Pathway for Polyarginine Entry into Mammalian Cells[†]

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Abstract

Cationic peptides known as protein transduction domains (PTDs) provide a means to deliver molecules into mammalian cells. Here, nonaarginine (R_9), the most efficacious of known PTDs, is used to elucidate the pathway for PTD internalization. Although R_9 is found in the cytosol as well as the nucleolus when cells are fixed, this peptide is observed only in the endocytic vesicles of live cells. Co-localization studies with vesicular markers confirm that PTDs are internalized by endocytosis rather than by crossing the plasma membrane. The inability of R_9 to enter living cells deficient in heparan sulfate (HS) suggests that binding to HS is necessary for PTD internalization. This finding is consistent with the high affinity of R_9 for heparin ($K_d = 109$ nM). Finally, R_9 is shown to promote the leakage of liposomes, but only at high peptide:lipid ratios. These and other data indicate that the PTD-mediated delivery of molecules into live mammalian cells involves: (1) binding to cell-surface HS, (2) uptake by endocytosis, (3) release upon HS degradation, and (4) leakage from endocytic vesicles.

Facilitating the delivery of molecules into mammalian cells is of substantial interest (1–6). Developing the means to do so can generate both insights into cellular function and potential applications in biomedicine. As early as 1965, Ryser noted that mixing polylysine or other cationic macromolecules with proteins greatly increases the uptake of those proteins by cells (7). In 1988, Frankel and Green independently discovered that the RNA-binding HIV[1] TAT protein was capable of crossing lipid bilayers (8,9). The dissection of HIV TAT function has revealed that a small cationic domain, residues 47–57, is responsible for transduction (10). Other cationic, nucleic acid-binding sequences are likewise capable of entering cells (11,12). Peptide-like molecules and even non-peptidyl polycations are capable of crossing lipid bilayers (11,13). These cationic "protein transduction domains" (PTDs) are not only capable of entering cells but also can be used to deliver molecular cargo (14–16), such as proteins, oligonucleotides, and small molecules (1,17–20).

What are the physicochemical characteristics of cationic PTDs? A natural backbone is not necessary for transduction, as D-amino acid (21), peptoid (18), oligocarbamate (22), β-peptide (23,24), and 6-aminocaproic acid (25) analogs are capable of entering cells. In contrast, the positive charge of cationic PTD is essential for transduction, and arginine is preferred over

[1]Abbreviations: CHO, Chinese hamster ovary; CS, condroitin sulfate; GAG, glycosaminoglycan; HIV, human immunodeficiency virus; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; MALDI–TOF, matrix-assisted laser desorption ionization–time-of-flight; PC, phosphatidyl choline; PTD, protein transduction domain; R9, nonaarginine; TAT, transactivator of transcription; TAMRA–R9, 5-(and 6-)carboxytetramethylrhodamine conjugated to nonaarginine.

lysine or histidine (13). Nonaarginine (R_9) is the most efficacious known PTD that is composed of natural L-amino acid residues (18).

Although the use of PTDs as macromolecular delivery devices has received much attention, the mechanism of PTD internalization remains controversial (4). To date, most mechanistic studies have been performed with fixed cells (11,13,22–28). Recently, Johansson, Lebleu, and their coworkers showed that cell fixation alters the transduction of cationic peptides (29,30). Internalization was found to be energy-dependent, and the internalized peptide was observed in the endocytic vesicles of living cells (30). Dervan, Johansson, and their coworkers have reported similar cell-fixation artifacts for the transduction of N-methylpyrrole/N-methylimidazole polyamides (31) and PTD–green fluorescent protein fusions (32).

There have also been conflicting reports on whether PTDs bind to cell-surface glycosaminoglycans (GAGs) (11,27,33,34). The cell surface is coated with polysaccharides such as HS and sialic acid, and these molecules are known to mediate the binding of many proteins to cells (34). GAGs are actively internalized from the cell surface in vesicles (35), and this turnover could allow for the passive uptake of interacting macromolecules. Tyagi has shown that the internalization of HIV TAT protein is dependent on the presence of heparan sulfate proteoglycans (HSPGs) (33). Subsequently, however, Sugiura reported that this constraint does not exist for polyarginine internalization into fixed cells (11).

Here, we examine the internalization of R_9 into both fixed and living cells. We also analyze R_9 internalization into living GAG-deficient Chinese hamster ovary (CHO) cells, and we extend those analyses with both qualitative and quantitative experiments on the binding of R_9 to heparin. Finally, we determine whether R_9 is able to promote the leakage of liposomes. Together, the data reveal provisions for the entry of a cationic PTD into mammalian cells.

EXPERIMENTAL PROCEDURES

Materials

Amino acids for peptide synthesis were from Novabiochem (Darmstadt, Germany). 5-(and 6-) Carboxytetramethylrhodamine (TAMRA), Hoescht 33342, and FM 1-43 were from Molecular Probes (Eugene, OR). Solvents were from Aldrich Chemical (Milwaukee, WI). Egg phosphatidylcholine (PC) and low-molecular weight heparin (M_r 3000) were from Sigma Chemical (St. Louis, MO). All other chemicals were from Fisher Scientific (Pittsburgh, PA) or Aldrich Chemical (Milwaukee, WI), and were used without further purification.

CHO-K1 cells were from the American Type Culture Collection (Manassas, VA). CHO–pgsD-677 cells (36) and CHO–pgsA-745 cells (37) were gifts of R. I. Montgomery. Cell culture medium and supplements were from Life Technologies (Gaithersburg, MD).

Peptide Synthesis

R_9 was synthesized by normal Fmoc-based solid-phase peptide synthesis with an Applied Biosystems Model 432A peptide synthesizer. R_9 was cleaved from the Rink acid resin by incubation for 4 h in trifluoroacetic acid/ethanedithiol/phenol/thioanisole (32:4:3:1) and precipitated in diethylether. R_9 was purified by reversed-phase HPLC on a C4 column using a gradient of water:acetonitrile containing trifluoroacetic acid (0.1% v/v), and analyzed by matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry using a Bruker Biflex III instrument (m/z 1424.97; expected: 1423.69).

Peptide Labeling

R_9 was labeled on resin by incubation for 4 h with TAMRA (4 equivalents) in dimethylformamide containing benzotriazole-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate (4 equivalents) and diisopropylethylamine (8 equivalents). The resin was washed with dimethylformamide and dichloromethane before deprotection as described above. TAMRA–R_9 was purified by reversed-phase HPLC and analyzed by MALDI–TOF mass spectrometry (*m/z* 1837.92; expected: 1837.13).

Liposomal Leakage

Egg phosphatidyl choline liposomes containing carboxyfluorescein (100 mM) were formed as described previously (38). Liposomes of 100-μm diameter were generated by passage (≥10 times) through a mini-extruder (Avanti Polar Lipids). Promotion of vesicular leakage by R_9 was monitored by an increase in fluorescence intensity at 515 nm (excitation: 495 nm) using a QuantaMaster 1 photon-counting fluorometer from Photon Technology International (South Brunswick, NJ). Experiments were performed in 20 mM HEPES–NaOH buffer, pH 7.2. Percent leakage (%L) was calculated as $100 \times (I - I_o)/(I_{detergent} - I_o)$ where I_o is the initial fluorescence intensity and $I_{detergent}$ is the intensity following the addition of Triton X-100 (to 0.1% v/v). Following each experiment, exact phospholipid concentrations were measured by using the method of Ames (39).

Cell Culture

CHO cell lines were cultured in Ham's F-12 medium supplemented with fetal calf serum (to 10% v/v) and antibiotics. Cells were grown in a $CO_2(g)$ (5% v/v) atmosphere at 37 °C.

Peptide Internalization

CHO cells were seeded in 4-well Lab-Tek-II chamber slides (Nalge Nunc International, Naperville, IL) in Ham's F-12 medium. Labeled peptide was added to each slide, which was then incubated at 37 °C. After 60 min, cells were washed (6 times) with PBS containing Ca^{2+} (0.1 g/L) and Mg^{2+} (0.1 g/L). Fixed cells were prepared according to the procedure that we described previously (24). Cellular localization was visualized using an Eclipse E800 fluorescence microscope from Nikon (Tokyo, Japan) equipped with an × 60 or × 100 lens.

Heparin-Affinity Chromatography

The interaction of R_9 and heparin was assessed qualitatively by affinity chromatography. A 1.0-mL HiTrap Heparin HP column from Amersham Pharmacia Biotech (Piscataway, NJ) was equilibrated with 50 mM sodium acetate buffer (pH 5.0). Labeled peptide (~2 mg) was loaded onto the column, and eluted with a linear gradient of NaCl (0–2 M) in the same buffer. Peptide elution was monitored by absorbance at 280 and 535 nm.

Binding of TAMRA–R_9 to Heparin

An increase in the fluoresence of TAMRA–R_9 at 590 nm (excitation: 531 nm) accompanies its binding to soluble heparin. This increase was used to quantitate the affinity of TAMRA–R_9 (1.0 nM) for heparin (M_r 3000) in 20 mM HEPES–NaOH buffer, pH 7.5, using an EnVision multilabel plate reader from PerkinElmer (Wellesley, MA).

RESULTS

R_9 Internalization in CHO Cells

Earlier this year, the intracellular localization of HIV TAT peptide in fixed cells was reported to differ from that in living cells (30). To explore whether this dichotomy is a general feature

of cationic PTDs, TAMRA–R$_9$ internalization was examined in living and fixed CHO-K1 cells. In living cells, TAMRA–R$_9$ was found in what appeared to be vesicles within the cell (Figure 1A). Upon fixing with paraformaldehyde (4% w/v), TAMRA–R$_9$ migrated to the nucleolus and diffused throughout the cytoplasm (Figure 1B). Similar results were observed when cells were fixed with acetone/methanol (1:1) (data not shown).

To confirm that TAMRA–R$_9$ did indeed localize to the vesicles of living cells, the localization of TAMRA–R$_9$ was compared to that of the dyes FM 1-43 (which is a marker for endocytotic vesicles) and Hoescht 33342 (which is a cell-permeable nuclear stain). Both TAMRA–R$_9$ and FM 1-43 appeared in punctate vesicular structures (Figure 2). No TAMRA–R$_9$ was visible in the nucleus of living cells.

R$_9$ Uptake by Glycosaminoglycan-Deficient CHO Cells

The CHO–pgsD-677 and CHO–pgsA-745 cell lines have mutations in the glycosaminoglycan (GAG) biosynthetic pathway. Specifically, CHO–pgsD-677 cells do not produce HS (36), and CHO–pgsA-745 cells produce neither HS nor chondroitin sulfate (CS) (37). TAMRA–R$_9$ was incubated with these mutant cells and wild-type CHO-K1 cells (positive control experiment), and its uptake was examined by fluorescence microscopy. Although TAMRA–R$_9$ was clearly visible in CHO-K1 cells, there was almost no detectable fluorescence from TAMRA–R$_9$ in GAG-deficient cell lines (Figure 3). These data indicate that interaction with GAGs on the cell surface is critical for TAMRA–R$_9$ internalization. Adding exogenous heparin to CHO-K1 cells prior to the addition of TAMRA–R$_9$ leads to results similar to that with the GAG-deficient cell lines (data not shown), presumably because the exogenous heparin competes with cell-surface GAGs for TAMRA–R$_9$.

Binding of TAMRA–R$_9$ to Heparin

The interaction of R$_9$ with heparin (Figure 4A), which mimics the GAGs on the surface of a mammalian cell,[2] was probed in both qualitative and quantitative assays. The qualitative assay used affinity chromatography with immobilized heparin. TAMRA–R$_9$ was found to bind extremely tightly to immobilized heparin, eluting at approximately 1.5 M NaCl in 50 mM sodium acetate buffer, pH 5.0 (Figure 4B). This affinity is comparable to that of the HIV TAT peptide under similar conditions (40).

The affinity of TAMRA–R$_9$ for soluble heparin was quantitated with a direct titration monitored by fluorescence spectroscopy. To determine the value of the equilibrium dissociation constant (K_d), binding data were fitted by nonlinear regression analysis to the equation: $K_d{}^n = [\text{heparin}][\text{TAMRA–R}_9]^n/[\text{heparin} \cdot n\text{TAMRA–R}_9]$ with the program Prism 4 from GraphPad (San Diego, CA). Because heparin (M_r 3000) and TAMRA–R$_9$ (M_r 1836) are essentially homopolymers of similar mass, the value of n is likely to be near unity. For $n = 1$, $K_d = 109 \pm 13$ nM (Figure 4C). Allowing the value of n to vary gave $n = 0.87 \pm 0.05$ and $K_d = 119 \pm 16$ nM, which is not distinguishable from the value of K_d calculated with $n = 1$.

Liposomal Leakage

Some cell-permeating peptides enter mammalian cells by interacting directly with the lipid bilayer (41). The ability of peptides to disrupt membrane integrity can be measured with a liposomal leakage assay (38). Carboxyfluorescein was encapsulated in liposomes at a high concentration (>100 mM), which quenches its intrinsic fluorescence. Upon vesicle disruption, leakage occurs, resulting in an increase in fluorescence. In a negative control experiment, no leakage leakage of carboxyfluorescein from egg PC vesicles was observed in buffer alone (data

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[2]Both HS and heparin are acidic glycosaminoglycans characterized by a repeating unit of α-D-glucosamine linked 1→4 to a uronic (α-L-iduronic or β-D-glucuronic) acid and modified to a variable extent by *O*- and *N*-sulfation, and *N*-acetylation.

not shown). R_9-Induced leakage in a peptide concentration-dependent manner (Figure 5A). This result indicates that R_9 is capable of disrupting the lipid bilayer. Yet, a peptide:lipid molar ratio of nearly 1:1 was required to induce leakage by R_9 (Figure 5B). In a positive control experiment, melittin (which forms pores in lipid bilayers (42)) caused more rapid leakage at 30 nM than did R_9 at 260 nM (data not shown).

DISCUSSION

The efficacy of nascent chemotherapeutics can be limited by their ability to enter mammalian cells (43,15). PTDs can expedite delivery (1–6). Determining the requirements for PTD entry into cells could enable the creation of even better molecular devices to effect internalization, as well as reveal new aspects of cellular biochemistry and biophysics.

Previous work has led to conflicting conclusions regarding the interaction of PTDs with cells. The mechanism by which peptide toxins, such as mellitin and magainin, interact with cells has been studied extensively, and these peptides are known to form pores in the plasma membrane (42). Penetratin, which is a cationic peptide corresponding to the third α-helix of the Antennapedia homeodomain, is capable of crossing membranes without causing vesicle disruption. In the presence of a lipid bilayer, penetratin appears to form an amphipathic α-helix, which facilitates its insertion into the bilayer (44,45). HIV TAT peptide has likewise be found in a helical structure by NMR spectroscopy, suggesting that HIV TAT and perhaps other PTDs could act in a manner similar to penetratin (46).

Early mechanistic studies on the cellular entry of PTDs were performed with fixed cells (11, 13,22–28). Fixation is believed to permeate membranes (30), and allows vesicle-entrapped peptides and proteins to travel to new locations (29,30,32). Likewise, we find that TAMRA– R_9 is found in different cellular locations in fixed and living cells. Specifically, TAMRA–R_9 localizes in the nucleolus and cytoplasm of fixed cells (Figure 1B). In living cells, however, TAMRA–R_9 is found primarily in vesicles (Figures 1A and 2). As a result, our efforts to elucidate the requirements for PTD internalization have focused on living cells.

In living cells, cationic proteins such as HIV TAT protein and some analogues of ribonuclease A (which is a small cationic protein (47)) are thought to bind to anionic carbohydrates on the cell surface (33,48). Moreover, polysaccharides such as heparan sulfate are believed to play important roles in cellular signaling by initiating the binding of certain proteins to their cellular receptors (34,49,50). On the cell surface, heparan sulfate is attached to either transmembrane proteins to form syndecans or GPI-anchored proteins to form glypicans (51,52). The HS side chains of these heparan sulfate proteoglycans (HSPGs) undergo hydrolytic degradation by heparanases *en route* to the lysosome (53).

A number of proteins are known to bind to specific HSPGs. For some of these proteins, HS is believed to act as the receptor for cellular entry (54). We find that the entry of a cationic PTD into living mammalian cells relies on the presence of HSPGs. Specifically, the entry of TAMRA–R_9 into CHO cells that are deficient in HS is decreased greatly relative to that into wild-type cells (Figure 3). Similar results have been observed with the intact HIV-1 TAT protein (54).

HS and heparin are analogous glycosaminoglycans (51,55).[2] Accordingly, heparin has been used as a surrogate for HS in a wide variety of biochemical experiments (56–58). We reasoned that if HSPGs mediate the cellular entry of PTDs, then cationic peptides such as TAMRA– R_9 should have a measurable affinity for heparin. Indeed, we find that TAMRA–R_9 binds strongly to immobilized heparin (Figure 4B), which mimics the HS side chains of cell-surface HSPGs. Moreover, the soluble heparin·TAMRA–R9 complex has a value of K_d near 0.1 μM (Figure 4C), which is similar to that of a typical receptor–ligand interaction.

How does R9 release from HS and escape from endocytic vesicles? HS is cleaved by heparanases, first in vesicles of neutral pH and then in acidic endosomes (34,53,59). HS cleavage would diminish its anion valency and should thus diminish its affinity for R9. We find that free R_9 is capable of disrupting a lipid bilayer and causing a liposome to leak (Figure 5). This leakage is maximal between pH 7.5 and 9.5 (data not shown), suggesting that R_9 could escape from endosomes prior to their acidification. Regardless, R_9-induced liposomal leakage occurs at a peptide:lipid molar ratio that is 10- to 100-fold greater than that for known pore-forming peptides (38). Thus, the disruption of lipid bilayers is likely to play a limited role in transduction at low concentrations of R_9, and a relatively high concentration could be necessary for efficient transduction.

Our findings are consistent with a four-step pathway for the entry of cationic PTDs into the cytoplasm of mammalian cells (Figure 6). First, peptides bind to HSPGs on the cell surface. These peptides are then taken up a cell by heparan sulfate-mediated endocytosis. Once in vesicles, HS is degraded by heparanases, releasing the PTD. Finally, unbound PTD escapes from the vesicles after achieving a concentration high enough to promote vesicular leakage. This pathway bears some analogy to those proposed for the cellular entry of other HS-binding proteins, such as bFGF (60) or follistatin (61). The identity of the rate-limiting step for PTD entry (Figure 6) is not known. Identifying that step could lead to more efficacious PTDs or to transduction agonists.

Acknowledgments

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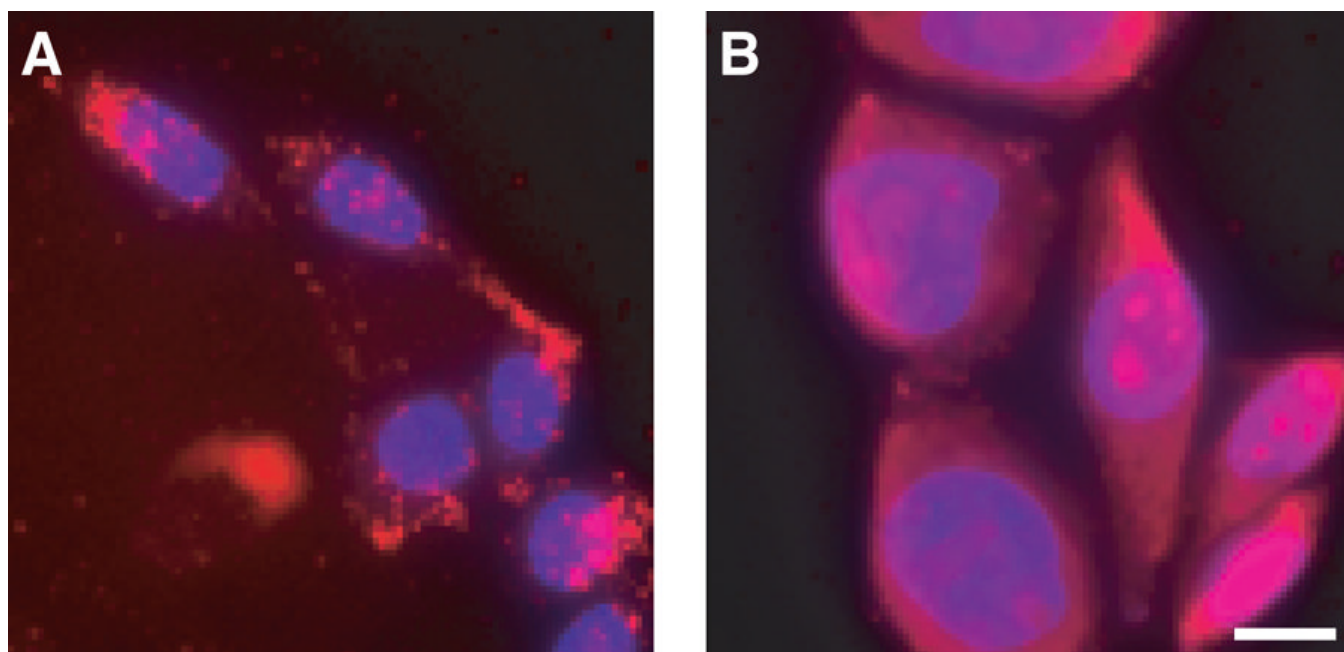
Figure 1.
Localization of TAMRA–R_9 in living and fixed cells. The internalization of TAMRA–R_9 (1.0 μM) was analyzed by fluorescence microscopy. Cells were counterstained with Hoescht 33342. (A) living CHO-K1 cells. (B) CHO-K1 cells fixed with paraformaldehyde. Bar, 10 μm.
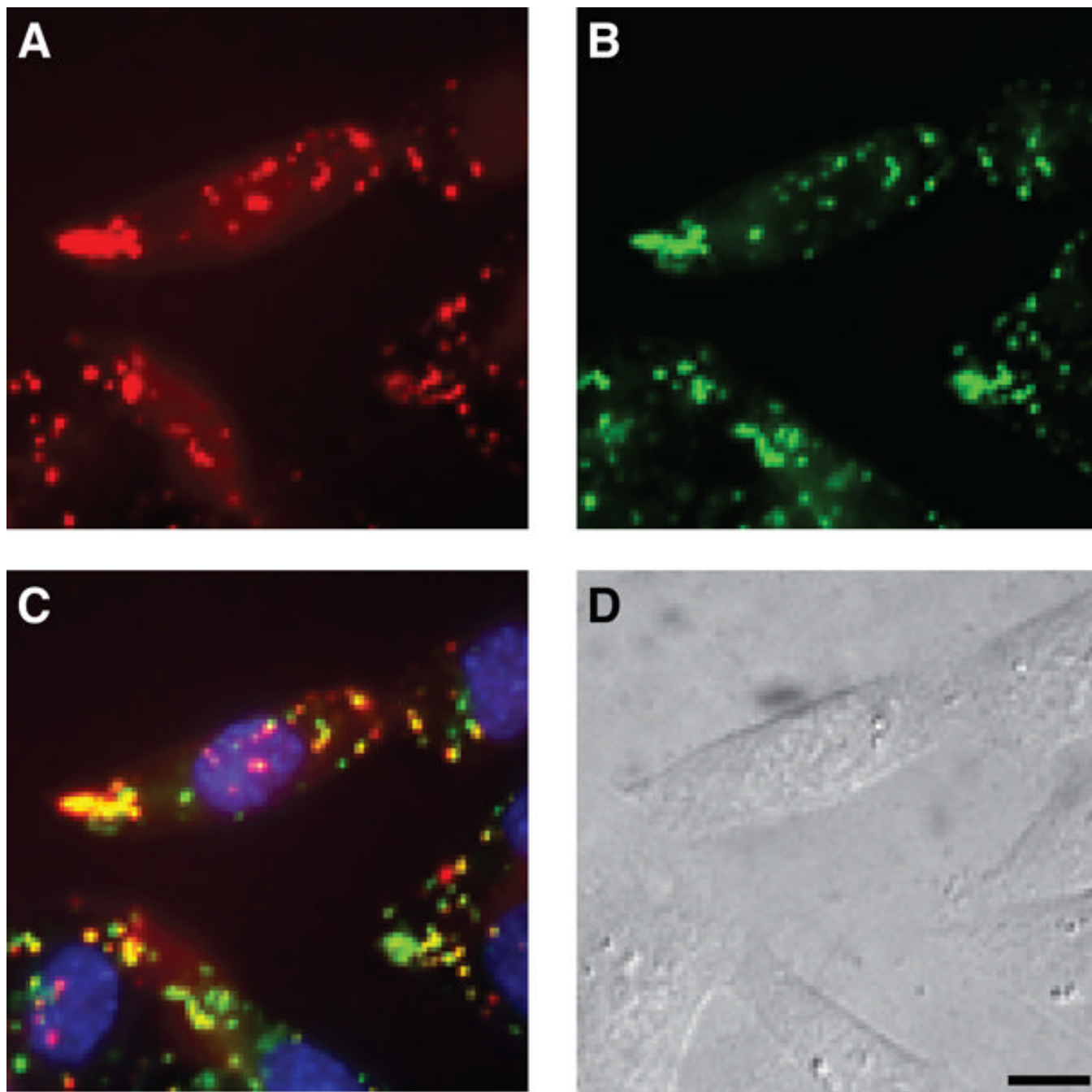
Figure 2.
Co-localization of TAMRA–R_9 in living cells. The internalization of TAMRA–R_9 (1.0 μM) and marker dyes into living CHO-K1 cells was analyzed by fluorescence microscopy. (A) TAMRA–R_9. (B) FM 1-43. (C) TAMRA–R_9, FM 1-43, and Hoescht 33342. (D) Bright-field image of CHO-K1 cells. Bar, 10 μm.
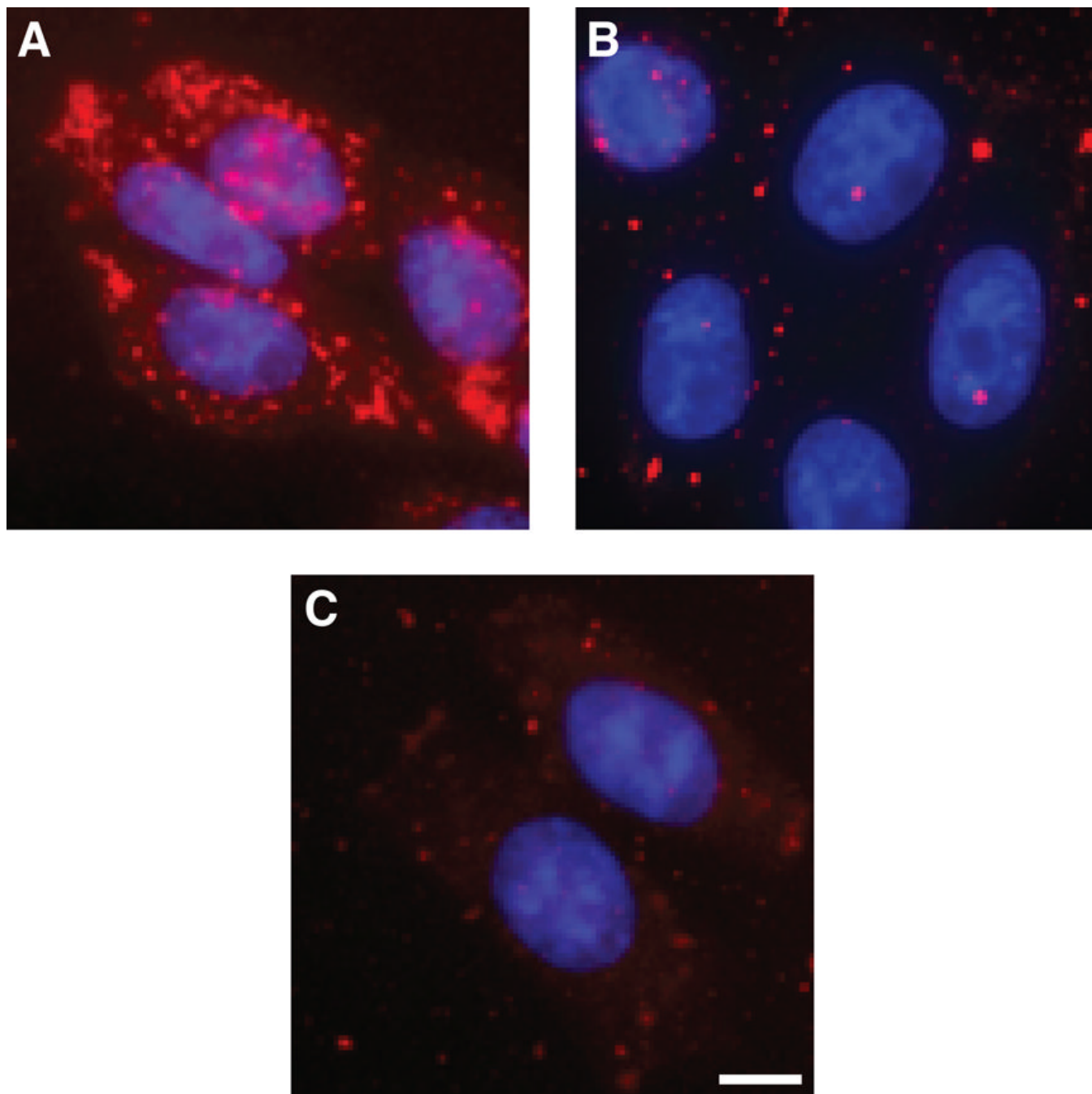
Figure 3.
Dependence of TAMRA–R_9 internalization into living cells on glycosaminoglycans. Internalization of TAMRA–R_9 (1.0 μM) into living wild-type and mutant CHO cells. (A) CHO-K1 cells (wild-type). (B) CHO–pgsD-677 cells (which lack HS). *C*, CHO–pgsA-745 cells (which lack HS and CS). Bar, 10 μm.
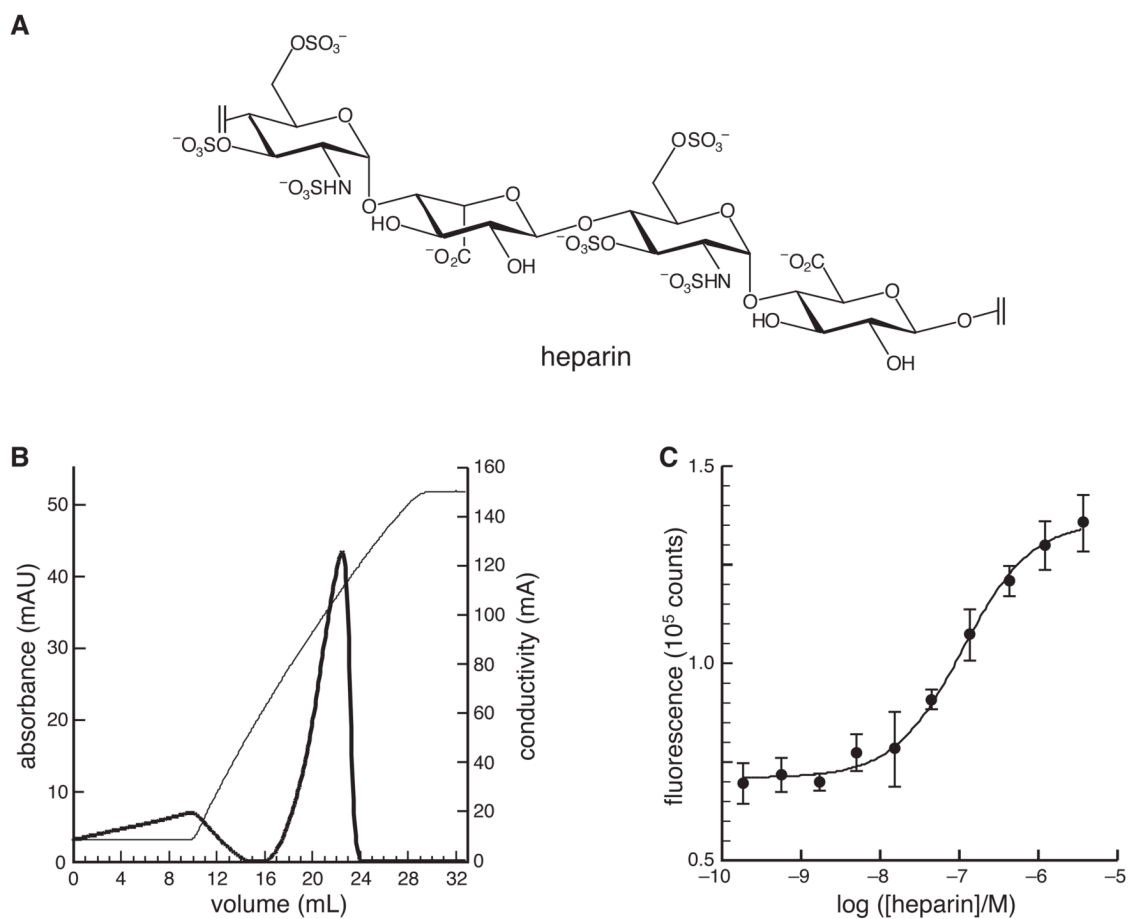
A

B   **C**

Figure 4.
Affinity of TAMRA–R$_9$ for heparin. (A) Prevalent structural motif in heparin.[2] (B) Elution profile from affinity chromatography of TAMRA–R$_9$ on immobilized heparin. TAMRA–R$_9$ was eluted with a linear gradient of NaCl (0–2 M) (conductivity, thin line), and monitored by its absorbance at 280 nm (thick line). (C) Fluorescence titration of TAMRA–R$_9$ (1.0 nM) with heparin (M_r 3000) in 20 mM HEPES–NaOH buffer, pH 7.5. The equilibrium dissociation constant is $K_d = 109 \pm 13$ nM.
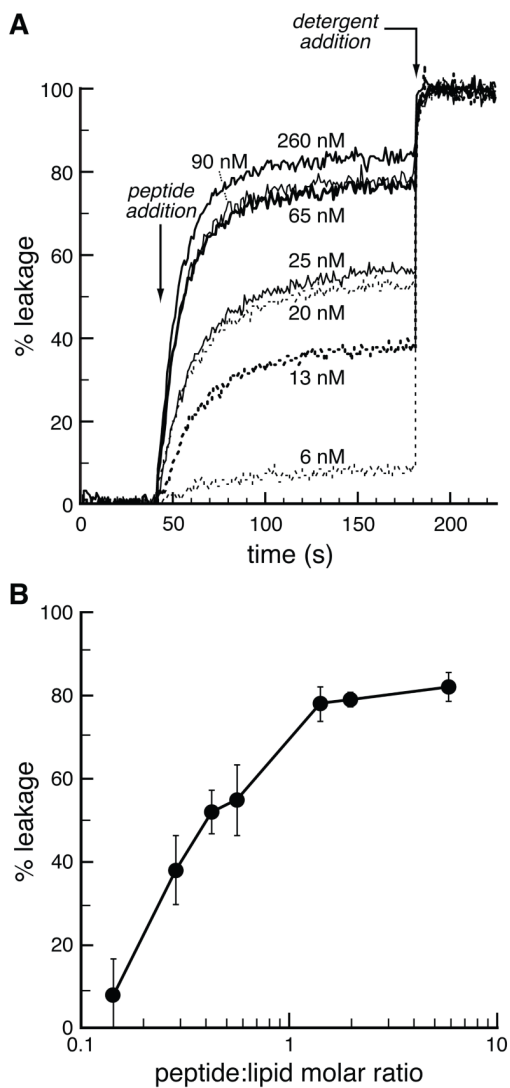
Figure 5.
R_9-Induced leakage of egg PC liposomes. (A) Time course for the leakage of carboxyfluorescein from egg PC liposomes (100-μm diameter) upon exposure to various concentrations of R_9. (B) Leakage of carboxyfluorescein from egg PC liposomes (100-μm diameter) as a function of peptide:lipid molar ratio. Data in panel B are derived from data in panel *A* at 180 s.
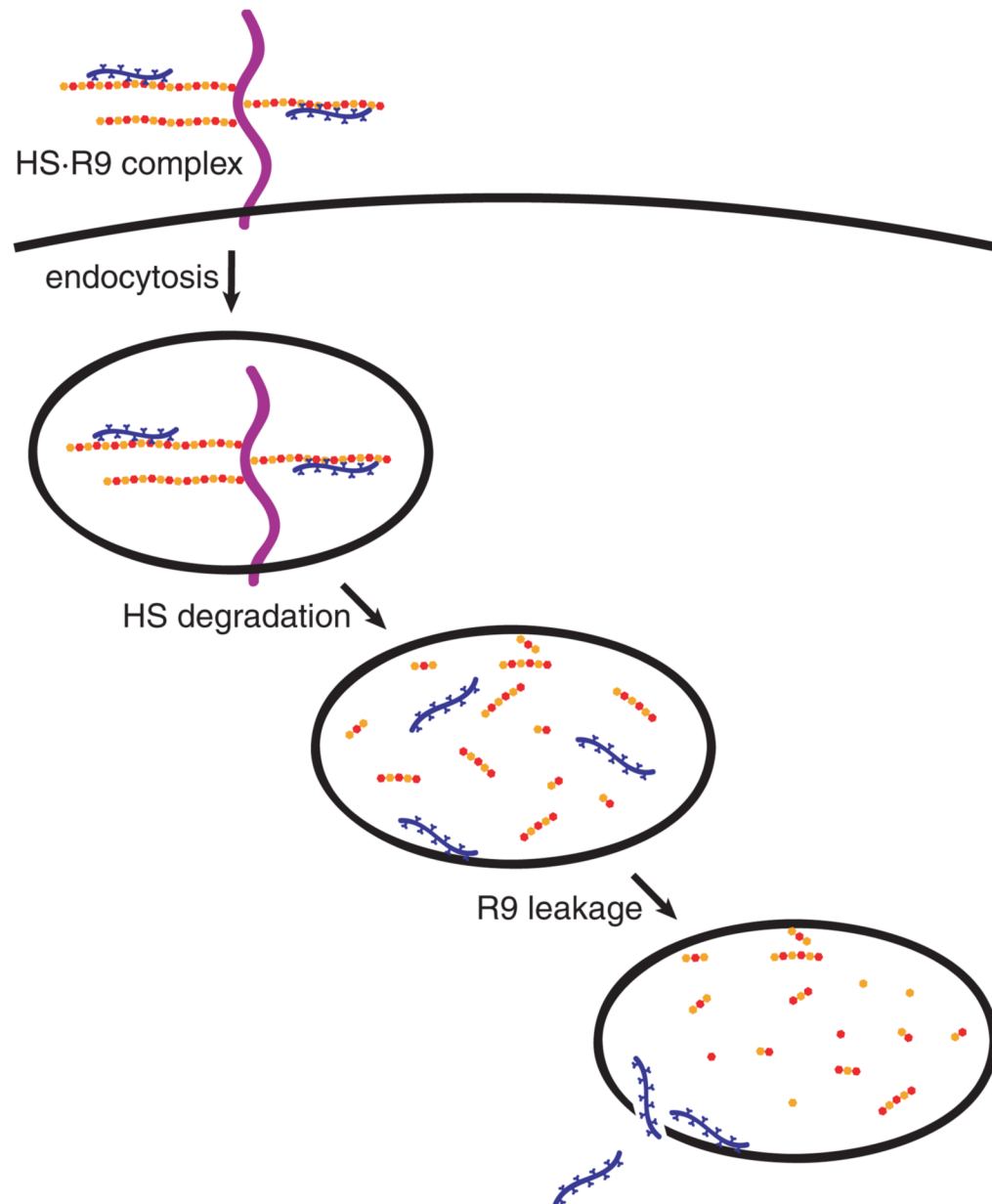
Figure 6.
Pathway for the transduction of R_9 into cells. Cationic PTDs such as R_9 bind to HSPGs on the cell surface. PTDs are internalization by endocytosis. HS is degraded by heparanases. Free PTDs leak from endocytic vesicles and enter the cytosol.