# REGULATED UPTAKE OF BIOPOLYMERS Role of cell surface proteoglycans Implications for drug and gene delivery

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# Nuclear Targeting of Macromolecular Polyanions by an HIV-Tat Derived Peptide

ROLE FOR CELL-SURFACE PROTEOGLYCANS*

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**New therapies based on gene transfer and protein delivery require a better understanding of the basic mechanisms of macromolecular membrane transport. We have studied cellular uptake of macromolecular polyanions, *i.e.* DNA and glycosaminoglycans, and a polybasic HIV-Tat derived peptide (GRKKRRQRRRPPQC) using fluorescence assisted cell sorting and confocal fluorescence microscopy. The transactivator of HIV transcription (Tat) peptide stimulated cellular uptake of both DNA and heparan sulfate in a time-, concentration-, and temperature-dependent manner. Peptide-polyanion complexes accumulated in large, acidic, cytoplasmic vesicles formed *de novo*. This was followed by transfer of polyanion into the nuclear compartment and subsequent disappearance of the endolysosomal vesicles. In the absence of polyanion the Tat peptide displayed rapid accumulation in the nuclear compartment. However, in the presence of polyanion the peptide was almost exclusively retained in cytoplasmic vesicles. Cell-surface proteoglycans played a pivotal role in the uptake of complexes exhibiting a relatively high peptide to polyanion ratio, corresponding to a net positive charge of the complexes. Uptake of polyanions *per se* or complexes with a relatively low peptide to polyanion ratio was favored by proteoglycan deficiency in the recipient cells, indicating the existence of distinct transport mechanisms. Moreover, expression of full-length HIV-Tat as well as exogenous addition of HIV-Tat peptide resulted in cellular accumulation of endogenous proteoglycans. We conclude that an HIV-Tat derived peptide efficiently targets extraneous DNA and glycosaminoglycans to the nuclear compartment and that proteoglycans serve a regulatory role in these processes, which may have implications for directed gene and drug delivery *in vivo*.**

The plasma membrane defines the border of living cells and constitutes a barrier to extracellular components. Advances in the molecular biology field have resulted in the development of novel therapeutic strategies, *e.g.* gene therapy and cellular protein delivery, which must rely on the entry of large, polyvalent molecules into the intracellular compartment. Although internalization of DNA has been demonstrated in various cell lines and tissues, it is a relatively inefficient and potentially cytotoxic process (1–2). Thus, substantial efforts should be focused on mechanistic studies of macromolecular membrane passage, which then form a basis for the construction of novel DNA and protein delivery vehicles.

Recently, two major classes of membrane-penetrating proteins have been identified, *i.e.* arginine/lysine-rich peptides and peptides containing the hydrophobic core region (h region) of signal peptides (see Refs. 3–4 for review). Viral proteins, *e.g.* VP22 and Tat (transactivator of HIV transcription) from herpes simplex and HIV,[1] respectively, contain protein transduction domains (PTD) that have been utilized for cellular delivery of fusion proteins or chemically cross-linked cargoes, *e.g.* the PTD of HIV-Tat displayed on the surface of λ particles facilitated transfer of phage-encapsulated marker genes into mammalian cells (5), and liposomes covalently attached to the same peptide were efficiently internalized via an energy-independent process (6). Notably, virtually all cell types display efficient uptake of PTD fusion proteins, suggesting a ubiquitous transport pathway (7). In AIDS pathology, HIV-infected cells can secrete Tat, which then enters surrounding cells and activates HIV-1 gene expression through a specific interaction with the viral TAR RNA target sequence. In this way Tat can stimulate a very high level of viral gene expression and replication (8–11). In several extensive studies, Presta and co-workers (11–13) have pursued the interaction between full-length HIV-Tat and various polyanions, *e.g.* heparin, suramin, and pentosan sulfate, and found an inhibitory role for these compounds in Tat internalization and transactivation activity, which may be utilized for the design of novel anti-HIV drugs. More recently a role for cell-surface heparan sulfate proteoglycans (HSPG) in cellular uptake of a recombinant GST-Tat-GFP fusion protein was shown by the same group (14).

PG encompasses a heterogeneous group of proteins substituted with long linear polysulfated, and thereby highly negatively charged, polymers named glycosaminoglycans (GAG) (see Refs. 15–17 for reviews). The GAG chains, *e.g.* chondroitin

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[1] The abbreviations used are: HIV, human immunodeficiency virus; CHO, Chinese hamster ovary; CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; HS, heparan sulfate; HTDP, HIV-tat derived peptide; PG, proteoglycan; PTD, protein transduction domain; XT-def, xylosyl transferase deficient; HSPG, heparan sulfate proteoglycans; GST, glutathione-*S*-transferase; Tat, transactivator of HIV transcription; GFP, green fluorescence protein; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; TAR, trans-acting-responsive.

sulfate (CS), dermatan sulfate (DS), and HS, consist of a common linkage region, GlcA-Gal-Gal-Xyl, where a glycosidic linkage is formed between the xylose residue of the GAG chain and a serine from the core protein. The linkage region is then extended with repeating disaccharides consisting of either GlcA-$\beta$-GalNAc or GlcA-$\alpha$-GlcNAc. The final PG is formed after complex modifications including sulfation reactions, which render the molecule anionic in its character. Because of their size and polyanionic character, GAG chains may serve as attachment sites for a vast number of polybasic compounds. Cell-surface PG are selective regulators of ligand-receptor encounters (see Ref. 18 for review) and facilitate binding and internalization of growth factors, lipoproteins, as well as of viruses and bacteria (19–22). We have previously studied binding of HS to growth-promoting polyamines (23) and found that HSPG facilitates polyamine uptake (24) and that combined inhibition of polyamine and HS synthesis attenuates tumor growth *in vivo* (25). Moreover, we and others have presented data indicating a role for PG in cationic lipid-mediated gene delivery (26–29).

The present study investigates nuclear targeting of polyanionic macromolecules by an HIV-Tat derived peptide in mammalian cells and the potential role for cell-surface PG in these processes.

## EXPERIMENTAL PROCEDURES

*Materials*—The HIV-Tat derived peptide (HTDP) (amino acid 48–60 plus a C-terminal cysteinamide, GRKKRRQRRRPPQC-amide) and Texas red-labeled HTDP were from Innovagen AB, Lund, Sweden. Peptide sequences were confirmed by reverse phase high pressure liquid chromatography and mass spectrometry. pcDNA3HIV-Tat-GFP plasmid was generously provided by Dr. Berkhout, University of Amsterdam, The Netherlands. HS and DS preparations were the same as described previously (27). LipofectAMINE reagent was from Invitrogen. Rhodamine (Molecular Probes) green- and red-labeled HS and DS were prepared as described (30). LysoTracker red DND-99 was from Molecular Probes, and Rhodamine red-labeled DNA plasmid was from Gene Therapy Systems Inc., United States. $Na_2{}^{35}SO_4$ (1310 Ci/mmol) was purchased from PerkinElmer Life Sciences. Chondroitin ABC lyase (chondroitinase ABC, EC 4.2.2.4) and heparitinase (alias heparinase III) were from Seikagaku Inc., Japan. PD-10, Superose 6 HR 10/30 and MonoQ HR 5/5 columns were all obtained from Amersham Biosciences, Sweden. Paraformaldehyde, bovine serum albumin, human placenta DNA, and all fine chemicals were from Sigma.

*Cell Culture*—CHO-K1, pgsA-745 (XT-def), pgsB-618, and human embryonic lung fibroblast (HFL-1) cells were obtained from the American Type Culture Collection. CHO cells were routinely cultured in F12K nutrient mixture supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin (growth medium) in a humidified 5% $CO_2$, 37 °C incubator. HFL-1 cells were maintained in Dulbecco's modified Eagle's medium with the same supplements as above.

*FACS Analysis*—Fluorophore-labeled DNA plasmid, HS, or DS was pre-incubated with HTDP at different concentrations in F12K for 30 min at room temperature. The mixture was then added to extensively rinsed subconfluent cell layers in 24-well plates, and the incubation was allowed to proceed for various periods of time at 37 °C, or in some cases 4 °C. After removal of incubation medium cells were treated with trypsin followed by extensive washing with ice-cold PBS 1% (w/v) bovine serum albumin to remove unspecific extracellular fluorophore. The cells were finally suspended in 0.5 ml PBS, 1% bovine serum albumin and analyzed for fluorescence in a FACS-Calibur (BD Biosciences) instrument integrated with PC-compatible Cell-Quest software.

*Fluorescence Microscopy*—Cells were seeded at low density in 4-well chamber slides and allowed to adhere over night. After extensive rinsing with PBS, cells were incubated with polyanions and/or HTDP for time periods and at concentrations as indicated in the respective figure legends. Cells were then separated from unspecific extracellular fluorophore by either of the following methods: 1) trypsin treatment, followed by consecutive rinsing in PBS 1% bovine serum albumin, and re-seeding on chamber slides in growth medium for 4–6 h; or 2) brief rinsing with 2 × 1 ml 2 M NaCl in PBS followed by rinsing with 3 × 1 ml PBS. In both cases cells were then fixed in 4% paraformaldehyde in PBS for 30 min. After fixation slides were washed with 2 × 1 ml PBS and 2 × 1 ml distilled water, and then air dried. LysoTracker red DND-99, which is able to enter the intracellular compartment and emits light in a pH-dependent manner, was present during the last 2 h of incubation. Cells were visualized using a Nikon Eclipse E800 microscope and a Bio-Rad MRC 1024 confocal laser scanning microscopy system. Collected data was analyzed using PC-compatible Laser-Sharp software.

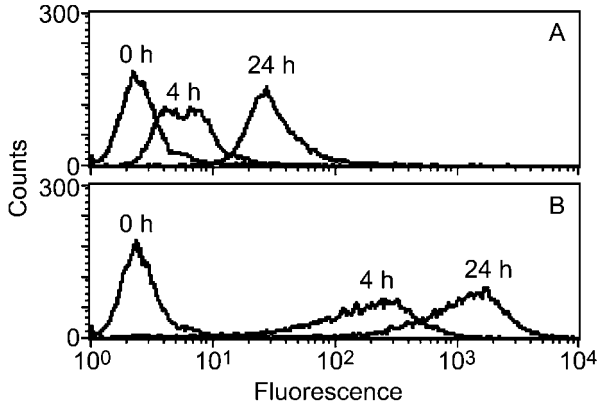
*Luciferase Gene Expression Assay*—Cells were plated in 24-well plates at $1 \times 10^5$ cells per well in 0.5 ml growth medium 24 h before transfection. To prepare HTDP-DNA complexes, pGL3-luciferase plasmid DNA (10 $\mu$g/ml) and the desired amount of HTDP (5–80 $\mu$g/ml) were mixed by inversion and incubated for 30 min at room temperature. The DNA-peptide mixture was then added to cells that had been rinsed twice with F12K. At the end of the 14-h incubation at 37 °C in 5% $CO_2$, the medium was aspirated and replaced with 0.5 ml of growth medium. After an additional period of incubation for 36 h, cells were washed twice with PBS and treated with 150 $\mu$l of cell lysis reagent containing 25 mM Tris-HCl, pH 7.8, 2 mM CDTA, 2 mM dithiothreitol, 10% glycerol, 1% Triton X-100 (Promega, Sweden) for 10 min at 4 °C followed by scraping. Luciferase expression was quantified in 25 $\mu$l of the cell lysate supernatant using a luciferase assay kit. Light emission was measured by integration over 15 s at 25 °C using BMG Fluostar Optima equipment.

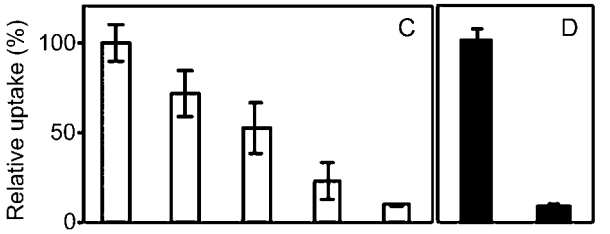
*Cell Treatment with Chlorate and Glycosaminoglycan Lyases*—CHO-K1 cells were treated with chlorate, chondroitin ABC, and heparitinase, essentially as described previously (24). Briefly, cell layers were incubated for 1 h at 37 °C with 0.5 ml of the digestion buffer (20 mM Hepes, pH 7.4, 0.5% bovine serum albumin) containing 0.6 milli-units of heparitinase or 25 milli-units of chondroitin ABC lyase. Another aliquot of heparitinase or ABC lyase was then added to bring the concentration to 4.8 milli-units/ml and 200 milli-units/ml, respectively, and the incubation was allowed to proceed for another 2 h. Following the removal of cell-associated GAG, cells were rinsed extensively with 0.5 ml of the digestion buffer, and Tat peptide-polyanion internalization was determined by FACS according to the usual protocol. For chlorate treatment, cells were incubated for 24 h with NaCl-free Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 25 mM $NaClO_3$ and appropriate amounts of NaCl to obtain physiological ionic strength. After extensive washing, Tat peptide-polyanion uptake was determined by FACS.

*Isolation and Characterization of Radiolabeled Cell Components*—Subconfluent cells were labeled with $Na_2{}^{35}SO_4$ (100 $\mu$Ci/ml) in 8 ml of fresh F12K medium for 24 h. The medium was then aspirated, and cell layers were washed extensively with PBS to remove free isotope. This was followed by another incubation period of 48 h in 10 ml of fresh F12K. Conditioned medium (CM) was aspirated and subjected to centrifugation at 300 × *g* to remove cell debris. CM was pre-incubated for 30 min at room temperature with or without 100 $\mu$g/ml HTDP and then added to fresh cells for a period of 4 h, at 37 °C. Cell medium was re-isolated, centrifuged to remove cell debris, and supplemented with 1 mM di-isopropyl fluorophosphate (DFP), and *N*-ethylmaleimide (NEM). After extensive rinsing in PBS, cell layers were extracted with 2.5 ml of 2% Triton X-100 (v/v) in PBS containing 10 mM EDTA, 10 mM NEM, and 1 mM DFP for 15 min at 4 °C. [$^{35}$S]sulfate-labeled material was analyzed by gel chromatography on Superose 6 and Mono-Q as described (28). Alternatively, confluent cell cultures were labeled with [$^{35}$S]sulfate (100 $\mu$Ci/ml), either with or without 100 $\mu$g/ml HTDP for 24 h, at 37 °C. Polyanion material was isolated by ion-exchange chromatography, desalted on PD-10, and then further processed as described above. Transfections with Lipofectamine were performed according to the instructions by the manufacturer. Cells were then incubated for another 40 h, followed by radio labeling with 100 $\mu$Ci/ml [$^{35}$S]sulfate for 24 h at 37 °C and then further analyzed as described above.

*Degradative Procedures and Gel Electrophoresis*—[$^{35}$S]sulfate-labeled material was subject to enzymatic cleavage by chondroitin ABC lyase overnight at 37 °C in 0.1 M Tris-HOAc, 10 mM EDTA, pH 7.3, or by chemical depolymerization of HS with nitrous acid, pH 1.5, for 10 min followed by neutralization with 2 M $Na_2CO_3$ as described (31). Samples were then subjected to polyacrylamide gel electrophoresis on a 20–30% gradient gel as described (23). The gel was visualized using a Fuji imaging system.

*Statistical Analyses*—Each data point in the dose activity and time course experiments is the mean ± S.D. ($n$ = 4–6) from two separate experiments. In some cases the error bars were smaller than the drawn symbols. Confocal fluorescence microscopy and FACS figures are representative of five or more separate experiments.




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HS | 10* | 10* | 10* | 10* | 10* | 0 | 200 |
| HTDP | 10 | 10 | 10 | 10 | 10 | 80 | 80 |
| DNA | 0 | 25 | 50 | 100 | 200 | 8* | 8* |

FIG. 1. **HIV-Tat peptide mediates DNA and HS uptake via a common pathway.** Uptake of Rhodamine green-labeled HS (20 $\mu$g/ml) into CHO cells, either in the absence (*A*) or presence (*B*) of HTDP (20 $\mu$g/ml) as determined by FACS analysis at the various time points indicated in the figure. *C*, the effect of DNA on HTDP-mediated HS uptake and (*D*) the effect of HS on Rhodamine red-labeled DNA-HTDP complex uptake was analyzed by FACS after co-incubation at the indicated concentrations. *, fluorophore label.

## RESULTS

*Membrane Passage and Intracellular Routing of Charged Macromolecules*—To investigate macromolecular membrane transport in cultured cells, we used fluorophore-tagged GAG (HS-Rhodamine green), DNA (DNA plasmid-Rhodamine red), and HTDP with the following structure: GRKKRRQRRRP-PQC-Texas red. The peptide, which contains 6 arginine and 2 lysine residues, corresponds to the PTD of HIV-Tat. Initial experiments investigated if HTDP could mediate cellular entry of DNA plasmid (approximately 5 kb) and HS (approximately 15 kDa). Chinese hamster ovary (CHO) cells were incubated with polyanion with or without HTDP and analyzed by FACS. Addition of HTDP resulted in a 32- to 43-fold increase in macromolecular polyanion uptake (*cf.* Fig. 1, *A* and *B*). Reciprocal inhibition of HTDP-mediated uptake of HS and DNA was demonstrated, indicating a common uptake mechanism of HTDP/DNA and HTDP/HS complexes (Fig. 1, *C* and *D*).

We next employed confocal fluorescence microscopy to study intracellular routing of peptide-polyanion complexes. At both time points (2 and 24 h), uptake of free HS was below the detection limit (*cf.* Fig. 2, *A* and *F*). HTDP displayed nuclear accumulation already at an early time point (Fig. 2*B*) and was still present at 24 h (Fig. 2*G*). In the presence of HTDP, HS chains initially accumulated in vesicular, cytoplasm-located compartments (Fig. 2*C*) and were then further transported to the nucleus (Fig. 2*H*). Employing a luciferase reporter gene assay it was demonstrated that HTDP caused a dose-dependent and dramatic increase in luciferase activity (up to 3000-fold) as compared with DNA plasmid alone, suggesting that HTDP delivers intact DNA plasmid to the nuclear compartment of recipient cells (results not shown). The polyanion-

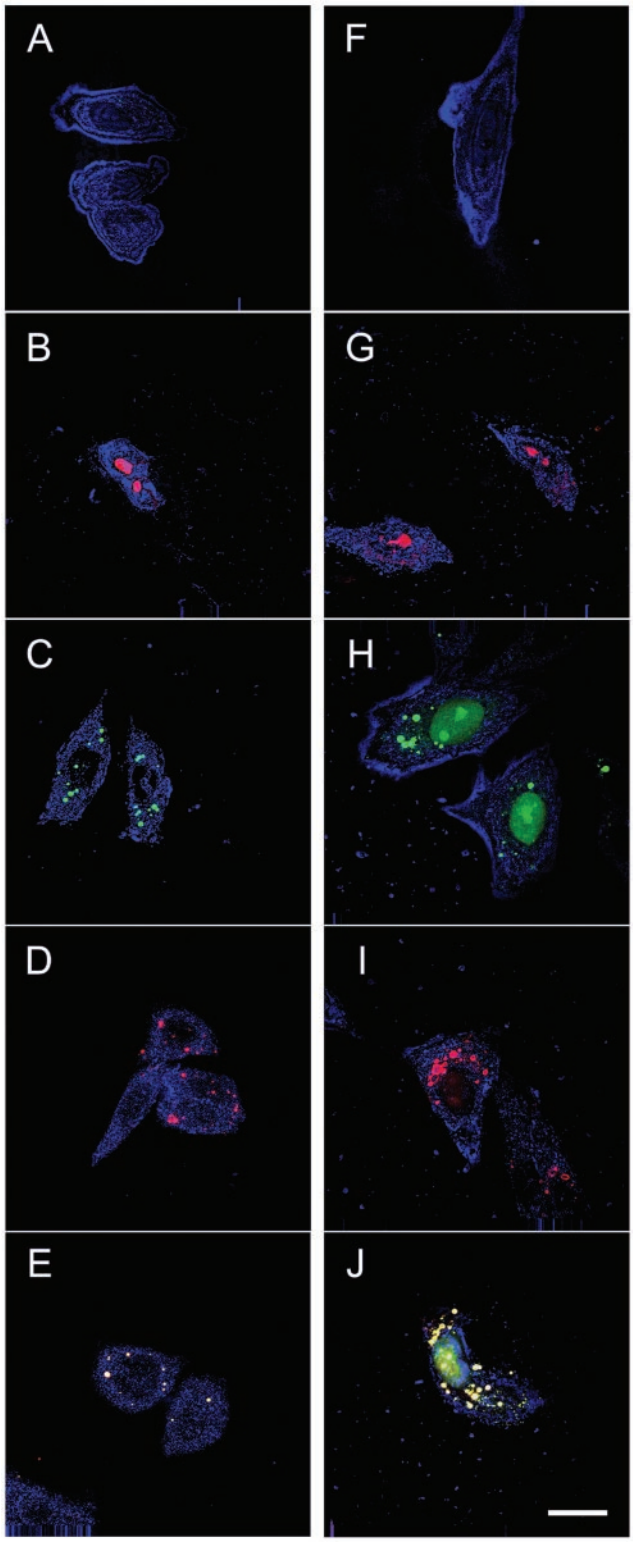



FIG. 2. **Nuclear transfer of macromolecular polyanions by HTDP.** Confocal fluorescence microscopy analysis of wild-type CHO cells incubated with Rhodamine green-labeled HS (*A* and *F*), Texas red-labeled HTDP (*B* and *G*), labeled HS and unlabelled HTDP (*C* and *H*), labeled HTDP and unlabelled HS (*D* and *I*). *Panels E* and *J* represent images of cells incubated with both labeled HTDP and labeled HS. Cells were analyzed after incubation for 2 h (*A–E*) or 24 h (*F–J*). In all cases, 20 $\mu$g/ml and 60 $\mu$g/ml of HS and HTDP, respectively, were used. *Scale bar* indicates 20 $\mu$m.

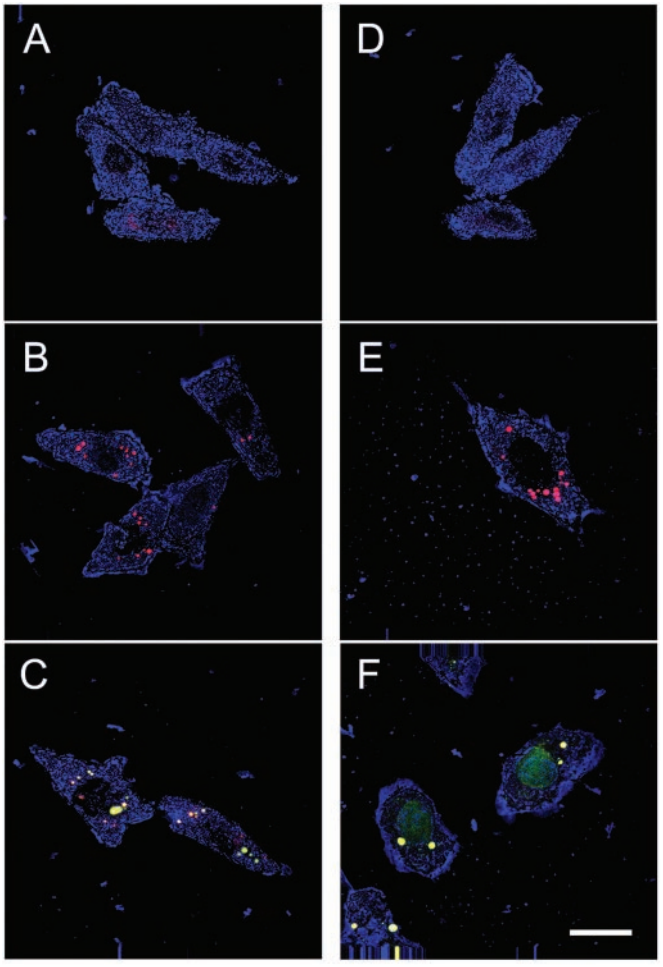



FIG. 3. **HTDP-polyanion complexes accumulate in endolysosomal vesicles.** Wild-type CHO cells were incubated for 2 h (*A–C*) or 24 h (*D–F*) in the presence of Lysotracker alone (*A* and *D*) or Lysotracker, unlabelled HTDP, and unlabelled HS (*B* and *E*) and analyzed by confocal fluorescence microscopy. *Panels C* and *F* represent merged images of cells co-incubated with Lysotracker, HTDP, and Rhodamine green-labeled HS. Lysotracker (1 $\mu$l/ml) was present during the final 2 h of the incubation time. Concentrations of HS and HTDP were 20 $\mu$g/ml and 100 $\mu$g/ml, respectively. *Scale bar* indicates 20 $\mu$m.

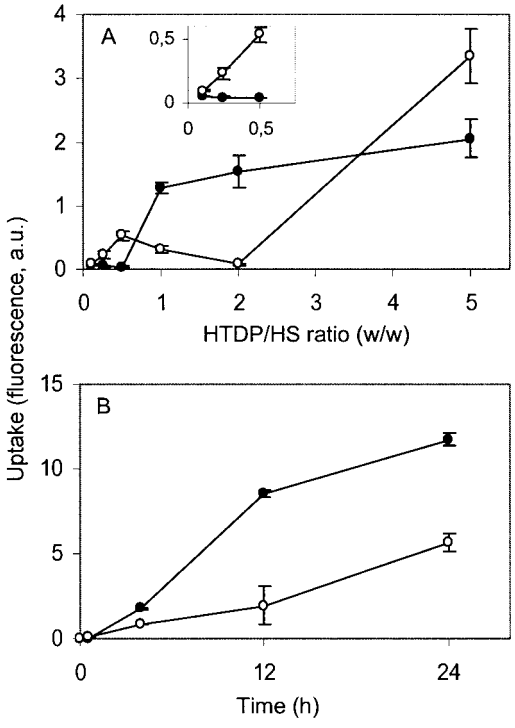



FIG. 4. **HTDP-polyanion complex uptake in wild-type and XT-def CHO cells.** *A*, wild-type (●-●) and XT-def (○-○) CHO cells were incubated with Rhodamine green-labeled HS (20 $\mu$g/ml) and 2, 5, 10, 20, 40, or 100 $\mu$g/ml HTDP. After incubation for 4 h, cell-associated fluorescence was determined by FACS analysis and presented *versus* the ratio HTDP to HS (w/w). *Inset*: uptake of complexes with a low HTDP:HS ratio. *B*, WT (●-●) and XT-def (○-○) cells were incubated with labeled HS (20 $\mu$g/ml) in the presence of HTDP (20 $\mu$g/ml) and analyzed by FACS after 30 min, 4 h, 12 h, and 24 h of incubation.

containing vesicles were dense and displayed considerable size heterogeneity upon inspection of refracted light images, both in CHO cells and human lung fibroblasts (results not shown). Interestingly, in the presence of HS HTDP was redistributed from the nucleus to cytoplasmic vesicles, both at an early (*cf.* Fig. 2, *B* and *D*) and late (*cf.* Fig. 2, *G* and *I*) time point. This was confirmed by merged confocal images, clearly demonstrating co-localization of HTDP and HS in vesicular structures (*yellow* in Fig. 2, *E* and *J*) and at a later time point a predominating signal from HS in the nucleus (*green* in Fig. 2*J*). From these results we conclude that exogenously added HTDP and HS or DNA are co-transferred to cytoplasmic vesicles formed *de novo*, that HTDP targets HS and DNA to the nucleus, and that macromolecular polyanions do not preclude cellular uptake of the HIV-Tat transduction domain, but rather abrogate nuclear localization of the peptide.

To further characterize the nature of cytoplasmic vesicles formed *de novo* upon exposure to HTDP-polyanion complexes, a pH-sensitive probe was employed, *i.e.* a fluorophore that emits light only at acidic conditions. In our hands acidic compartments were diffusely distributed with a faint pH-probe signal in untreated cells (Fig. 3, *A* and *D*). However, upon addition of HTDP-HS complexes large vesicular structures with a strong signal from the pH-probe appeared already at 2 h, indicating rapid acidification of these compartments (Fig. 3*B*). Localization of polyelectrolyte complexes to these acidic compartments, which conceivably correspond to late endosomes/lysosomes, was demonstrated by the merged confocal images (Fig. 3*C*). After 24 h of incubation, localization of polyelectrolyte complexes to acidic vesicles was still evident, and release of HS into the nucleus had begun (*green* in Fig. 3*F*). The formation of lysosomal vesicles was a reversible phenomenon, as indicated by the disappearance of Lysotracker staining upon exit of the polyelectrolyte complexes (results not shown).

*Mechanisms of Macromolecular Plasma Membrane Translocation*—There seems to be no consensus as to whether HIV-Tat and its PTD enter cells via endocytosis or by alternative pathways, as both temperature-dependent (32–33) and -independent (6, 34) uptake have been reported. Employing quantitative FACS methodology, it was revealed that internalization of HTDP-polyanion complexes is a concentration- and time-dependent event (Fig. 4, *A* and *B*, *filled symbols*) and that uptake was insignificant at 4 °C (data not shown). Altogether our results indicate that HTDP mediates DNA and HS uptake into endolysosomal vesicles via a saturable and temperature-dependent pathway, consistent with endocytosis. Similar results were obtained with human fibroblasts, COS cells, and a bladder carcinoma cell line (data not shown).

Based on previous observations, indicating a role for cell-surface PG in cellular transport of polybasic compounds, *e.g.* polyamines (24–25) and full-length HIV-Tat (14), an involvement of PG in HTDP-polyanion complex uptake may be hypothesized. To study the possible role for cell-surface PG in HTDP-mediated macromolecular internalization, the following experiments were performed with wild-type and mutant CHO

TABLE I
*HTPD-polyanion uptake is dependent on CS/DS- and HSPG*

Uptake of Rhodamine green-labeled HS (20 μg/ml) in the presence of HTDP (40 μg/ml) for 4 h was determined in either untreated wild-type cells (Control), untreated PG-deficient mutant cells (pgsB-618), or wild-type cells pre-treated with chondroitin ABC lyase (Ch'ase ABC), or heparitinase (Hep'ase), or 25 mM chlorate (Chlorate), as described under Experimental Procedures.

| | Uptake |
|---|---|
| | *% of control ± S.D., n = 6* |
| Control | 100 |
| PgsB-618[a] | 1.0 ± 0.6 |
| Ch'ase ABC[a] | 63 ± 39 |
| Hep'ase[a] | 59 ± 8.6 |
| Chlorate[a] | 2.4 ± 0.9 |

[a] Statistically different from Control, $P < 0.05$.

cells deficient in xylosyl transferase (XT-def). XT catalyzes the first step in PG assembly, *i.e.* the linkage of xylose to specific serine residues in the core protein. XT-def cells express ~5% PG as compared with wild-type cells (35). As shown in Fig. 4*A* (*inset*), uptake of complexes with a relatively low peptide to polyanion ratio (approx. 1:2 w/w) was favored by PG-deficiency in the recipient cells. Upon increased HTDP to polyanion ratios (approx. 2:1 w/w), complex uptake was reduced to baseline levels in XT-def cells, whereas uptake was dramatically enhanced in wild-type cells (Fig. 4*A*). The inability of PG-deficient cells to internalize HTDP-polyanion complexes under these conditions was independent of mutant genotype as another strain, pgsB-618, that expresses approximately 10% PG as compared with wild-type cells (35), also exhibited insignificant complex uptake (Table I). Furthermore, pretreatment of wild-type cells with chlorate, which inhibits GAG sulfation, resulted in dramatically decreased complex uptake (Table I). Previous studies have described specific binding of full-length HIV-Tat to HS and very limited binding to galactosaminoglycans (11, 14). Interestingly, pretreatment of wild-type cells with either chondroitin ABC lyase to eliminate CS/DSPG or heparitinase to cleave HS chains significantly reduced complex uptake, indicating that HSPG as well as CS/DSPG participate in HTDP-mediated polyanion uptake in CHO cells (Table I). Altogether, the results presented in Fig. 4 and Table I show that the alteration in complex internalization was caused by a reduction of PG rather than any secondary mutations that may have occurred in the mutants.

At even higher HTDP concentrations, complex uptake reached similar levels in wild-type and XT-def cells, suggesting PG-independent transport mechanisms at a high excess of peptide (Fig. 4*A*). Internalized polyelectrolyte complexes seemed to accumulate in intracellular compartments, as cell fluorescence increased almost linearly up to at least 24 h (Fig. 4*B*), which is consistent with the results presented in Figs. 2 and 3. Notably, this is in contrast with uptake of free full-length HIV-Tat that is transduced into cells in a rapid process, achieving maximum intracellular concentration in less than 15 min (7).

*Effects of HTDP and Full-length HIV-Tat on Endogenous PG Turnover*—From the results presented above it may be concluded that endogenous PG regulates cellular entry of HTDP-polyanion complexes. To gain more insight into the possible interplay between HTDP and endogenous PG, cells were metabolically labeled with [$^{35}$S]sulfate, which is predominantly incorporated into sulfated GAG, either in the presence or absence of HTDP. PG was isolated from cell media (Fig. 5*A*) as well as cell extracts (Fig. 5*B*) and analyzed by gel filtration chromatography. In the presence of HTDP (Fig. 5, *A* and *B*, *filled symbols*) redistribution of large secreted PG to large and small cell-associated PG/GAG was demonstrated. We next investigated the effect of endogenous, full-length HIV-Tat on PG

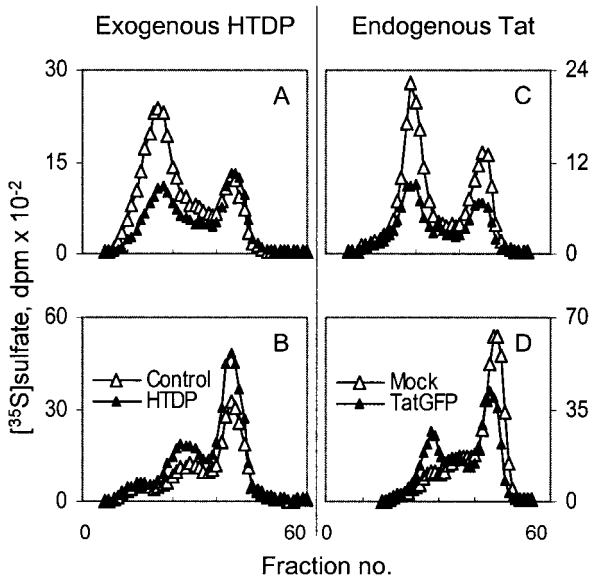



FIG. 5. **Effects of HTDP and HIV-Tat expression on proteoglycan turnover.** *A* and *B*, wild-type CHO cells were metabolically labeled with [$^{35}$S]sulfate as described under "Experimental Procedures," either in the absence (△-△) or presence (▲-▲) of 100 μg/ml HTDP. In (*C* and *D*) wild-type CHO cells were either mock transfected (△-△) or transfected with pcDNA3HIV-TatGFP plasmid (▲-▲) and then [$^{35}$S]sulfate-labeled without further treatments. [$^{35}$S]sulfate-labeled polyanionic material was recovered by ion-exchange chromatography from both the media (*A* and *C*) and cell extracts (*B* and *D*) followed by gel-filtration chromatography on Superose 6. 1-min fractions were collected and analyzed for radioactivity by β-scintillation counting.

turnover. Wild-type cells were either mock-transfected (Fig. 5, *C* and *D*, *open symbols*) or transfected with HIV-TatGFP cDNA (Fig. 5, *C* and *D*, *filled symbols*). TatGFP expression was confirmed by FACS analysis (results not shown). In agreement with the experiments presented above, redistribution of large secreted PG to cell-associated PG was shown in TatGFP-expressing cells. The results presented in Fig. 5 could be explained by direct effects of HTDP/HIV-Tat on PG biosynthesis and/or by co-internalization with medium PG and/or by inhibition of degradation of intracellular PG. In the following experiments, fresh cells were incubated with [$^{35}$S]sulfate-labeled conditioned medium, either in the presence or absence of HTDP. Cell media (Fig. 6, *A* and *B*) and detergent cell extracts (Fig. 6, *C* and *D*) were separately analyzed by ion exchange and gel filtration chromatography. Again, in the presence of HTDP redistribution of large secreted PG to large/small cell-associated PG was demonstrated, indicating co-internalization of HTDP-PG complexes from the extracellular environment. In the absence of HTDP, insignificant amounts of medium PG were internalized (Fig. 6, *C* and *D*, *open symbols*). Further analyses of internalized PG material from HTDP-treated cells (Fig. 6, *C* and *D*, *filled symbols*) demonstrated the almost exclusive existence of CS/DSPG (Fig. 7*A*), further reinforcing the notion that HTDP interacts with this type of GAG. Accordingly, efficient uptake of DS in the presence of HTDP was demonstrated by FACS experiments, as in Fig. 7*B*. Moreover, confocal microscopy experiments demonstrated vesicular accumulation of DS after 2 h of incubation (Fig. 7*C*), which is similar to the results obtained with HS (Fig. 2*C*). However, after 24 h of incubation DS was still retained in vesicular structures (Fig. 7*D*), and no nuclear localization was observed even after 48 h (results not shown).

## DISCUSSION

Here we demonstrate that an HIV-Tat derived peptide targets the macromolecular polyanions DNA and HS to the nu-

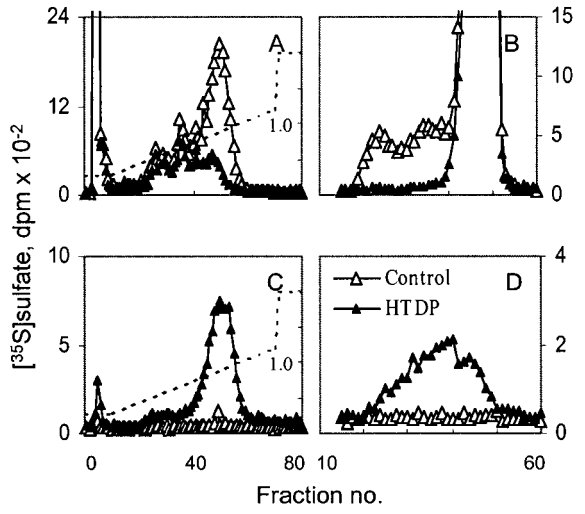



FIG. 6. **HTDP induces intracellular accumulation of secreted proteoglycans.** [$^{35}$S]sulfate-labeled conditioned medium was isolated as described under Experimental Procedures and added to wild-type CHO cells in the presence (▲-▲) or absence (△-△) of 100 $\mu$g/ml HTDP. Cell media (*A* and *B*) and cell extracts (*C* and *D*) were re-isolated and analyzed by either anion-exchange chromatography on Mono-Q (*A* and *C*) or gel filtration chromatography on Superose 6 (*B* and *D*). Radioactivity in collected fractions was determined by $\beta$-scintillation counting. In (*A* and *C*) a linear gradient of 0.3–1.2 M NaCl was applied (*dotted lines*).

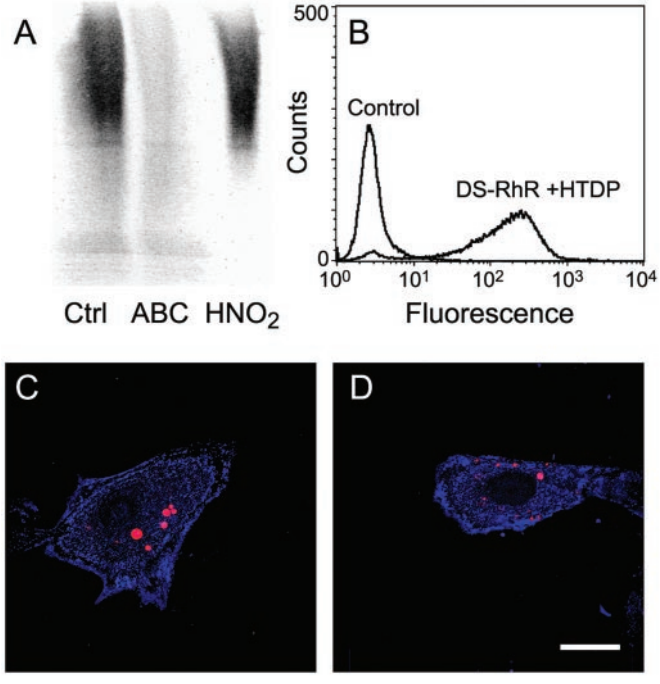



FIG. 7. **Internalization of CS/DS by HTDP.** *A*, re-isolated material corresponding to fractions 40–55 in Fig. 6*C* was further analyzed on a 20–30% gradient PAGE, either untreated (*Control*), or after treatment with chondroitin ABC lyase (*ABC*), or after deaminative cleavage at pH 1.5 (*$HNO_2$*). *B*, FACS analysis of wild-type CHO cells incubated with Rhodamine red-labeled DS (20 $\mu$g/ml), and HTDP (100 $\mu$g/ml) for 4 h. *C* and *D*, confocal fluorescence microscopy analysis of wild-type CHO cells incubated with Rhodamine red-labeled DS (20 $\mu$g/ml) and unlabelled HTDP (100 $\mu$g/ml) for 2 h (*C*) or 24 h (*D*). *Scale bar* indicates 20 $\mu$m.

clear compartment of mammalian cells and that cell-surface PG either counteracts or facilitates uptake, depending on the composition of the peptide-polyanion complexes. To our knowledge this is the first paper to demonstrate that a 14-amino acid peptide from HIV-Tat efficiently delivers macromolecules via electrostatic interactions rather than, as previously shown,

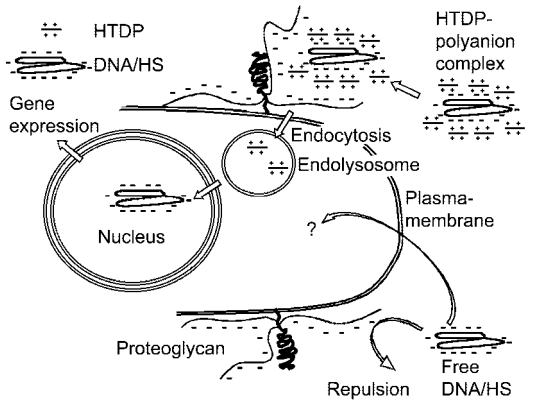



FIG. 8. **Proposed model of the regulatory role for PG in cellular uptake of charged macromolecules.** In the absence of HTDP the polyanions DNA and HS are repulsed by the negatively charged GAG chains of endogenous PG. Accordingly, uptake of free polyanion is favored by PG-deficiency in the recipient cells. However, in the presence of HTDP an electrostatic complex is formed between HTDP and DNA or HS that efficiently traverses the plasma membrane via a PG-dependent and endocytosis-like process. The complexes co-localize to endolysosomal vesicles followed by nuclear transfer of the polyanion.

delivery of covalently attached molecules. Because of their biochemical properties, DNA and GAG do not readily pass plasma membranes. A dramatic shift in HS uptake was demonstrated at an HTDP:HS ratio of ~1:1 (w/w). Assuming an average molecular weight of 15 kDa and negative net charge density of 65/HS chain for the HS preparation used in this study (36) and that HTDP exhibits a net charge of +8, an HTDP:HS ratio of 1:1 (w/w) corresponds to a global charge ratio of approximately +1.4. PG-expressing wild-type cells displayed substantially increased polyanion uptake under such conditions (approximately 40-fold as compared with control), whereas, interestingly, PG-deficient cells (pgsA-745) displayed less uptake as compared with "naked" polyanion. Another PG-deficient mutant (pgsB-618) also displayed dramatically decreased uptake. Moreover, treatment of wild-type cells with either chondroitin ABC lyase or heparitinase significantly reduced Tat/HS complex uptake, indicating that HSPG as well as CS/DSPG are involved.

In the case of naked DNA or HS, as well as with HTDP-polyanion complexes with a global negative charge, internalization was favored by PG-deficiency in the recipient cells, suggesting significant repulsion of negatively charged complexes by PG in wild-type cells. These data strongly support the view that cell-surface PG regulate transfer of macromolecular complexes from the extracellular milieu to the intracellular compartment via electrostatic interactions (see Fig. 8). Several models for PG-mediated processing of extracellular ligands have been suggested (37). The simplest is endocytosis directly by the PG. An alternative model involves initial binding of ligands to PG followed by transfer to other receptor-proteins (*e.g.* lipoprotein receptors) that then mediate ligand internalization (20). Bearing in mind that HS-binding growth factors as well as PG core proteins have been isolated from the nuclear compartment, co-transduction to the nucleus may be postulated (38–39). Recent studies by our group have demonstrated recycling of the glucosylphosphatidylinositol linked cell-surface HSPG glypican-1 and that interference with glypican-1 recycling results in diminished polyamine uptake (Ref. 40 and references therein). Ongoing studies investigate the specific role for recycling glypican-1 in plasma membrane translocation and nuclear targeting of peptide-DNA complexes.

We further demonstrate that HTDP and DNA or HS are co-distributed to large endolysosomal vesicles formed *de novo* followed by dissociation of the complex and nuclear translocation of the polyanion. The first step, *i.e.* passage over the

plasma membrane and vesicular accumulation, was a relatively rapid process (~30 min), whereas nuclear accumulation occurred after 4–24 h. Interestingly, in the presence of HS, HTDP was retained for degradation in endolysosomes and was not further transported to the nuclear compartment as was the case with free HTDP. Thus, HS could both reduce total uptake of HIV-Tat as previously reported (11–14) and preclude further nuclear transfer of internalized Tat as suggested by our results, which would efficiently inhibit Tat-dependent viral gene expression. It has been stated that heparin, *i.e.* over-sulfated HS, is not internalized either in the absence or in the presence of HIV-Tat, thus ruling out the possibility that heparin could interfere with intracellular processing of Tat (12). Other studies (19, 39) have shown that cell-surface HSPG mediates uptake of basic fibroblast growth factor into CHO cells and that the presence of the growth factor altered the distribution of HS degradation products from endosomal/lysosomal compartments to the nucleus. It was suggested that HS fragments accompany the growth factor to the nuclear compartment. From our study it is evident that both DNA and HS accumulate in the nuclear compartment in the presence of the HIV-Tat peptide. Moreover, extracellular addition of HTDP as well as ectopic expression of full-length HIV-Tat caused redistribution of endogenous PG from the medium to the intracellular compartment. Interestingly, endogenous CS/DSPG was involved, and HTDP-mediated membrane passage of this type of GAG could be confirmed by FACS experiments. These results suggest that HTDP binds efficiently to both HS and galactosaminoglycans, as opposed to full-length HIV-Tat that was reported to strictly interact with HS (11). However, as shown in this study, further intracellular routing of DS, which was retained in cytoplasmic vesicles, differed from that of HS and DNA, both of which accumulated in the nucleus, suggesting a certain degree of specificity in nuclear transfer of internalized polyanions.

Tyagi *et al.* (14) suggested a requirement of HSPG for cellular uptake of a GST-Tat-GFP fusion protein. We have demonstrated the ability of a 14-amino acid peptide derived from HIV-Tat to efficiently deliver DNA, HS, and DS into mammalian cells via electrostatic binding and that both CS/DS and HSPG are involved in peptide-polyanion complex uptake, as opposed to uptake of full-length HIV-Tat that specifically required HSPG (14). Importantly, our results suggest that PG either facilitates or inhibits membrane transport, depending on the net charge of the complexes, *i.e.* uptake of naked polyanion was greater in PG-deficient cells than in wild-type cells. Moreover, HTDP uptake was not precluded by GAG, which was the case with uptake of full-length Tat (14). Instead, HTDP was re-distributed from the nuclear compartment to cytoplasmic vesicles in the presence of the polyanion. Using a pH-sensitive fluorophore, we further show that peptide-polyanion complexes accumulate in acidic vesicles and that polyanions are transferred to the nucleus. We have also studied the effects of extraneous HIV-Tat peptide and ectopic expression of full-length HIV-Tat on endogenous PG turnover, providing biochemical evidence for the interplay between HTDP/Tat and endogenous PG in live cells. Our observation that HIV-Tat induces intracellular accumulation of PG may have important implications in AIDS-associated pathologies. Several *in vitro* and *in vivo* models have shown that HIV-Tat exerts cytotoxic effects independently of HIV infection, and that HTDP mediates the deleterious effects (41–43). In some cases, HIV-Tat assembly in affected tissues was accompanied by accumulation of HSPG or the amyloid precursor protein, which is a CSPG (33, 44). From the results presented here it may be postulated that HIV-Tat-PG complexes accumulate in vesicular and nuclear compartments *in vivo*. Future studies should examine possible cytotoxic effects exerted by such aggregates and whether endogenous PG biosynthesis could be manipulated to counteract the cytotoxic effect.

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REFERENCES

1. Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990) *Science* **247,** 1465–1468
2. Hengge, U. R., Walker, P. S., and Vogel, J. C. (1996) *J. Clin. Investig.* **97,** 2911–2916
3. Derossi, D., Chassaing, G., and Prochiantz, A. (1998) *Trends Cell Biol.* **8,** 84–87
4. Hawiger, J. (1999) *Curr. Opin. Chem. Biol.* **3**, 89–94
5. Eguchi, A., Akuta, T., Okuyama, H., Senda, T., Yokoi, H., Inokuchi, H., Fujita, S., Hayakawa, T., Takeda, K., Hasegawa, M., and Nakanishi, M. (2001) *J. Biol. Chem.* **276,** 26204–26210
6. Torchilin, V. P., Rammohan, R., Weissig, V., and Levchenko, T. S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8786–8791
7. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) *Science* **285,** 1569–1572
8. Green, M., and Loewenstein, P. M. (1988) *Cell* **55**, 1179–1188
9. Frankel, A. D., and Pabo, C. O. (1988) *Cell* **55**, 1189–1193
10. Cullen, B. R. (1991) *FASEB J.* **5,** 2361–2368
11. Rusnati, M., Coltrini, D., Oreste, P., Zoppetti, G., Albini, A., Noonan, D., d'Adda di Fagagna, F., Giacca, M., and Presta, M. (1997) *J. Biol. Chem.* **272,** 11313–11320
12. Rusnati, M., Tulipano, G., Urbinati, C., Tanghetti, E., Giuliani, R., Giacca, M., Ciomei, M., Corallini, A., and Presta, M. (1998) *J. Biol. Chem.* **273,** 16027–16037
13. Rusnati, M., Tulipano, G., Spillmann, D., Tanghetti, E., Oreste, P., Zoppetti, G., Giacca, M., and Presta, M. (1999) *J. Biol. Chem.* **274,** 28198–28205
14. Tyagi, M., Rusnati, M., Presta, M., and Giacca, M. (2001) *J. Biol. Chem.* **276,** 3254–3261
15. Kjellén, L., and Lindahl, U. (1991) *Annu. Rev. Biochem.* **60,** 443–475
16. Bernfield, M., Götte, M., Park, P. -W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) *Annu. Rev. Biochem.* **68,** 729–777
17. Esko, J. D., and Selleck, S. B. (2002) *Annu. Rev. Biochem.* **71,** 435–471
18. Park, P. W., Reizes, O., and Bernfield, M. (2000) *J. Biol. Chem.* **275,** 29923–29926
19. Tumova, S., and Bame, K. J. (1997) *J. Biol. Chem.* **272,** 9078–9085
20. Mahley, R. W., and Ji, Z. S. (1999) *J. Lipid Res.* **40,** 1–16
21. van Putten, J. P., and Paul S. M. (1995) *EMBO J.* **14,** 2144–2154
22. Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999) *Cell* **99,** 13–22
23. Belting, M., Havsmark, B., Jönsson, M., Persson, S., and Fransson, L. -Å. (1996) *Glycobiology* **6**, 121–129
24. Belting, M., Persson, S., and Fransson L. -Å. (1999) *Biochem. J.* **338**, 317–323
25. Belting, M., Borsig, L., Fuster, M. M., Brown, J. R., Persson, L., Fransson, L. -Å., and Esko, J. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 371–376
26. Mislick K. A., and Baldeschwieler, J. D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 12349–12354
27. Belting, M., and Petersson, P. (1999) *J. Biol. Chem.* **274,** 19375–19382
28. Belting, M., and Petersson, P. (1999) *Biochem. J.* **342,** 281–286
29. Wiethoff, C. M., Smith, J. G., Koe, G. S., and Middaugh, C. R. (2001) *J. Biol. Chem.* **276,** 32806–32813
30. Cheng F., Petersson P., Arroyo-Yanguas Y., and Westergren-Thorsson, G. (2001) *J. Cell. Biochem.* **83,** 597–606
31. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* **15,** 3943–3950
32. Mann, D. A., and Frankel, A. D. (1991) *EMBO J.* **10,** 1733–1739
33. Liu, Y., Jones, M., Hingtgen, C. M., Bu, G., Laribee, N., Tanzi, R. E., Moir, R. D., Nath, A., and He, J. J. (2000) *Nat. Med.* **6,** 1380–1387
34. Vives, E., Brodin, P., and Lebleu, B. (1997) *J. Biol. Chem.* **272,** 16010–16017
35. Esko, J. D., Stewart, T. E., and Taylor W. H. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 3197–31201
36. Fransson, L. -Å., Sjöberg, I., and Havsmark, B. (1980) *Eur. J. Biochem.* **106,** 59–69
37. Fuki, I. V., Iozzo, R. V., and Williams, K. J. (2000) *J. Biol. Chem.* **275,** 25742–25750
38. Liang, Y., Häring, M., Roughley, P., Margolis, R. K., and Margolis, R. U. (1997) *J. Cell Biol.* **139,** 851–864
39. Tumova, S., Hatch, B. A., Law, D. J., and Bame, K. J. (1999) *Biochem. J.* **337,** 471–481
40. Ding K., Sandgren S., Mani K., Belting M., and Fransson L. -Å. (2001) *J. Biol. Chem.* **276**, 46779–46791
41. Kopp, J. B., Klotman, M. E., Adler, S. H., Bruggeman, L. A., Dickie, P., Marinos, N. J., Eckhaus, M., Bryant, J. L., Notkins, A. L., and Klotman, P. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 1577–1581
42. Sabatier, J. M., Vives, E., Mabrouk, K., Benjouad, A., Rochat, H., Duval, A., Hue, B., and Bahraoui, E. (1991) *J. Virol.* **165,** 961–967
43. Weeks, B. S., Lieberman, D. M., Johnson, B., Roque, E., Green, M., Loewenstein, P., Oldfield, E. H., and Kleinman, H. K. (1995) *J. Neurosci. Res.* **42,** 34–40
44. Giometto, B., An, S. F., Groves, M., Scaravilli, T., Geddes, J. F., Miller, R., Tavolato, B., Beckett, A. A., and Scaravilli, F. (1997) *Ann. Neurol.* **42,** 34–40