Cytoplasmic and Nuclear Delivery of a TAT-derived Peptide and a β-Peptide after Endocytic Uptake into HeLa Cells*

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Terra B. Potocky‡, Anant K. Menon§, and Samuel H. Gellman‡‡

From the Departments of ‡Chemistry and §Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Several highlycationic peptides are able to enter the cytoplasm and nucleus of cells from the extracellular medium. The mechanism of entry is unknown. A number of fluorescence-based studies suggested that these molecules cross the plasma membrane by an energy-independent process, directly gaining access to the cytoplasm. Recent reports have questioned this conclusion, attributing the prior observations to artifacts resulting from fixation procedures used to prepare cells for fluorescence microscopy. These studies analyzed live cells and showed that the peptides entered through endocytosis and accumulated in endocytic vesicles, without necessarily entering the cytoplasm. To resolve this controversy and to extend the analyses to non-natural β-peptide sequences, we studied the cytoplasmic and nuclear delivery of a fluorescein-labeled 9-residue sequence derived from the human immunodeficiency virus transactivator of transcription (TAT) peptide, TAT-(47–57), as well as a similarly labeled 12-residue β-peptide, β-(VRR)₄, in live cells. Using fluorescence confocal microscopy, we show that when added to cells, both peptides are found in endocytic vesicles containing the transferrin receptor as well as in the cytoplasm and nucleus (TAT-(47–57)) or nucleolus (β-(VRR)₄). The cells were verified to be intact through all experimental procedures by demonstrating their ability to exclude propidium iodide. Endocytic entry of the peptides was blocked by the energy poisons sodium azide and 2-deoxy-D-glucose, whereas staining of the nucleus (nucleolus), but not endocytic vesicles, was abrogated by treating the cells with ammonium chloride. Our observations are consistent with the proposal that TAT-(47–57) and β-(VRR)₄ enter cells by endocytosis and then exit an endosomal compartment to enter the cytoplasm by means of a mechanism requiring endosome acidification.

There is widespread interest in the use of protein-derived peptides (1–3), designed peptides (4–7), and oligomers constructed from unnatural building blocks (8–12) as vectors to deliver molecular “cargo” to targets within the cytoplasm and nucleus of eukaryotic cells (13–21). Delivery requires translocation of the peptide across the plasma membrane or an intracellular membrane of a living cell, a behavior that has been ascribed to arginine-rich peptides and related oligomers (22, 23). The prototypical sequences that constitute these so-called protein transduction domains are derived from the human immunodeficiency virus (HIV)¹ transactivator of transcription (TAT) protein, the antennapedia (Antp) homeodomain, and the herpes simplex virus (HSV) type 1 DNA-binding protein VP22, all of which are rich in arginine residues (13, 15, 16). The mechanism by which these highly charged peptides are able to translocate from the extracellular milieu into the cytoplasm and nucleus without breach of the plasma membrane permeability barrier is unknown. One possible translocation mechanism is direct transfer of the peptide across the plasma membrane by means of a reverse micelle, which would ensure that the peptide remains in a hydrophilic environment during membrane transit (1, 13, 15). Although functional studies leave little doubt that TAT and Antp peptides can carry cargo into cells, resulting in a biological effect (23, 24), recent reports have called into question the fluorescence-based methodologies that are commonly used to assess peptide translocation (25–27). These concerns are highlighted in a study by Richard et al. (26) that describes artifacts in analyses based on fluorescence microscopy of fixed cells and fluorescence-activated cell sorting; both of these techniques are widely used to characterize translocation of fluorescently labeled peptides.

Fluorescence microscopy studies of peptide translocation into the cytoplasm and nucleus have commonly involved exposing cells to the fluorescently tagged peptide of interest, washing to remove residual peptide, fixing the cells, and examining them via confocal fluorescence microscopy to determine intracellular peptide distribution (1, 2, 4). Richard et al. (26) studied fluorescently labeled TAT-(48–60) (GRKKRRQRRRPP) and arginine nonamer (R₉) by this method and found that the observed translocation of the peptides to the nucleus results from a fixation artifact. In live cells that were shown to be impermeable to propidium iodide, TAT-(48–60) and R₉ accumulated in vesicles containing known endocytic markers such as transferrin receptor and the lipophilic dye FM 4–64 but showed no fluorescence in the cytoplasm or nucleus (26). Studies conducted at low temperature or after depletion of cellular ATP with azide and 2-deoxy-D-glucose showed no evidence of uptake, implying that the entry process is energy-dependent, as expected for endocytosis. Similar conclusions of endocytic uptake have been reported for peptide cargo conjugates such as TAT-green fluorescent protein (28) and complexes such as fluorescein isothiocyanate-Avidin/TAT (29). No cytoplasmic or nuclear staining was observed in either case. It has also been demonstrated that TAT-(44–57) and Antp-(43–58) are not able to translocate across liposomal membranes, providing further evidence that transfection involves endocytosis.

1 The abbreviations used are: HIV, human immunodeficiency virus; TAT, transactivator of transcription; Antp, antennapedia; Flu, fluorescein; PBS, phosphate buffered saline; PI, propidium iodide; TR-TF, Texas Red-labeled transferrin, HBS, Hepes-buffered saline; TRF, transferrin receptor.
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evidence for an energy-dependent pathway of internalization (30–32). Endocytic uptake of Antp [45–58] and SynB5, an analogue of protegrin 1, has also been reported [31]. These peptides were also unable to enter liposomes. A recent critical review of the literature supports an endocytic mechanism of basic peptide uptake [24]. In contrast, a report from Thorén et al. [27] contains somewhat different conclusions. This work showed that analogues of TAT [48–60] and hepta-arginine containing one tryptophan residue (designated TAT PS6W and R,W, respectively) were taken up into live PC-12 and Chinese hamster ovary cells. However, unlike other reports, [26, 28, 29], Thorén et al. [27] observed weak fluorescence in the cytoplasm and nucleus of cells after incubation with these peptides. In addition, use of low temperature or depletion of cellular ATP with rotenone and 2-deoxy-D-glucose slowed but did not stop uptake, which led the authors to conclude that uptake occurs at least in part via an energy-independent process, i.e. a process other than endocytosis. Zaro and Shen [33] have used a cell fractionation protocol to try to distinguish peptide entry via endocytosis from direct entry in the cytoplasm across the plasma membrane (the latter process is designated “transduction”). These authors concluded that the majority of TAT-[47–57] enters Chinese hamster ovary cells via transduction rather than endocytosis.

Here we report fluorescence microscopy studies that extend recently reported conclusions [26, 28–30] in several important dimensions. Working with the TAT-[47–57] sequence, we show that fluorescently labeled peptide can gain access to the cytoplasm and ultimately the nucleus in this way. The integrity of the plasma membrane permeability barrier was confirmed throughout our experimental procedures by demonstrating the ability of the cells to exclude propidium iodide. In addition, we show that a basic β-amino acid oligomer (“β-peptide”) displays comparable or better uptake behavior than TAT itself. For both TAT-[47–57] and β-(VRR)4, experiments with ammonium chloride suggest that acidification plays an essential role in escape from endosomes to the cytoplasm and nucleus/nucleolus.

MATERIALS AND METHODS

Synthesis of Fmoc-β Amino Acid Monomers—Fmoc-protected β-HArg(PMC)-OH and β-Val-OH were synthesized via Arndt-Eistert homologation [52] using a modified procedure described by Muller et al. [53]. Fmoc-protected α-amino acids were obtained commercially.

Peptide Synthesis and Labeling with Fluorescein (Flu)—Synthesis of Flu-α-TAT and Flu-β-(VRR)4 were performed on an Applied Biosystems Model 432A Synergy peptide synthesizer using the standard Fmoc solid-phase strategy with O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) activation and 1-hydroxybenzotriazole (HOBT) additive. Two-hour coupling times and extended deprotections were employed to synthesize β-(VRR)4. A β-homoglycine residue was incorporated at the N terminus of the peptide; the free N-terminal was then conjugated to 6-carboxyfluorescein manually using N,N'-dicyclohexylcarbodiimide. After a 12-h coupling, the resin was washed with 3× N,N-dimethylformamide. A 3× methylene chloride, and 3× diethyl ether, and the peptide was then cleaved from resin using a solution of 95% trifluoroacetic acid, 5% thioanisole, and 2.5% ethanethiol. Peptides were purified by preparative reverse-phase high pressure liquid chromatography and characterized by matrix-assisted laser desorption ionization time-of-flight analysis. The purified peptides were lyophilized and resuspended in deionized distilled water. Peptide concentrations were determined by UV-visible spectroscopy at 494 nm. Peptide stock solutions were stored frozen at –70 °C.

Cell Culture—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified incubator containing 5% CO₂ (gas).

Confocal Microscopy—HeLa cells that had been grown on 90-mm plates to sub-confluence were dissociated from the plates by treatment with Trypsin/EDTA for 15 min at 37 °C. An aliquot of 1 × 10⁵ cells was plated on 30-mm glass-bottom culture dishes (MatTek Corp.) and cultured overnight in Dulbecco’s modified Eagle’s medium to allow the cells to adhere. The medium was removed and the cells were washed with phosphate buffered saline (PBS), pH 7.3. The cells were then incubated for 15 min at 37 °C with 1 ml Opti-MEM containing 7 μm peptide (Flu-TAT or Flu-β-(VRR)4) and 8 μg/ml propidium iodide (PI). The medium was again discarded, and the cells were washed 3 × 5 min with 2 ml PBS. Each wash solution contained 8 μg/ml PI. PBS (2 ml) containing 8 μg/ml PI was added to the cells, and the cells were then placed on ice. The cells were then viewed by confocal microscopy using a Bio-Rad MRC-1024 laser scanning confocal microscope.

Co-localization Studies—Cells, grown and plated as described above, were incubated for 15 min at 37 °C with 1 ml of Opti-MEM containing 1.5 μm or 7 μM Flu-TAT and Flu-β-(VRR)4, 8 μg/ml PI, and either 25 μg/ml Texas Red labeled transferrin (TR-Tf) or 1.25 μM Syto-60. The cells were washed after the incubation period and placed on ice as above.

Fluorescence Bleed-through Controls—Control cells that had been incubated with only peptide or only the costain (TR-Tf or Syto-60) were used to determine appropriate microscope parameters that would prevent bleed-through among the far-red, red, and green fluorescence channels. These parameters were used to view cells that had been incubated with peptide, PI, and the desired costain.

Energy Poison Studies—Cells were plated at a density of 1 × 10⁵ cells/well in glass-bottom plates as described above, then incubated with 1 ml of glucose-free HEPES buffered saline (HBS) (pH 7.4) containing 50 mM 2-deoxy-D-glucose and 10 mM sodium azide (NaN₃) for 30 min at 37 °C. Peptide (7 μM), 25 μg/ml TR-Tf, and 8 μg/ml PI were added to the HBS solution. The cells were incubated for an additional 15 min at 37 °C, washed with 3 × 2 ml of glucose-free HBS containing 2-deoxy-D-glucose and NaN₃, and viewed by confocal microscopy. Treated cells were compared with mock-treated cells that had been incubated with HBS that did not contain NaN₃ or 2-deoxy-D-glucose.

Ammonium Chloride Studies—Cells plated to a density of 1 × 10⁵ cells/well in glass-bottom plates were incubated with 1 ml of Opti-MEM containing 50 mM NH₄Cl for 30 min at 37 °C (34, 35). Peptide (7 μM) was added, and the cells were incubated for 15 min at 37 °C, washed with 3 × 2 ml of PBS containing 50 mM NH₄Cl, and viewed by confocal fluorescence microscopy. Mock-treated cells that had been incubated with Opti-MEM that did not contain NH₄Cl were viewed in parallel. The difference in uptake efficiencies in the presence and absence of NH₄Cl was determined by cell counting of nuclear staining.

RESULTS

The peptides used in this study are shown in Fig. 1. Flu-TAT is a fluorescein-labeled, 9-amino acid sequence corresponding to residues 47–57 (YGRKKRRQRRR) of HIV TAT; Flu-β-(VRR)₄ is a fluorescein-labeled 12-residue β-peptide. HeLa cells were incubated with Flu-TAT and Flu-β-(VRR)₄ for 15 min at 37 °C, washed, and examined by confocal fluorescence microscopy. All assays were conducted in small glass-bottom dishes, thus allowing the cells to be viewed in buffered solution instead of in mounted preparations. PI (8 μg/ml) was included during the incubation with peptide, as well as in all wash buffers, to establish cell viability. Because PI is membrane impermeant it cannot enter viable cells; non-viable cells with a compromised plasma membrane are readily detected because PI enters these cells and fluoresces strongly upon binding to nucleic acid. All of the data described in this paper are derived from an examination of live cells, i.e. those that excluded PI. Fig. 2A illustrates uptake of Flu-TAT into the cytoplasm and nucleus of a live cell; the fluorescence image also shows a non-viable cell displaying PI staining of the nucleus.

Flu-TAT Is Distributed in Endocytic Vesicles as Well as in the Nucleus and Cytoplasm.—When 7 μM Flu-TAT was incubated with HeLa cells for 15 min at 37 °C, the majority of the cells took up the peptide. A small fraction of cells (5–10%) showed no detectable fluorescence. Three distinct fluorescence patterns were observed (Fig. 2B). Some cells showed only punctate fluorescence (Fig. 2B, left panel), whereas others showed a combination of punctate fluorescence and more diffuse fluorescence in the cytoplasm and nucleus (Fig. 2B, center panel). A third group of cells showed only cytoplasmic and nuclear staining.
with little or no punctate fluorescence (Fig. 2B, right panel). The TAT peptide did not localize to nucleoli as reported in previous studies on fixed cells (3), but instead showed a diffuse nuclear staining that tended to exclude nucleoli. Upon incubation with a lower concentration of Flu-TAT (1.5 μM), the majority of cells showed only punctate fluorescence as in Fig. 2B, left panel. At 7 μM peptide, ~50% of the cells showed nuclear fluorescence (resembling the images in Fig. 2B, center and right panels), whereas the remaining cells showed only a punctate distribution with a faint, diffuse fluorescence background (as in Fig. 2B, left panel).

To test whether the punctate distribution of Flu-TAT was due to endocytosis of the peptide, we performed a double-labeling experiment using a TR-Tf conjugate (Molecular Probes) and Flu-TAT (Fig. 2C). TR-Tf binds to the cell surface transferrin receptor (TfR) and is taken up by receptor-mediated endocytosis, thus providing a well characterized marker for the endocytic pathway (36). Prior to imaging double-labeled cells, fluorescence images were taken of cells incubated with Flu-TAT or TR-Tf alone to determine the appropriate microscope parameters to minimize fluorescence bleed-through (Fig. 2D). Fig. 2C shows the top and equatorial views of cellular distribution of Flu-TAT (Fig. 2C, green, left panel), TR-Tf (Fig. 2C, red, center panel), and an electronically merged image (Fig. 2C, right panel) showing the overlap (yellow) between the Flu-TAT and TR-Tf distributions. The merged image clearly shows that there is considerable overlap between the punctate structures seen in the Flu-TAT image and the endocytic vesicles defined by TR-Tf, but that there are also TR-Tf-positive vesicles that do not contain Flu-TAT and a few Flu-TAT positive vesicles that do not contain TR-Tf. This result is consistent with other reports of endocytic uptake of the TAT peptide (26, 28). The TR-Tf-positive, Flu-TAT-negative structures may correspond to endosomes committed to the recycling pathway that is responsible for returning TfR to the cell surface (36).

**Intracellular Localization of β-(VRR)₄ to Endocytic Vesicles, Cytoplasm, and Nucleoli**—Cells incubated with 7 μM Flu-β-(VRR)₄ showed three distinct patterns of fluorescence, varying from endocytic uptake to diffuse cytoplasmic and nuclear staining, with over 75% of cells showing cytoplasmic and nuclear staining (Fig. 3A). About 5–10% of cells showed no detectable fluorescence, as also seen with Flu-TAT, whereas a few cells showed strong surface labeling (Fig. 3A, right panel). Unlike Flu-TAT which shows a diffuse nuclear staining (Fig. 2B, middle and right panels), Flu-β-(VRR)₄ accumulated in nucleoli (Fig. 3A, three right panels), which was supported by its ability to co-stain with Syto 60, a far-red nuclear stain (Fig. 3B).

Co-localization studies with the endocytic marker TR-Tf (Fig. 3C) showed that Flu-β-(VRR)₄ shares endosomal compartments with TfR (merged images in Fig. 3B, right panels) while enjoying a larger cytoplasmic and nucleolar distribution (Fig. 3, B and C) from which TR-Tf is excluded. We conclude that Flu-β-(VRR)₄, like Flu-TAT, enters the endocytic pathway in live HeLa cells and also gains access to the cytoplasm and nucleus.

**Entry of Flu-TAT and Flu-β-(VRR)₄ into Cells Is Blocked by Energy Poisons**—Cells were treated with sodium azide and deoxyglucose to determine whether translocation of Flu-TAT and Flu-β-(VRR)₄ is energy-dependent. Incubation of cells with 10 mM sodium azide and 50 mM 2-deoxyglucose completely blocked entry of Flu-TAT and Flu-β-(VRR)₄ into endocytic vesicles as well as into the nucleocytoplasmic compartment (Fig. 4A). The energy-dependent endocytic uptake of TR-Tf was also blocked under these conditions (data not shown), establishing the effectiveness of our ATP-depletion protocol.

**Flu-TAT and Flu-β-(VRR)₄ Are Endocytosed but Unable to Access the Nucleus in Cells Treated with Ammonium Chloride**—The distribution of peptides into endosomal and nucleocytoplasmic compartments that we observed may represent two independent internalization events. Alternatively, because peptide entry into both the endocytic and nucleocytoplasmic compartments of the cell is blocked in energy-poisoned cells, it is possible that endocytosis is a necessary prelude to nucleocytoplasmic delivery. Thus peptides may be endocytosed and then escape the endosomal vesicles to enter the cytoplasm. To investigate this idea, we tested whether endosome acidification influences delivery of peptides to the nucleocytoplasmic compartment. Cells were incubated in the presence of 50 mM NH₄Cl, a weak base that increases the pH of acidic organelles, including endocytic vesicles (35). This procedure is often used to block the cytoplasmic delivery of viruses and toxins that use the endocytic pathway to gain access to the cytoplasm (34, 37, 38). After incubation with NH₄Cl, Flu-TAT and Flu-β-(VRR)₄ were found only in endocytic vesicles. No nuclear staining was observed, consistent with the possibility that endosome acidification may be necessary for these two cationic peptides to enter the cytoplasm and nucleus. The ammonium chloride-treated cells showed a faint diffuse fluorescence background in addition to the distinct punctate fluorescence pattern characteristic of endosomes. This diffuse fluorescence may be due to a small amount of peptide within the cytoplasm, although we consider this possibility unlikely because no fluorescence was detected in the nucleus. Also, similar “diffuse” fluorescence is often seen in images of TR-Tf-treated cells, suggesting that this “background” is a result of out-of-focus fluorescence. This background could also be attributed to fluorescence from the surface of the well, as these cationic peptides have a high affinity for glass. Although endosome-only morphologies are evident in cells incubated with 7 μM peptide without prior ammonium chloride treatment (Fig. 2B, left panel), they represent a minor fraction of the cells; the majority of cells (50% in the case of Tat and 75% in the case of β-(VRR)₄, Fig. 4C) display peptide fluorescence in the cytoplasm and nucleus, as well as in endosomes (Fig. 2B, center and right panels; Fig. 3C). In contrast, only 1% of the ammonium chloride-treated

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**Fig. 1. Structure of Flu-TAT-(47–57) and Flu-β-(VRR)₄.** A, structure of Flu-TAT-(47–57). B, structure of Flu-β-(VRR)₄.
cells displayed peptide in the nucleus/nucleolus upon treatment with the same peptide concentration (Fig. 4C) as determined by cell counting.

**DISCUSSION**

Our data for Tat and β-(VRR)$_4$ support the model for peptide entry shown in Fig. 5. We propose that Flu-TAT and Flu-β-(VRR)$_4$ bind to the cell surface and are taken up into the cells by endocytosis. Cell surface binding, as suggested by others (24, 39–41), may be caused, in part, by ionic interactions between the positively charged peptides and negatively charged cell surface heparan sulfate proteoglycans. Examples of cell surface-bound peptide are shown in Fig. 3, A and B. Evidence for endocytic uptake of peptides presented in this paper includes observation of the characteristic TR-Tf-positive punctate endocytic structures occupied by the Flu-peptides (Figs. 2C and 3C), as well as data indicating that all uptake is blocked when cells are treated with sodium azide and 2-deoxyglucose to reduce ATP levels (Fig. 4A). These results are in accordance with previous studies of TAT-(48–60) (26) and of TAT conjugated to cargo (28, 29). However, unlike previous confocal studies on TAT-derived peptides, our data also clearly show that the fluorescein-labeled peptides can access not only TR-Tf-containing endosomal compartments but also the cytoplasm and nucleus (in the case of Flu-TAT) or nucleolus (in the case of Flu-β-(VRR)$_4$) in live cells. Treatment of cells with ammonium chloride blocks entry into the nuclear compartment without blocking endocytic uptake (Fig. 4B). We explain this effect by proposing that peptides enter the cytoplasm by translocating across endosomal membranes (Fig. 5) by means of an unknown mechanism that requires endosome acidification. Alternatively, it is possible that peptide transport into endosomes and into the cytoplasm and nucleus result from two independent processes, with the latter representing direct ATP-dependent translocation across the plasma membrane, and that only this latter process is inhibited by ammonium chloride. We favor the former hypothesis over the latter, however, because it is more consistent with the known effect of ammonium chloride on a variety of membrane transport processes, including entry of viral capsids and bacterial toxins into the cytoplasm (34, 38, 42, 43).

Our proposal differs from other explanations of translocation behavior (24, 26, 28, 29, 31) because we propose movement of internalized peptides from endosomes to the cytoplasm, and because of the necessity of acidification for this movement. Our image of the same two cells, one of which is stained with propidium iodide. Right, transmission image of the two cells. Bar, 10 μm. B, HeLa cells were incubated with 7 μM Flu-TAT (47–57) and 8 μg/ml propidium iodide for 15 min at 37 °C. Left, image of double fluorescence, indicating endocytic uptake. Center, punctate and diffuse cytoplasmic and nuclear fluorescence. Right, cytoplasmic and nuclear fluorescence (note that the nucleus appears to exclude fluorescein and can be seen as a dark spot in the brightly stained nucleus). Bar, 10 μm. C, HeLa cells were incubated with 7 μM Flu-TAT and 25 μg/ml TR-Tf for 15 min at 37 °C. Top row, images taken near the top of the cell; bottom row, same cell visualized at the equatorial plane. Left, green fluorescent image shows internalization of Flu-TAT. Center, red fluorescent image shows internalization of Texas-Red transferrin. Right, merged image of green and red fluorescent images showing co-localization of Flu-TAT with TR-Tf. Bar, 10 μm. D, bleed-through controls were performed to determine the appropriate microscope parameters for co-localization studies of Flu-TAT and TR-Tf. All images were taken using the same microscope parameters. Upper panel, left, green fluorescent image of a HeLa cell incubated with 7 μM Tat for 15 min at 37 °C; center, red fluorescent image of the same cell at levels that prevent bleed-through; right, transmission image of cell. Lower panels, left, green fluorescent image of a cell incubated with 25 μg/ml TR-Tf for 15 min at 37 °C; center, red fluorescent image of the cell, showing transferrin uptake; right, transmission image of cell. Bar, 10 μm.
data show that this explanation applies to the TAT sequence as well as to a guanidinium-rich oligomer with an unnatural backbone. The details of the model are open to testing using standard molecular cell biological approaches, especially through the use of genetic rather than chemical or pharmacological blocks of the various proposed steps.
Other recent studies suggest that basic peptides can access the cytoplasm and nucleus of live cells but by means of a process other than endocytosis (27, 33). The data presented here show that Flu-TAT-(47–57) (also Flu-TAT-(48–60), data not shown) and Flu-β-(VRR)₄ accumulate in the cytoplasm and nucleus of live HeLa cells. The inability of Richard et al. (26) to detect labeled TAT-(48–60) in the nucleocytoplasmic compartment could have arisen because these authors used a lower peptide concentration (1 μM versus 7 μM used here; cell densities in the studies were similar). Indeed, in our hands, incubation of cells with 1.5 μM TAT-(47–57) or TAT-(48–60) showed a mainly endocytic uptake profile as in Fig. 2B, left panel. Cell fractionation studies by Zaro and Shen (33) show that the TAT peptide gains access to the cytoplasm. These authors attributed this cytoplasmic presence to direct transduction of peptide across the cell membrane. An alternative explanation, consistent with our model, is that the peptides arrive in the cytoplasm via endosomal escape.

The concentration dependence of nucleocytoplasmic uptake of Flu-TAT and Flu-β-(VRR)₄ is interesting. It is possible that, at lower concentrations, there is insufficient accumulation of peptide in the cytoplasm or nucleus to be detected by confocal fluorescence microscopy. However, we feel this explanation is unlikely because the nucleus/nucleolus is effectively a negatively charged trap in which cationic peptide accumulation should be easily detected. An alternate possibility is that the peptide must reach a critical (threshold) concentration within the endosome for escape to occur. Further studies are needed to test this hypothesis.

The transport model that we propose (Fig. 5) indicates that peptides translocate across the endosomal membrane instead of the plasma membrane. Rather than attributing this behavior to compositional differences between endosomal membranes and the plasma membrane, we suggest that peptide translocation critically depends on the low pH environment provided by the endosomal compartment. This conclusion is supported by our finding that peptides are unable to enter the nucleus of ammonium chloride-treated cells (Fig. 4). Treatment with ammonium chloride is known to increase the pH of endosomal compartments (35) and affect the cytoplasmic entry of viral capsids (34, 38) and bacterial toxins (42–44). For example, in the case of diphtheria toxin, current models suggest that the low pH of endosomes causes a conformational change in the protein generating a membrane-active, translocation-competent species (42–44). Thus it is possible that low pH activates an as yet unknown peptide translocation cofactor or somehow promotes the formation of peptide conformations or aggregates that are membrane active. Our hypothesis that peptides move into the cytoplasm from an endosomal compartment rather than across the plasma membrane makes it possible to consider peptide translocation models requiring pore formation or other forms of membrane disruption, because these processes could occur in an intracellular compartment without necessarily affecting cell viability.

We are interested in β-peptides with cell permeation ability because β-amino acid oligomers are resistant to proteolytic degradation (45) and form well defined secondary structures (46–50). β-peptide secondary structure can be specified via monomer selection (50), and appropriately designed β-peptides form stable secondary structures at shorter oligomer lengths than do conventional peptides, i.e. oligomers of α-amino acids. Therefore, β-peptides should be valuable for determining whether the three-dimensional arrangement of guanidinium side chains exerts a significant effect on translocation activity. Conventional α-peptides are too flexible to provide incisive information on this point (51). The studies reported here rep-

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An attempt was made to conduct live cell studies as part of the previous study (10), but subsequent experiments have revealed that the conditions employed led to rapid cell death. Rueping et al. (11) have reported cell entry by a β-homoarginine oligomer, and Garcia-Echeverria and Ruetz (12) have reported entry by β-homolysine oligomers.
resent a first step toward these objectives. Our data show that the β-peptide Flu-β-(VRK)4 behaves similarly to Flu-TAT, with the intriguing difference that Flu-β-(VRK)4 accumulates in nucleoli, whereas TAT accumulates in the nucleus and seems to be excluded from nucleoli. The basis for this variation in distribution is currently unknown, but could potentially be exploited for the specific delivery of molecular cargo to domains within the nucleus.

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