GE Healthcare). Purity and concentration of proteins were confirmed by SDS-PAGE, and Cre activity was confirmed by in-cell transduction and *in vitro* recombination assays. Recombinant Cre and TAT PTD-Cre were labeled with Alexa Fluor 546 by reaction with the succinimidyl ester (Invitrogen) for 1 h under slightly basic conditions according to the company's protocol. Labeled protein was concentrated and repurified by ion exchange fast protein liquid chromatography and confirmed by gel electrophoresis.

TAT-Cre Transduction and Recombination Assays—Stable CHO-K1, pgsA, and Lec2 LoxP-STOP-LoxP (LSL)-GFP clones were seeded overnight onto 24-well plates at a density of 20,000 cells/well. Cells were washed and incubated in serum-free medium for 1 h at 37 °C. When pharmacological inhibitors (Sigma) were used, these were added with the serum-free medium and added again with recombinant protein. Cells were then treated with Cre/TAT PTD-Cre for 1 h in serum-free medium at 37 or 4 °C as indicated. Cells were washed with phosphate-buffered saline (PBS), trypsinized, and replated. At 24 h after Cre addition, cells were trypsinized and assayed for GFP expression on an LSRII flow cytometer equipped with FACSDiva acquisition and analysis software (BD Biosciences). The live cell population was gated by forward scatter/side scatter, and 10,000 live cells were used for each analysis. Histograms were generated using FlowJo software (Tree Star).

For trypsin depletion of cell-surface proteins, cells were treated with trypsin/EDTA (Invitrogen) or EDTA-based cell dissociation solution (Sigma) for 15 min at 37 °C, followed by washes with PBS and 1× soybean trypsin inhibitor (10 mg/ml in PBS; Sigma). Cells were then treated with TAT PTD-Cre for 1 h at 37 °C in serum-free medium with 1× soybean trypsin inhibitor, followed by trypsinization and replating. Cells were analyzed for recombination at 24 h as described above.

TAT PTD-FITC and Labeled TAT PTD-Cre Cell Association and Extracellular Binding—CHO-K1, pgsA, and Lec2 cells (pZ/EG LSL-GFP clones were used interchangeably with identical results) were seeded overnight onto 24-well plates at a density of 75,000 cells/well. Cells were washed and incubated in serum-free medium for 1 h at 37 °C. For cell association, cells were then treated with TAT PTD-FITC, TAT PTD-TMR, or TAT PTD-Cre-Alexa Fluor 546 for 1 h in serum-free medium at 37 or 4 °C as indicated. Cells were placed on ice, washed several times with PBS, washed with 0.5 mg/ml heparin (Sigma) in PBS, trypsinized, and placed on ice for FACS. Cell association was quantitated by FACS as described above. For extracellular binding, cells were placed on ice and incubated for 15 min with labeled TAT PTD peptide or TAT PTD-Cre in serum-free medium. Cells were then washed several times with ice-cold PBS, washed with 0.5 mg/ml heparin in PBS, removed from the plate with cell dissociation solution, and placed on ice for immediate FACS analysis. For microscopic visualization of cell association, cells were plated on glass coverslips and treated as described above with 0.5 μM TAT PTD-Cre-Alexa Fluor 546 for 1 h in serum-free medium at 37 °C. Cells were washed several times with PBS, washed with 0.5 mg/ml heparin in PBS, and visualized on an Axiovert 200M microscope equipped with a live cell incubation chamber and CCD camera (Zeiss). Images were acquired using Axiovision 4.5 software (Zeiss) and processed for publication using Photoshop CS2 (Adobe).

Macropinocytotic Uptake of Neutral Dextran—For flow cytometric analysis, CHO-K1, pgsA, and Lec2 cells (LSL-GFP clones were used interchangeably with identical results) were seeded overnight onto 24-well plates at a density of 50,000 cells/well. Cells were washed and incubated in serum-free medium for 1 h at 37 °C. Cells were then incubated for 1 h at 37 °C with

0.5 mg/ml 70-kDa neutral dextran-Texas Red with the indicated concentrations of unlabeled TAT PTD peptide or TAT PTD-Cre protein. Cells were placed on ice, washed several times with PBS, washed with 0.5 mg/ml heparin in PBS, trypsinized, and placed on ice for FACS. Cells were analyzed by FACS as described above. For microscopy, cells were plated on glass coverslips overnight and incubated in serum-free Ham's F-12 medium for 1 h at 37 °C. Cells were then incubated for 1 h at 37 °C with 0.5 mg/ml 70-kDa neutral dextran-TMR and 1 μM unlabeled TAT PTD peptide, followed by washes with PBS and 0.5 mg/ml heparin/PBS and microscopy as described above.

Heparinase and Sialidase Depletion of Glycans—CHO-K1, pgsA, and Lec2 LSL-GFP cells or Tex-LoxP-EGFP cells were seeded overnight on 24-well plates and treated with the indicated concentrations of heparinase-3 (Sigma) or sialidase (neuraminidase from *Arthrobacter ureafaciens*; E-Y Laboratories) in serum-free medium for 1 h at 37 °C. After washing with PBS, cells were assayed for TAT PTD-Cre recombination, cell association, or dextran uptake as described above. To determine the efficiency of HS depletion, cells were placed on ice and treated with 1 μg/ml biotinylated fibroblast growth factor 2 (from the laboratory of Jeffrey Esko) for 15 min, followed by washes with ice-cold PBS, a 15-min incubation with streptavidin-phycoerythrin-Cy5 (BD Biosciences), and washes with PBS. Following removal from the plate with cell dissociation solution, cells were analyzed by FACS. To assay SA depletion, cells were incubated with FITC-conjugated wheat germ agglutinin (Sigma) on ice for 15 min, followed by PBS washing and FACS.

RESULTS

Fluorescently Labeled TAT PTD Actively Associates with Cells Lacking Glycans—To obtain a quantitative measure of transduction efficiency and to reconcile our preliminary data with those of previous researchers, we measured, via flow cytometry, the cell association of fluorescently labeled TAT PTD peptide and TAT PTD-Cre in wild-type and glycan-deficient CHO cells. Cells treated with fluoresceinated TAT PTD peptide (TAT PTD-FITC) exhibited differences in cell association, with less TAT peptide association in the glycan-deficient cells, particularly at 5 μM, where there was a 30–40% reduction in cell association (Fig. 1*A*). Also notable were differences in the dynamics of TAT PTD-TMR uptake (Fig. 1*B*). Incubation of cells at 4 °C is known to inhibit macropinocytotic uptake of TAT PTDs (8–10). We detected differences between wild-type CHO cells and HS- and SA-deficient cells in the cell-surface binding of TAT PTD peptides at 4 °C (Fig. 1*C*) that correlated with differences in TAT PTD-FITC cell association, indicating a reduction in extracellular binding capacity in glycan-deficient cells. We also examined uptake differences of a TAT PTD fusion protein, namely TAT PTD-Cre (37 kDa). Consistent with the TAT PTD peptide results above, cells lacking HS and SA also had reduced TAT PTD-Cre-Alexa Fluor 546 cell association relative to wild-type glycan-positive CHO cells (Fig. 2*A*). Cells treated with TAT PTD-Cre-Alexa Fluor 546 also exhibit punctate macropinocytotic staining as visualized by live cell microscopy (Fig. 2*B*). Extracellular binding of labeled TAT PTD-Cre at 4 °C (Fig. 2*C*) revealed similar differences in the

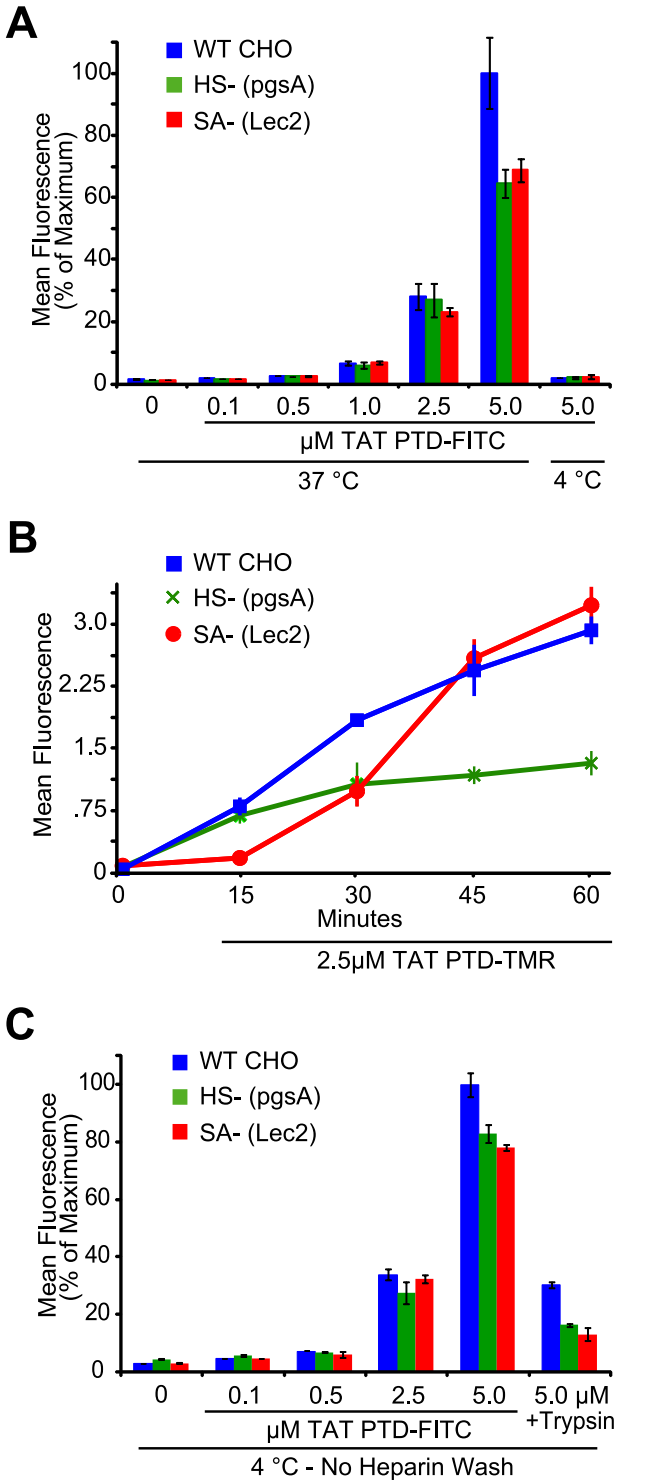

FIGURE 1. **TAT PTD peptide cell association and cell-surface binding in glycan-deficient cells.** *A*, TAT PTD peptide cell association in parental CHO-K1 and derivative glycan mutant pgsA and Lec2 cell lines. Cells were treated with the indicated concentrations of fluoresceinated TAT PTD peptide (TAT PTD-FITC) at 37 °C for 1 h, followed by washes with PBS, heparin, and trypsin to remove extracellular peptide. Cells were placed on ice and immediately analyzed by flow cytometry. *B*, time course of TAT PTD cell association in parental and glycan mutant cell lines. Cells were treated with 2.5 μM TAT PTD-TMR as described for *A* for the indicated times. *C*, TAT PTD peptide cell-surface binding at 4 °C. Cells were treated for 15 min with TAT PTD-FITC peptide, followed by washes with PBS, nonenzymatic cell dissociation, and flow cytometry. *WT*, wild-type.

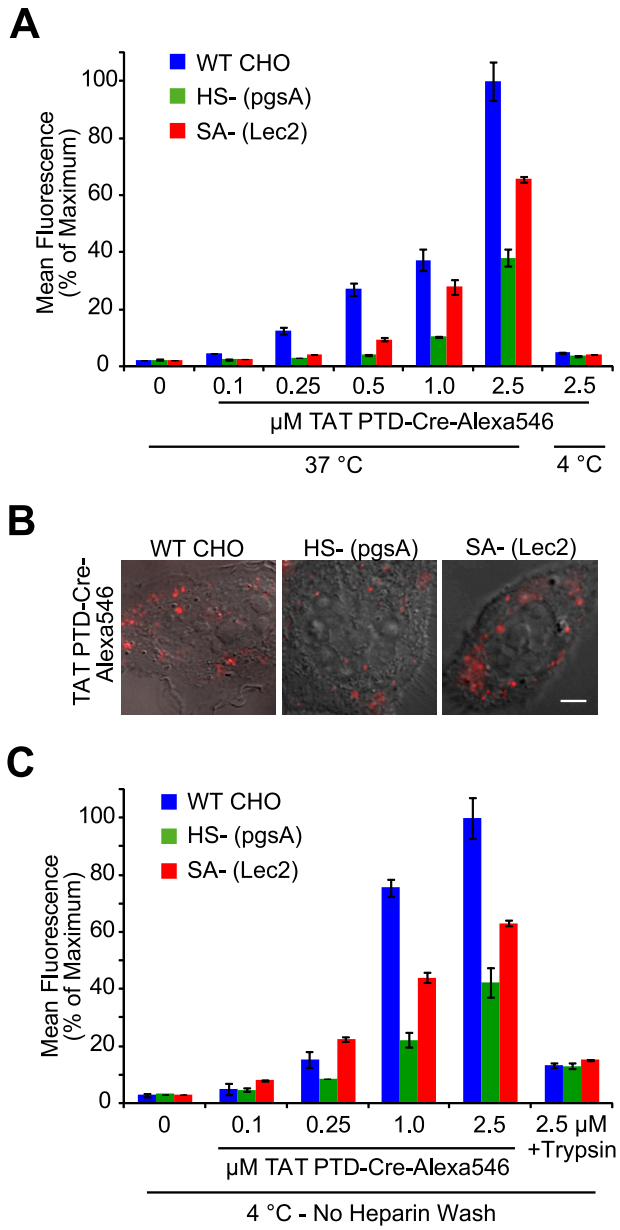

FIGURE 2. **TAT PTD-Cre fusion protein cell association and cell-surface binding in glycan-deficient cells.** *A*, flow cytometric analysis of cells treated for 1 h at 37 °C at the indicated concentrations of recombinant TAT PTD-Cre labeled with Alexa Fluor 546. Cells were washed thoroughly with PBS, heparin, and trypsin to remove extracellular TAT fusion protein. *B*, live cell photomicrographs of the indicated cell lines treated with 1 μM TAT PTD-Cre-Alexa Fluor 546 for 1 h at 37 °C. TAT PTD-Cre treatment was followed by washes with PBS and heparin. *Scale bar* = 5 μm. *C*, TAT PTD-Cre-Alexa Fluor 546 cell-surface binding at 4 °C. Cells were treated for 15 min with TAT PTD-Cre-Alexa Fluor 546, followed by PBS wash, nonenzymatic cell dissociation, and flow cytometry. *WT*, wild-type.

inability of the glycan-deficient cells to bind TAT PTD-Cre and TAT PTD-FITC, further indicating that the differences seen in cell association may be due to differential extracellular binding capacities rather than a defect in transduction *per se*.

TAT PTD-Cre Transduction and Recombination Occur Efficiently in CHO Glycan Mutant Cell Lines—Analysis of cell association and uptake of fluorescently labeled PTD peptides and fusion proteins may be incomplete and potentially misleading. To avoid these pitfalls, we assayed HS- and SA-deficient cells for induction of a cellular phenotype that is dependent on PTD cargo escape into the cytoplasm/nucleus. Previously, we devised a TAT PTD-Cre-LSL-GFP reporter system as a readout for TAT PTD-Cre transduction. Entrance of TAT PTD-Cre into the nucleus results in recombination and elimination of the LSL-GFP transcriptional terminator, resulting in expression of GFP. Unlike visual assays employing fluorescently labeled proteins or peptides that may inadvertently measure cell-surface binding and endocytotic uptake (in the absence of endosomal escape), TAT PTD-Cre-induced recombination and GFP expression are an unequivocal measure of transduction (8). Because of its stringency, this system may underestimate the efficiency of the transduction phenomena being observed, yet it gives an unparalleled phenotypic measure of transduction with minimal potential for false positives or misinterpretation.

We analyzed TAT PTD-Cre transduction in stable LSL-GFP clones of parental glycan-positive wild-type CHO-K1 and derivative glycan-deficient pgsA and Lec2 cell lines. Treatment of each of these cell lines with TAT PTD-Cre induced GFP expression in a dose-dependent manner as observed by flow cytometry (Fig. 3, *A* and *C*) and microscopy (Fig. 3*B*). This demonstrates the ability of TAT PTD-Cre to enter cells and transit to the nucleus to recombine the LSL genetic element in the absence of HS and SA. Although cells lacking glycans showed a slight shift of the dose-response curve, both HS- and SA-deficient cells reached the same maximal response at 2 μM. Similar results were obtained using recombinant Arg_8-PTD-Cre fusion protein (data not shown). In contrast, control recombinant Cre protein (no PTD) showed no induction of GFP above background levels (Fig. 3*A*). Inhibition of macropinocytosis by cytochalasin D resulted in a dramatic reduction in TAT PTD-Cre-mediated recombination and GFP induction (Fig. 3, *A* and *B*).

TAT PTD-mediated Transduction Occurs via Macropinocytosis in Wild-type and Glycan-deficient Cells—We measured the effect of several macropinocytotic chemical inhibitors on TAT PTD-Cre transduction. Wild-type, HS-deficient, and SA-deficient CHO cells were treated with three different macropinocytotic inhibitors: amiloride (a proton pump inhibitor that specifically affects macropinocytosis) (Fig. 4*A*), wortmannin (a kinase inhibitor) (Fig. 4*B*), and cytochalasin D (an inhibitor of F-actin polymerization) (Fig. 4*C*). Treatment with all three macropinocytotic inhibitors resulted in dramatic reductions of TAT PTD-Cre transduction into cells as measured by a dose-dependent decrease in TAT PTD-Cre-mediated recombination of the LSL-GFP reporter in the absence of significant cellular toxicity (Fig. 4, *A–C*, *insets*). In addition, enzymatic removal of cell-surface proteins by trypsin also potently inhibited TAT PTD-Cre-mediated transduction into cells (Fig. 4*D*). In contrast, nonenzymatic release of cells from tissue culture dishes by cell dissociation solution did not alter TAT-Cre uptake.

We have shown previously that TAT PTDs stimulate their own cellular uptake by increasing the basal rate of macropinocytosis (8, 9). Treatment of wild-type, HS-deficient, and SA-deficient CHO cells with a known macropinocytosis marker, fluorophore-labeled 70-kDa neutral dextran, in combination with unlabeled TAT PTD peptide resulted in a dose-dependent

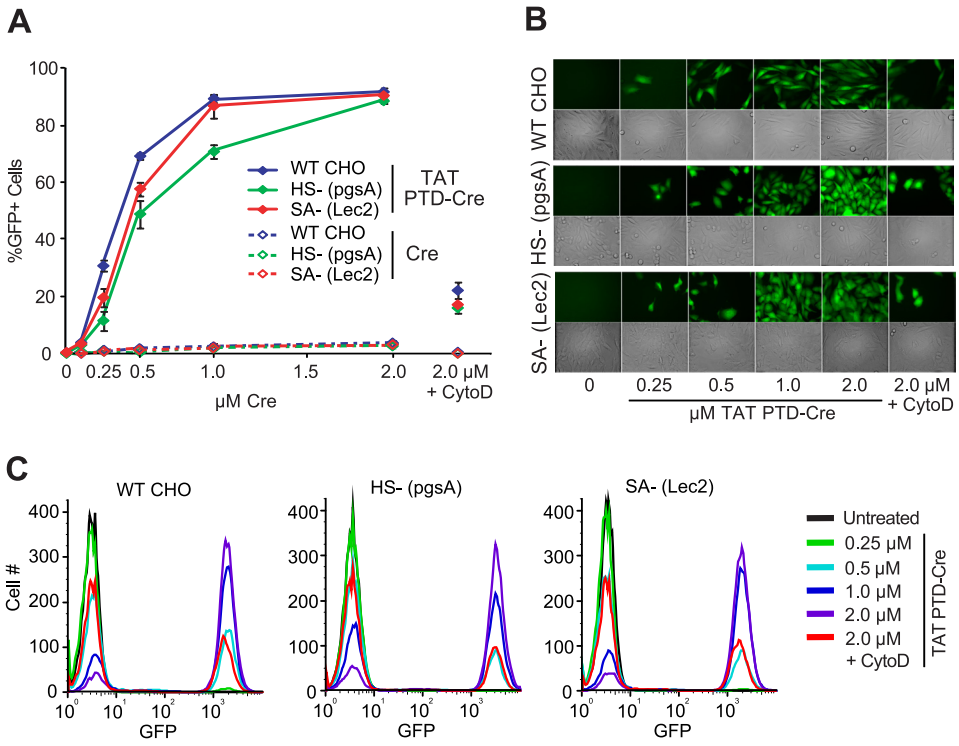

FIGURE 3. **TAT PTD-Cre transduction occurs in the absence of glycosaminoglycans and SAs in parental CHO and glycan-deficient cell lines.** *A*, TAT PTD-Cre recombination in CHO-K1 and derivative glycan mutant pgsA and Lec2 cells with a stably integrated Lox-STOP-Lox-GFP expression construct. Cells were treated with purified recombinant TAT PTD-Cre fusion protein or control recombinant Cre (no TAT PTD) at the indicated concentrations for 1 h, followed by trypsinization and replating. Flow cytometry was performed at 24 h after Cre addition. As indicated, control cells were treated with cytochalasin D to inhibit macropinocytosis for 1 h at 37 °C immediately preceding and during TAT PTD-Cre treatment. *B*, photomicrographs of TAT PTD-Cre recombination-induced GFP expression in parental and glycan mutant cells with stably integrated LSL-GFP construct. *C*, FACS profiles of representative samples from the experiment in *A*. *WT*, wild-type.

increase in dextran uptake in response to TAT PTD in all three cell types, although HS-deficient cells showed a lesser induction (Fig. 5*A*). Consistent with this observation, microscopic visualization also showed the presence of labeled dextran in TAT PTD-induced macropinosomes in all three cell types (Fig. 5*B*), suggesting that TAT PTD stimulation of macropinocytosis occurs independently of both HS and SA. Furthermore, enzymatic depletion of HS and SA with heparinase and sialidase had no effect on dextran uptake (Fig. 5, *C* and *D*), indicating that cells lacking both HS and SA in combination are capable of TAT PTD-induced macropinocytosis.

Enzymatic Removal of Glycans Has a Negligible Effect on Transduction—To confirm our assays using genetically glycan-deficient cell lines, we sought to determine whether the same results would be obtained by enzymatic depletion of HS and SA. Our observations with mutant CHO cell lines could be due to compensation for a deficiency that is evident only after acute depletion, or a strong phenotype might be evident only in cells lacking HS and SA in combination. Efficient removal of cell-surface HS and SA was accomplished using heparinase and sialidase enzymes (supplemental Fig. S1, *A* and *B*). Pretreatment of cells with these enzymes had little to no effect on TAT-Cre transduction and recombination efficiency (Fig. 6, *A* and *B*). Likewise, there was no substantial effect on TAT-FITC peptide cell association (Fig. 6, *C* and *D*). In contrast, binding of fibroblast growth factor 2 and wheat germ agglutinin, ligands known to bind specifically to HS and SA, respectively, was greatly reduced in the treated cells (supplemental Fig. S1, *A* and *B*). We also noted that treatment of murine T cells (Tex-LoxP-EGFP) with heparinase did not alter transduction of TAT-Cre (Fig. 6*E*). Taken together, these observations demonstrate that cationic TAT PTD-mediated cellular delivery of a Cre recombinase cargo and induction of macropinocytosis occur independently of HS and SA.

DISCUSSION

Based primarily on microscopic visualization, previous studies concluded that cells lacking proteoglycans are refractory to cellular transduction by several PTDs, including TAT PTD (13, 16, 17, 25–27), whereas other researchers obtained conflicting results (14, 15, 17). We originally intended to use HS-deficient mutants as transduction-null cells to facilitate our research into the mechanism of transduction. Our original hypothesis was that a HS proteoglycan served as a receptor for TAT PTD and was responsible for stimulating macropinocytotic uptake. However, to our surprise, experiments using a cellular phenotypic assay, not merely visualization, demonstrated that cells lacking HS or SA are fully competent to efficiently transduce TAT PTD-Cre into cells.

To further explore our unexpected preliminary findings, we first looked at cell association of fluorescent TAT PTD-FITC peptide and TAT PTD-Cre-Alexa Fluor 546 in wild-type and glycan-deficient cells. Our data corroborate the work of previous researchers indicating a reduction in cell association of labeled TAT PTD peptide and labeled TAT PTD-Cre protein in glycan-deficient cell lines (16, 17). However, the differences we see are not so dramatic as to warrant the conclusion that transduction is not occurring in these cells: the cell association of TAT PTD-FITC and TAT PTD-Cre-Alexa Fluor 546 is well above the control in both HS- and SA-deficient cells. Nevertheless, there are clear and consistent differences in the magnitude of transduction we see in glycan-deficient cells that likely explain the conclusions of previous authors that transduction does not occur in cells lacking HS. The differences we see in cell association in cells lacking glycans correlate well with differences in extracellular binding of TAT PTD peptide and TAT PTD-Cre protein. This indicates that the minor differences seen in cell association are due to a reduced extracellular TAT-PTD-binding capacity of glycan-deficient cells rather than a deficiency in transduction pathways.

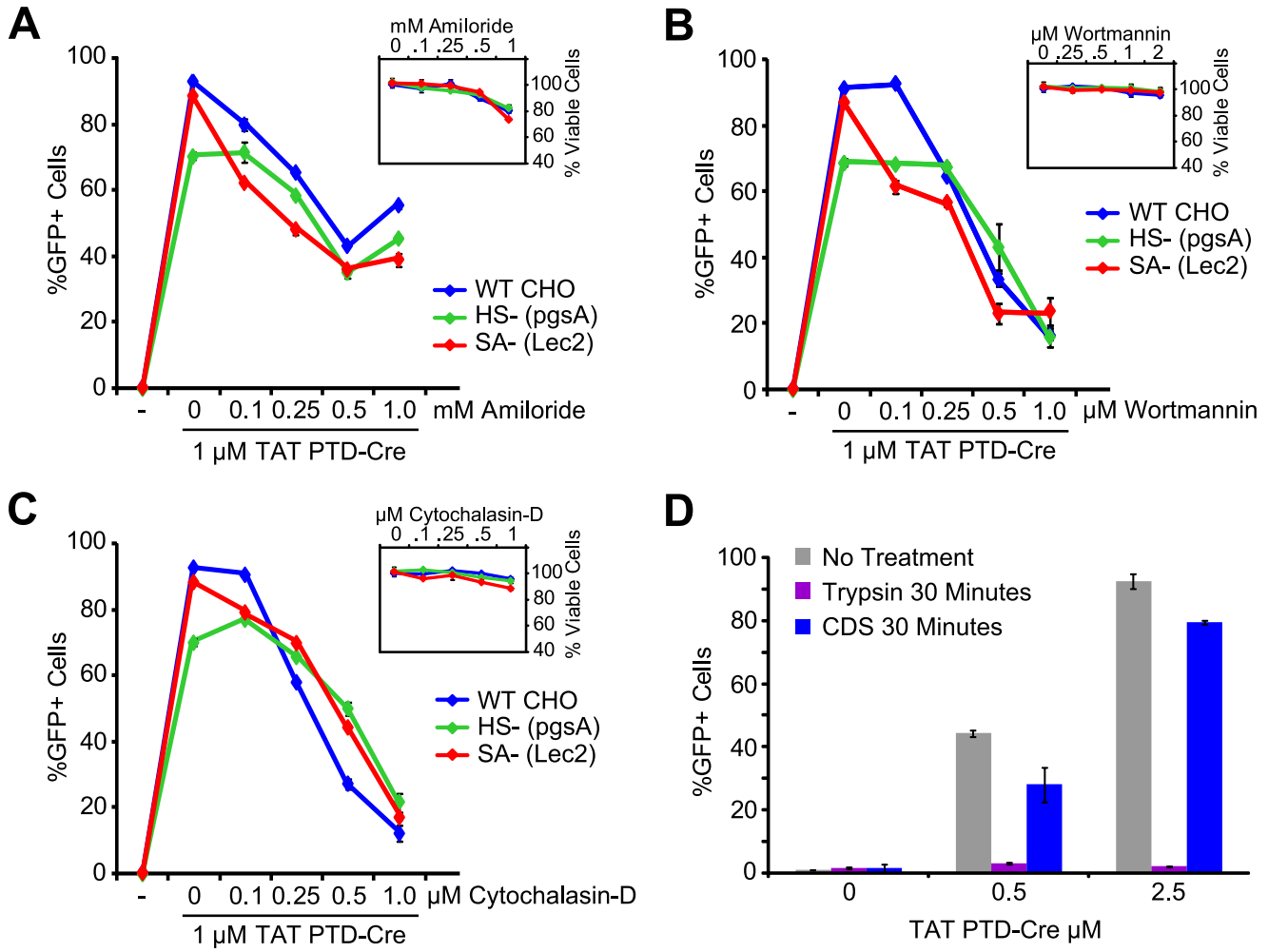

FIGURE 4. **Macropinocytotic inhibitors and TAT PTD-Cre transduction in glycan-deficient cells.** *A*, CHO-K1, pgsA, and Lec2 cells with a stably integrated LSL-GFP construct treated with the indicated concentrations of amiloride, an inhibitor of macropinocytosis, for 1 h. Cells were then treated with TAT PTD-Cre protein in the presence of amiloride for 1 h, followed by trypsinization and replating. GFP expression was assayed by flow cytometry at 24 h after TAT PTD-Cre addition. *Inset*, cell viability as assayed by flow cytometry immediately following TAT PTD-Cre treatment. *B*, treatment with wortmannin, a kinase inhibitor, as described for *A*. *C*, treatment with cytochalasin D, an inhibitor of F-actin and macropinocytosis, as described for *A*. *D*, depletion of cell-surface proteins by trypsin. CHO-K1 LSL cells were treated with trypsin or cell dissociation solution (*CDS*) for 30 min, followed by washes with PBS and trypsin inhibitor and treatment with TAT PTD-Cre for 1 h. Cells were then washed, trypsinized, and replated. GFP expression was assayed by FACS at 24 h following TAT PTD-Cre treatment. *WT*, wild-type.

To further explore transduction in the absence of glycans, we sought to generate a transduction data set based on a phenotypic assay rather than using cell association of labeled TAT as an approximation of transduction. To accomplish this, we generated stable Cre-responsive cell lines from CHO-K1, pgsA, and Lec2 cells using a LSL-GFP reporter gene. As we have shown previously (8), cell lines with an integrated LSL-GFP construct do not express GFP in the absence of Cre recombinase. However, after treatment of LSL-GFP cells with exogenous recombinant TAT PTD-Cre protein, GFP expression is induced. Using this system, we can determine the relative efficiency of TAT PTD transduction without the caveats of visualization based on labeled peptides and proteins. Assays that rely on fluorescent tags to measure cellular uptake cannot discriminate between endosomal and cytosolic fractions of the cell and may grossly overestimate the actual fraction of peptide and cargo that has transduced into the cell (escaped from the endosome). We prefer to refer to these measures as cell association because they do not sufficiently distinguish cells that have fluorescent label in endocytic vesicles or bound to the cell surface from cells where the peptide and cargo have actually escaped endosomes into the cytoplasm. This distinction is important because endosomal escape is requisite for transduction; the majority of labeled TAT PTD taken up by cells is present in endosomes, and most researchers would agree that only a small proportion escapes to the cytoplasm. In addition, for the purposes of most therapeutic interventions, the inside of the endosome is essentially the outside of the cell; no biological response will be elicited simply by uptake into vesicles. The importance of escape is further illustrated by the increase in transduction efficiency seen with endosome-disrupting peptides (8, 28).

Treatment of Cre-responsive cells with TAT PTD-Cre revealed a trivial difference between wild-type and HS- or SA-deficient cells. If transduction were not occurring in these cells, we would expect to see a reduction to the base line as seen with recombinant Cre lacking TAT PTD or treatment with cytochalasin D. The efficient manner with which TAT PTD-Cre enters glycan-deficient cells is strong and convincing evidence that

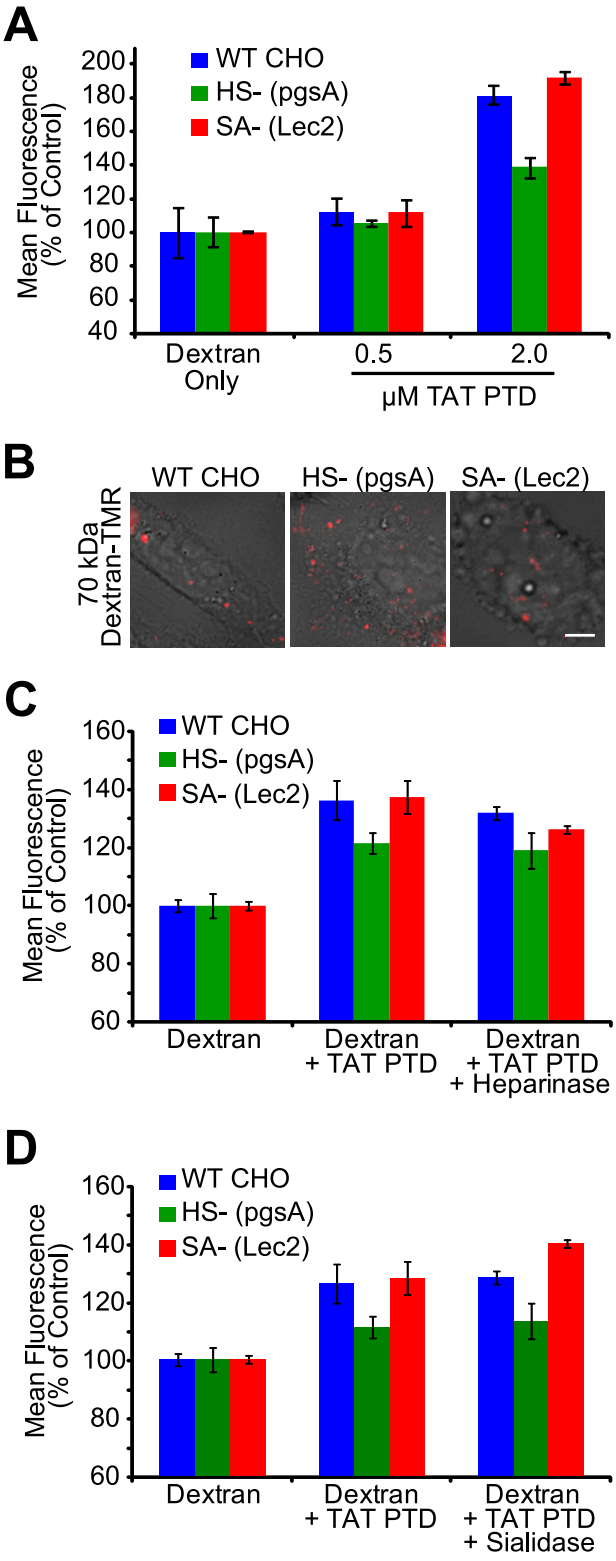

FIGURE 5. **TAT PTD-induced macropinocytotic fluid-phase uptake is intact in glycan-deficient cells.** *A*, parental CHO and glycan mutant cells treated with unlabeled TAT PTD peptide in the presence of 70-kDa neutral dextran-Texas Red for 1 h at 37 °C. After washing and trypsinization, cells were assayed for dextran uptake by FACS. *B*, live cell photomicrographs of the indicated cell lines following treatment with 1 μM TAT PTD in the presence of 70-kDa dextran-TMR. *C*, TAT PTD-induced uptake of 70-kDa dextran-Texas Red following treatment with 200 milliunits of heparinase for 1 h at 37 °C. The TAT PTD concentration was 1 μM. *D*, cells treated with 200 milliunits of sialidase followed by dextran and TAT PTD as described for *C*. *WT*, wild-type.

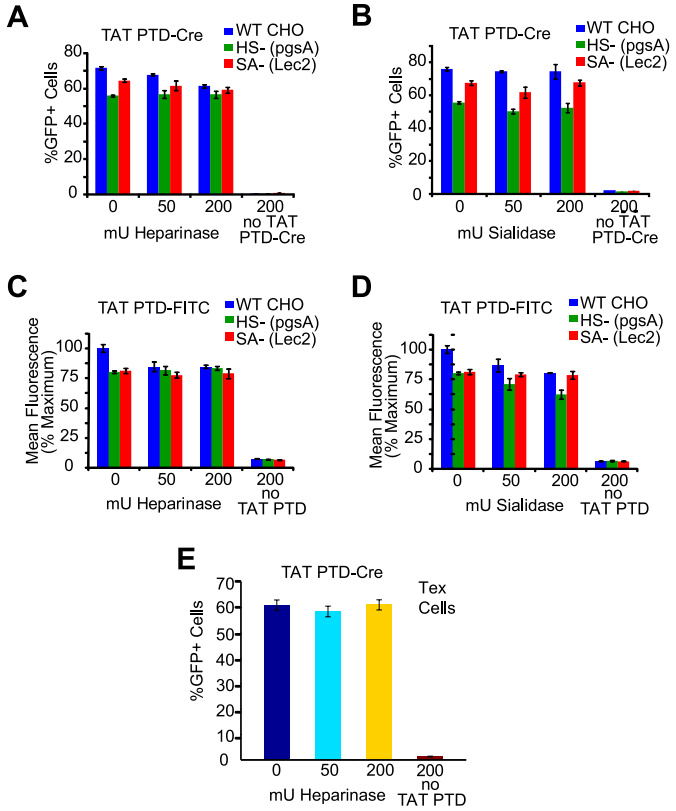

FIGURE 6. **Enzymatic depletion of HS and SAs does not impair TAT PTD transduction into parental or glycan-deficient cells or murine T cells.** *A* and *B*, the indicated Lox-STOP-Lox-GFP stable cell lines were treated for 1 h with heparinase or sialidase as indicated at 37 °C, followed by 1 μM TAT PTD-Cre for 1 h at 37 °C, trypsinization, and replating. GFP expression was assayed by flow cytometry 24 h after TAT PTD-Cre treatment. *C* and *D*, the indicated cell lines were treated with heparinase and sialidase enzymes as described for *A* and *B*, followed by treatment with 1 μM TAT PTD-FITC peptide for 1 h at 37 °C, immediately followed by PBS, heparin, and trypsin washes and flow cytometry. *E*, heparinase treatment does not affect transduction efficiency in the Tex-LoxP-EGFP murine thymoma cell line. *WT*, wild-type.

transduction occurs in the absence of extracellular glycosaminoglycans and SAs.

Further evidence that transduction is occurring in these cells is the existence of TAT PTD-induced macropinocytotic uptake of 70-kDa neutral dextran in glycan-deficient pgsA and Lec2 cells. If these cells were refractory to transduction or if there were another pathway for entry, we would not expect to see dextran uptake increased in response to TAT PTD. Furthermore, the mode of uptake is macropinocytosis in wild-type and glycan-deficient cell lines, as evidenced by the similar reductions in transduction efficiency seen after treatment with chemical inhibitors of macropinocytosis.

We have shown here, in an extensive exploration, that TAT PTD-mediated transduction does not require HS or SAs; it occurs efficiently in their absence. As shown by our very stringent Cre-LoxP system and by more conventional fluorescent visualization and cytometry, TAT PTD enters cells lacking HS or SA and cells that have been depleted of both in combination. Furthermore, TAT PTD-induced macropinocytotic fluid-phase uptake is intact in glycan-deficient cells, indicating that TAT PTD does not require these glycans to stimulate endocytosis. By contrast, depletion of cell-surface proteins with protease results in drastically reduced transduction efficiency, indi-

cating that a cell-surface protein is necessary for TAT PTD transduction (although not necessarily as a receptor). These data suggest that cationic peptides like TAT PTD may induce macropinocytotic uptake and transduction via binding to a protein on the cell surface rather than through interactions with glycans or direct interaction with the membrane.

Together, our findings are consistent with the hypothesis that the dense forest of extracellular glycans forms a pool of negative charge that TAT PTD binds on the cell surface. Differences in this charge pool affect the efficiency of TAT transduction to varying degrees but are independent of the ability of PTDs to transduce cells or to induce macropinocytotic uptake. Further study to determine the extracellular proteins required for transduction will offer important insights into the mechanism of transduction of TAT PTD as well as other PTDs.

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