Antennapedia and HIV Transactivator of Transcription (TAT) “Protein Transduction Domains” Promote Endocytosis of High Molecular Weight Cargo upon Binding to Cell Surface Glycosaminoglycans*§

Received for publication, February 19, 2003, and in revised form, June 26, 2003

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Protein transduction domains (PTDs) are short basic peptide sequences present in many cellular and viral proteins that mediate translocation across cellular membranes. PTDs have become widely used as tools for the delivery of high M₉ polypeptides, polynucleotides, or nanoparticles to cells in culture; and even the transfer of cargo molecules to the tissue of live animals has been reported. These cell-permeable peptides are functional when fused in-frame to recombinant polypeptides or when chemically coupled to their cargo. The mechanism responsible for PTD-mediated membrane translocation is controversially discussed and may vary among the various PTDs reported in the literature. Thus direct physical interaction with membrane lipids resulting in vectorial delivery to cells has been proposed for the Antennapedia (Antp) PTD, whereas uptake by the retroviral TAT (transactivator of transcription) protein PTD seems to require cell surface-expressed glycosaminoglycans. The view that PTD-mediated cellular uptake is energy-independent has been dismissed recently as an artifact resulting from fixation of cells. The data reported here agree with and further extend this work. They support the idea that certain PTDs promote cellular uptake via endocytosis and require the expression of negatively charged glycosaminoglycans on the surface of the target cells. Uptake of Antp PTD conjugates or peptide-derivatized liposomes was blocked by heparan sulfate proteoglycans, whereas TAT-mediated uptake was inhibited by both heparin and dextran sulfate. Mutant cells defective for glycosaminoglycan synthesis showed dramatically reduced Antp- or TAT-mediated transmembrane transport confirming the role of these complex polysaccharides in PTD-mediated cellular uptake. The fact that PTDs selectively interact with distinct glycosaminoglycan species has implications for therapeutic applications and may allow targeting of selective tissues that differ in their surface-expressed glycosaminoglycan patterns.

Uptake of charged molecules or high molecular weight compounds into live cells is hampered by the lipophilic nature of the plasma membrane. Thus high amounts of drugs are often required to maintain pharmacologically active concentrations in the target tissue of an organism. The human immunodeficiency virus (HIV)1-encoded transactivator of transcription (TAT) protein rapidly crosses the plasma membrane of live cells (1, 2). More recently, additional cellular and viral proteins with similar features have been described, such as VP22, a herpesvirus protein involved in regulating transcription, and Antennapedia (Antp) a transcription factor discovered first in insect cells. In all of these proteins, the activity of translocating proteins, nucleic acid polymers, or nanoparticles (such as liposomes) to cells (reviewed in Refs. 3–7).

The mechanism responsible for PTD-dependent membrane translocation is controversially discussed and may vary among the various PTDs. In recent papers, it has been proposed that Antp peptide-mediated membrane translocation is an energy-independent process and may be a direct consequence of PTD association with membrane lipids (8, 9). This mechanism would require the formation of transient micelles that are subsequently transferred to the cytoplasmic side of the membrane where cargo molecules are then released. This concept has been questioned in the meantime, and it has been demonstrated that PTD translocation at low temperature is an artifact resulting from the fixation of cells (10, 11). Other data suggest that, at least for the TAT peptide, membrane translocation is mediated by cell surface-expressed heparan sulfate glycosaminoglycans (HPSGs) (12–16). A detailed study of the role of HPSGs in TAT-mediated intracellular transport showed that TAT basic peptide also interferes with the release of newly synthesized HPSGs from cells (17).

Following this line of work, we investigated the uptake mechanism of Antp and TAT PTD protein complexes. The transport to cells by both PTDs occurred only at 37 °C and

* This work was supported by Grant SKL 1220-02-20002 from Schweizerische Krebsliga (to S. C.) and by a grant from Krebsliga Zürich (to C. M.). C. M. was supported by a fellowship from Krebsliga Zürich. S. C. was supported by a fellowship from Krebsliga Schweiz. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. A–C with legends.

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Printed in U.S.A.
**PTD-mediated Cellular Uptake**

Materials—The antibodies for staining lysosomes were G1/139 against human Lamp1 (18) and G1/221 against human transferrin receptor (19). Fluorescent avidin and streptavidin and secondary antibodies were from Southern Biotech (Birmingham, AL). Glycosaminoglycans and dextran sulfate were from Sigma, except for heparin, which was a kind gift from Dr. H. P. Wessel (Hoffman-La Roche). Soybean phosphatidylcholine (SPC) was obtained from L. Meyer (Hamburg, Germany). Cholesterol (Chol) was purchased from Fluka (Buchs, Switzerland), 2-dipalmityloyl-sn-glycerol-3-phosphatidylethanolamine (DPPE) from Sygena (Liestal, Switzerland), and 3,3′-dioctadecylxycarboxyanine perchlorate (DiO) from Molecular Probes (Leiden, The Netherlands). Sulfo-SMCC was purchased from Pierce (Lausanne, Switzerland).

PTD peptide sequences derived from TAT and Antp were synthesized on a Milligen 9050 automated peptide synthesizer (continuous flow), employing the fluorenlymethoxycarbonyl strategy and protocols from our laboratory reported previously (22, 23). After completion of the syntheses, the peptide resin was simultaneously cleaved and deprotected with trifluoroacetic acid/water/ethanedithiol (76:4:20, v/v/v) for 3 h at room temperature, and the crude compounds were purified by reverse-phase medium chromatography on a C18 column (0.1% trifluoroacetic acid-water/acetonitrile gradient). The purity of the peptides was verified by reverse-phase analytical high pressure liquid chromatography, and the identities of the final products were assessed by amino acid and mass spectral analyses (matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF) analyses). The analytical linear gradient was applied over 2.5 min consisting of acetonitrile, 0.09% trifluoroacetic acid and water, 0.1% trifluoroacetic acid from 1:19 to 2:2 on a Chromolith SpeedROD RP-18e column (50 × 4.6 mm), at a flow rate of 4.0 ml/min; detection at 215 nm; single peak at retention time 2.6 min (Antp peptide). Quanitation and mass spectrometry analysis on the peptides revealed the expected amino acid compositions. MALDI-TOF (negative-ion mode) found 2031.5 (calculated 2031.5) for the TAT peptide and 2718.2 (calculated 2718.4) for the Antp peptide.

Internalization of TAT and Antennapedia Peptide Complexes in HeLa Cells—HeLa cells were cultured on coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Internalization of peptide complexed with either fluorescein isothiocyanate (FITC)-labeled avidin or streptavidin was performed as follows. A 1-μM solution of biotinylated peptide was preincubated with 1 μM fluoresecently labeled avidin or streptavidin at room temperature for 30 min with or without inhibitory glycosaminoglycans (50 μg/ml) in Opti-MEM I. Peptide complexes were washed with PBS to wash HeLa cells on coverslips in Opti-MEM I at 4°C for 30 min. The cells were fixed in 3.7% formaldehyde or incubated in fresh Opti-MEM I prewarmed to 37°C and incubated with or without 20 μg/ml poly saccharide at room temperature for 15 min. The cells were incubated with the complex in serum-free Opti-MEM I at 37°C. Complexes were removed, and the cells were cultured in growth medium as described above for 90 min at 37°C. The cells were washed with PBS (0.137 mM NaCl, 2.68 mM KCl, 8.09 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4), detached from the plates with 10 mM EDTA in PBS, washed in PBS, and either used directly or after fixation in 3.7% formaldehyde in PBS for flow cytometric analysis. Internalization of PTD complexes or liposomes was determined in a FACScan flow cytometer (BD Biosciences). 10,000 cells were analyzed per data point, and all experiments were carried out in triplicate.

RESULTS

PTD peptide sequences derived from TAT and Antp were SGRYGRKRRQRCC and SGRQIKIWFQNRRMKWKKC, respectively. They were both chemically synthesized, biotinylated at the N terminus, and carried a C-terminal cysteine residue for chemical coupling to cargo molecules. The Ser-Gly dipeptide at the N terminus was used as a spacer between the peptide and biotin. These peptides were efficiently delivered to the cell and could be detected with FITC-labeled avidin or streptavidin in fixed and permeabilized cells as described earlier (8) (data not shown).

PTD-mediated cellular uptake was next investigated with FITC-labeled avidin or streptavidin conjugated with biotinylated PTD peptide. Intracellular transport of this complex was studied in a variety of cell lines such as HeLa, B16 melanoma, and CHO cells. Complexes were rapidly taken up by all cell types tested. Figs. 1 and 2 show representative data for HeLa cells. FITC-labeled avidin or streptavidin was first preincubated for 15 min with either biotinylated Antp or TAT peptide followed by incubation with cells at 0°C. The labeled material accumulated in small patches on the surface of the cells (Figs. 1A and 2A). FITC-labeled avidin or streptavidin did not associate with cells in the absence of PTD peptide or when bound to a peptide lacking a PTD domain (Figs. 1O, 2O, and data not shown). This indicates that membrane binding was a specific event mediated by PTD peptide.

Cells incubated with FITC-avidin-peptide complexes were then washed and further incubated at 37°C in growth medium to induce internalization. As seen in Figs. 1B and 2B, fluorescently labeled material was internalized within minutes and accumulated in vesicular structures resembling early endosomes. These vesicles partially colocalized with TRITC-labeled dextran added during the chase period at 37°C as a marker for fluid-phase endocytosis (data given in supplemental Fig. A). A marker protein for early endosomes, the transferrin receptor, showed similar localization as avidin-PTD complexes (data given in supplemental Fig. B). After incubation for 1–3 h at 37°C, the material was confined to intracellular vesicles (Figs. 1C and 2C). Antp complexes showed partial colocalization with...
lysosomes (data given in supplemental Fig. B), whereas TAT accumulated in vesicular structures located at the cell periphery (data given in supplemental Fig. C).

To prove that PTD complexes were internalized during the chase period at 37 °C, cells were analyzed by confocal microscopy. Z-sections through cells (Figs. 1 and 2, Z/X and Z/Y) showed that the material was predominantly associated with the extracellular face of the plasma membrane before the chase (Figs. 1D and 2D). Upon extended incubation at 37 °C, both Antp (Fig. 1, F and G) and TAT (Fig. 2, F and G) complexes were transferred to intracellular vesicular structures, whereas after a 10-min chase, most of the material remained on the extracellular surface. Taken together, our data show that both PTDs initially bind to the cell surface in a temperature-independent way where they cluster in small patches on the extracellular side of the plasma membrane. Upon shifting the cells to 37 °C, the material was internalized in intracellular vesicular structures by a mechanism resembling fluid-phase endocytosis.

We next investigated the mechanism responsible for PTD-mediated cellular uptake. Chinese hamster ovary (CHO) cells were incubated with FITC-labeled streptavidin:PTD complex for 90 min at 37 °C, washed, removed from tissue culture plates with EDTA solution, washed again in PBS, and submitted to flow cytometric analysis. Fig. 3A shows that heparin inhibited association with and internalization of TAT complexes by 60–70%, whereas uptake of streptavidin:Antp complex was reduced by 40–50%. Dextran sulfate inhibited uptake of TAT but not of Antp complexes. Other glycosaminoglycans such as chondroitin sulfate A, B, and C and hyaluronic acid had no effect on PTD-mediated cellular uptake (data not shown). No cellular uptake was observed in controls performed with FITC-labeled streptavidin in the absence of PTD peptide (Fig. 3A). It has recently been shown that fixation of cells promotes artifactual
association of PTD peptides with cells (10). To rule this out for our experiments, we analyzed cells incubated with PTD complex before and after fixation by flow cytometry. As shown in Fig. 3, the amount of PTD complex bound to cells and inhibition by heparin was not changed.

Similar experiments were then performed with PTD-derivatized phosphatidylcholine liposomes. The PTD peptides were coupled via a cysteine residue to maleimide derivatized lipids (24). As shown in Fig. 3, uptake of TAT peptide-modified liposomes was increased 10-fold, whereas Antp-derivatized liposomes were internalized 50 times more efficiently than control liposomes modified with cysteine instead of a PTD peptide. The difference in uptake efficiency presumably results from the fact that TAT-derivatized liposomes tend to form large aggregates that are less well internalized. Uptake of liposomes into CHO cells was blocked by heparin and dextran sulfate but not by other glycosaminoglycans such as chondroitin A (Fig. 3B) or chondroitins B and C and hyaluronic acid (data not shown). Taken together, these data suggest that cell surface-expressed glycosaminoglycans, in particular HPSGs, are required for TAT and Antp PTD-mediated cellular uptake of proteins and liposomes. Dextran sulfate polymers and heparan sulfates inhibited uptake by TAT, whereas Antp-dependent protein uptake was blocked only by heparan sulfates. This suggests differential binding of these peptides to negatively charged extracellular glycosaminoglycans and also indicates that TAT and Antp PTDs rely on different oligosaccharides for initial membrane binding. PTD-derivatized liposome uptake was indiscrimi-
nately blocked by inhibitory polysaccharides such as dextran sulfate and heparin.

To corroborate these findings, we investigated PTD uptake into mutant CHO cells deficient in glycosaminoglycan synthesis. Neither Antp nor TAT peptide complexes were efficiently internalized in the mutant cell line (pgs A-745) lacking UDP-N-acetylglucosaminyltransferase and glucuronosyltransferase (21) (Fig. 4). These cells are deficient in the synthesis of all proteoglycans. A second mutant (pgs A-677) expressing reduced amounts of HPSG because of an 10-fold reduction in N-acetylgalactosaminyltransferase showed a reduction in the uptake of Antp but not of TAT-streptavidin complexes. This suggests that the low amount of HPSG expressed by these cells still allowed PTD-mediated cellular uptake.

**DISCUSSION**

The data presented here show that the major route for TAT and Antp peptide-mediated cellular uptake of protein molecules and nanoparticles is endocytosis. Uptake by these PTDs was temperature-dependent, indicative of a step requiring energy. The material that initially accumulated on the cell surface in small patches (a process that was temperature-independent) was then followed by internalization in intracellular vesicular structures. The nature of these patches is unclear; they may represent plasma membrane microdomains such as lipid rafts that are enriched in particular membrane proteins carrying, for instance, HPSG polysaccharide chains. The route and kinetics of uptake are similar to those followed by markers for fluid-phase endocytosis such as fluorescent dextran conjugates.

Uptake of avidin- or streptavidin-PTD complexes prepared with either Antp or TAT peptide was blocked by negatively charged high 

heparin and dextran sulfate. The fact that cells deficient in glycosaminoglycan synthesis showed reduced PTD uptake further supports this notion. Specificity of the various PTDs for different types of glycosaminoglycans may arise from the positive charge distribution in various PTDs; in other words, high affinity interaction with negatively charged sulfated polysaccharides expressed on the cell surface may require proper alignment with the positive charges on a particular PTD. The possibility that PTDs discriminate between different surface-expressed glycosaminoglycans has been discussed earlier for TAT (12–14, 16), and the role that heparan sulfate oligosaccharides play in receptor binding and internalization has also been investigated in other molecules, e.g. decorin (25). Finally, Sandgren et al. (17) recently published work in which they investigated the role of HPSGs in TAT-mediated cellular uptake in detail. Differential interaction of PTDs with glycosaminoglycans might open new possibilities for the delivery of reagents to specific tissues using more selective PTD sequences. Further optimization of PTD peptides might thus improve their translocation efficiency and may even allow the development of tissue-specific PTDs (26).

The exact localization of cell-associated PTD complexes has not always been critically evaluated in earlier work. We therefore analyzed confocal micrographs such as those shown in Figs. 1 and 2 with an image-processing software. Z-sections of confocal data sets show that fluorescent material initially accumulated in patches on the cell surface, in particular at sites of cell-cell contact. As early as 10 min after shifting cells to a high temperature, fluorescent material appeared in intracellular vesicular structures resembling endosomes. Partial colocalization of PTD complexes with a marker for fluid-phase endocytosis further suggests that uptake is through endocytosis, as proposed earlier (1, 2). 1–2 h after incubation at 37 °C, PTD-cargo complexes accumulated in intracellular vesicular structures. In the case of TAT, these vesicles were localized close to the cell periphery, whereas Antp PTD complexes showed perinuclear staining partially overlapping with a lysosomal marker. The route of cell entry and the turnover of TAT-cargo complexes inside cells have also been investigated in detail recently (17). These authors showed that cellular uptake of TAT-PTD complexes was via endocytosis and that the complexes were subsequently targeted to the nucleus. Together with our data, this suggests that TAT and Antp PTDs target their cargo to different cellular organelles.

Cellular uptake by Antp PTD sequences has not been investigated in detail so far. It has been proposed that Antp peptide directly interacts with membrane lipids (6, 9), a finding that we and others have not been able to confirm (27). We cannot exclude the possibility, however, that the exact route of cell entry and the transfer to intracellular vesicles may differ for various PTDs. It will be of crucial importance for the further development of PTD technology to dissect these pathways for all PTD sequences, as each PTD might be destined for a specific cellular compartment.

Liposomes are useful for the delivery of fragile reagents that are rapidly degraded in the bloodstream. The drawback is that the release of the liposomal content into target cells is limited by the slow uptake of these vehicles into cells, and little is known about the mechanism of liposome-mediated drug delivery. It is generally assumed that liposomes release their content upon fusion with intracellular membranes. We show here that uptake of PTD-tagged liposomes is dramatically accelerated when compared with control liposomes. This is in agree-

ment with earlier work where similarly modified liposomes have been shown to be efficiently internalized in cells even at low temperature (28). A detailed analysis of the intracellular localization of liposomes was, however, not performed in this study. Based on the data shown here and on recently published work (10), it is possible that liposomes associated with cells at low temperature (as shown in that study) were actually bound to the cell surface rather than being translocated to cells.

In summary, our study extends recently published work (10, 11, 17) and shows that PTD peptides derived from either HIV Tat or Antennapedia promote cellular uptake of cargo through endocytosis. PTD complexes initially accumulate on the surface of target cells and are subsequently translocated across the plasma membrane upon incubation of cells at 37 °C but not at 0 °C. The earlier proposal that PTD peptides penetrate membranes upon direct interaction with the lipid bilayer, although not formally excluded, seems not to apply for uptake of the high M₀ cargo molecules used here (Ref. 29, and reviewed in Ref. 6).

Acknowledgment—We thank Dr. H. P. Hauri (Biozentrum Basel) for antibodies, Dr. H. P. Wessel (Hoffmann-La Roche, Basel, Switzerland) for heparin samples, R. Wille for technical assistance, and Dr. J. Jiricny for continuous support of our work.

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