Mechanism of Uptake of C105Y, a Novel Cell-penetrating Peptide*

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C105Y, a synthetic peptide (CSIPPEVFKNPFVYLI) based on the amino acid sequence corresponding to residues 359–374 of α1-antitrypsin, enhances gene expression from DNA nanoparticles. To investigate how this enhancement occurs, C105Y was fluorescently labeled to study its uptake and intracellular trafficking. When human hepatoma cells (HuH7) were incubated with fluorescently labeled C105Y for as little as 3 min, C105Y displayed nuclear and cytoplasmic staining with enrichment of fluorescent signal in the nucleus and nucleolus. Uptake and nucleolar localization were observed with the short sequence PFVYLI, but not with SIPPEVKF, and the D-isomer was readily taken up into cells but not into the nucleus. We found that the C105Y peptide is routed to the nucleolus very rapidly in an energy-dependent fashion, whereas membrane translocation and nuclear localization are energy-independent. When we tested the involvement of known endocytosis pathways in uptake and trafficking of this peptide, we demonstrated that C105Y peptide is internalized by a clathrin- and caveolin-independent pathway, although lipid raft-mediated endocytosis may play a role in peptide intracellular trafficking. Efficient energy-independent cell entry with rapid nuclear localization probably accounts for enhancement of gene expression from inclusion of C105Y into DNA nanoparticles.

Cell-penetrating peptides (CPP) are short peptide sequences that are rapidly internalized by cells through a receptor-independent process. These peptides can be categorized into three subgroups according to their origin (1–3). The first group is composed of CPPs derived from natural proteins, such as the human immunodeficiency virus, type 1 transactivator of transcription (TAT) and Antennapedia (Antp) proteins that were able to rapidly translocate across the cell membrane when added to cells in culture (4, 5). Specific amino acid sequences were identified within these proteins that appeared to mediate their cellular uptake. When these peptides were slightly modified from their naturally occurring sequences, they gave rise to the second class of CPPs. These CPPs include transportan, a synthetic peptide adapted from the neuropeptide galanin (6), and SynB, a peptide derived from the antimicrobial protein protegrin (7). By screening random phage display libraries for their ability to induce translocation, a third class of CPPs has been identified, which needs further characterization (2). Although CPPs are derived from a variety of sources, they have some common features. Some peptides are α-helical in structure, and some contain arginine or lysine rich motifs, whereas others have a hydrophobic core sequence. It appears that these motifs are important in mediating the translocation of CPPs across cell membranes. For example, the hydrophilic arginine residues within TAT appear to mediate its cell penetrating properties, whereas the hydrophobic core of penetratin is necessary to mediate its entry into cells (2, 8).

Proteins, peptides, and small molecules can enter cells through a variety of mechanisms. For example, small molecules such as amino acids, sugars, and ions can traverse the plasma membrane through pumps or channels. Macromolecules, however, must be transported into the cell by endocytosis. Endocytosis occurs by multiple mechanisms that fall into two distinct categories. Phagocytosis involves the uptake of large particles and is usually restricted to specialized mammalian cells, such as macrophages and neutrophiles. Pinocytosis occurs in all cell types and involves the uptake of fluids and solutes. Pinocytosis is mediated by four identified mechanisms: macrophagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-, caveolae-, and dynamin-independent endocytosis (9). It is not fully understood how CPPs enter cells, but the process seems to differ from peptide to peptide. In general, cells rapidly internalize most CPPs in an energy-independent manner, and their uptake is unaffected by most inhibitors of classical endocytic pathways. Furthermore, it does not appear that these peptides are internalized by a receptor-mediated process, because identical transduction properties are observed for their D-isomers (1). These data suggest that many CPPs enter cells by a non-classical endocytic pathway. Recently published data suggest that some CPPs enter cells by fluid phase macrophagocytosis, a specialized form of endocytosis that is independent of caveolae, clathrin, and dynamin (10, 11). Consequently, it appears that some peptides may use a distinct mechanism of translocation or that they have the ability to enter cells through alternative pathways (12). Such a pathway has been suggested for penetratin, which enters cells not by an endocytic pathway but by translocation across lipid bilayers (13).

CPPs have tremendous potential in biotechnology because of their ability to increase uptake and/or nuclear targeting of biologically active proteins or molecules by cells. CPPs may be able to deliver a wide variety of therapeutic compounds. A variety of cargoes have been successfully linked to the protein transduction domains of CPPs, including small and intermediate molecules, peptides, full-length proteins and liposomes (14). To achieve this potential, it will be important to understand how CPPs cross a biological membrane and localize to specific intracellular compartments.

Work in our laboratory has focused on developing efficient ways to deliver DNA into cells for gene therapy. We have used poly-L-lysine (polyK) condensed DNA complexes to effectively deliver DNA to cells.

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2 The abbreviations used are: CPP, cell-penetrating peptide(s); polyK, poly-L-lysine; α1-AT, α1-antitrypsin; sec-R, serpin enzyme complex receptor; EGF, enhanced green fluorescent protein; AF, Alexa Fluor; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; FCS, fetal calf serum; HTE, human trachea epithelia; FITC, fluorescein isothiocyanate; Cav, caveolin; MijCD, methyl-β-cyclodextrin.
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both in vitro and in vivo. To target and increase cellular uptake of these complexes, we covalently attached a small peptide ligand, called C105Y, to polyK before DNA condensation. This peptide was initially identified as an amino acid sequence within the C-terminal tail of α1-antitrypsin (α1-AT) that could mediate binding to the serpin enzyme complex receptor (sec-R). Presumably, when α1-AT binds to elastase, it undergoes conformational change, exposing this peptide sequence, which is then available to its receptor to promote cellular uptake of the α1-AT-elastase complex (15). This receptor was characterized mainly by biochemical means as a cell surface binding site on human hepatoma (HepG2) cells and blood monocytes and, as of yet, has not been cloned. The synthetic peptide ligand C105Y, based on amino acids 346–374 of α1-AT, competed with the natural ligand for binding to the sec-R and provided gene transfer into cells when it was conjugated polyK-DNA complexes (16, 17). These particles, which average 25 nm in diameter by electron microscope, can provide gene transfer of the cystic fibrosis transmembrane regulator gene in vivo to airway epithelial cells from cystic fibrosis transmembrane regulator-deficient mice. This gene transfer is sufficient to partially correct the chloride transport defect and to reverse the down-regulation of NOS-2 that is also a characteristic of cystic fibrosis mouse nasal epithelium (18). Other studies in mice show significant enhancement of gene transfer in the lung, liver, and spleen by inclusion of C105Y in the complex (19). In vitro studies show that the enhancement depends on the density of ligand substitution as well as chain length of the poly-l-lysine. In these studies C105Y increased both the intensity and the duration of reporter gene expression (20, 21).

To determine why conjugating C105Y peptide to polyK condensed DNA complexes increased the efficacy of gene transfer, we investigated the mechanism by which this peptide enters cells. C105Y by itself could penetrate the cell membrane rapidly, enter the cytoplasm, and localize to the nucleus and nucleolus of live HuH7 cells. In this study we describe the mechanism by which C105Y enters cells and travels to the nucleus. Our data suggest that C105Y does not undergo receptor binding but rather is a novel cell-penetrating peptide.

MATERIALS AND METHODS

Constructs and Reagents—The constructs encoding for EGFP and EGFP-DIII (Eps15 mutant) were a gift from Alexandre Benmerah (Faculté Necker-Enfants Malades, Paris, France). Alexa Fluor (AF)-488 conjugated transferrin (used at 50 μg/ml), Hoechst 33258 (used at 50 ng/ml), FM1–43X and FM4–64 (used at 5 μg/ml), and AF-594 labeling reagent were obtained from Molecular Probes (Eugene, OR). Antibodies caveolin-1 (used at 1:1000), caveolin-2 (1:200), actin (used at 1:200), and TRITC-labeled nucleolin (used at 1:100) were obtained from Santa Cruz Biotechnology. Sephadex-G25 columns were obtained from Amersham Biosciences.

Peptide Synthesis and Labeling—Fluorescein-labeled peptides and unlabeled C105Y were synthesized by solid phase method, purified, and subjected to high pressure liquid chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy by BIO-WORLD (Dublin, OH) and the Cleveland Clinic Foundation (Cleveland, OH), respectively. The peptides were dissolved in 5% Me2SO and PBS, and the stocks were periodically tested by matrix-assisted laser desorption ionization for peptide integrity. To attach AF-594 to unlabeled C105Y, peptide was dissolved in 0.1 M sodium bicarbonate buffer, pH 8.3, and incubated with AF-594 labeling reagent for 1 h at room temperature. The conjugate was separated from untreated labeling reagent using Sephadex G-25 columns.

Cells and Cell Culture—Human Hepatoma cells (HuH7) were maintained as previously described in RPMI medium (17) with 10% fetal calf serum (FCS). HuH7 cells were previously shown to bind the sec-R ligand C105Y (17). Fresh medium was added every second day. C6 and C6-Cav-1 glioma cells were provided by Bryan L. Roth (Case Western Reserve University) and were maintained in RPMI medium containing 10% FCS alone or 10% FCS and 0.6 mg/ml G418, respectively (22). 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS. Primary HTE cells were isolated from human tracheas, grown on filters in an air liquid interface, and maintained in medium containing Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium in a 1:1 ratio substituted with 2% Ultrase G.

Stable Cell Line Generation—Stable cell lines HuH7-GFP and HuH7-ΔEps15 expressing EGFP or EGFP-DIII, respectively, were generated by transfecting 70% confluent HuH7 cells grown on standard six-well tissue culture plates with 1.5 μg/well EGFP or EGFP-DIII DNA combined with FuGENE. DNA plasmids encoding EGFP or EGFP-DIII were given to us by Dr. Benmerah from (Faculté Necker-Enfants Malades) (23). Forty-eight hours after transfection, the cells were washed and supplemented with media containing G418 (0.65 mg/ml) as the selectable marker. The surviving colonies were examined for GFP expression by fluorescence microscopy and grown to confluence before further passages.

Peptide Uptake and Colocalization Studies—For experiments on peptide uptake, HuH7, HuH7-GFP, HuH7-ΔEps15, 3T3, HTE, C6, and C6-Cav-1 cells were grown to 50–70% confluence on chambered coverglass slides prior to incubation with peptide. To study intracellular peptide localization 1 μM FL-C105Y, PFVYLI, SCRAM, ΔFYVLI, or D-C105Y were incubated with HuH7 cells or 5 min in PBS with Ca2+/Mg2+ at room temperature. The cells were fixed with 2% paraformaldehyde and analyzed by fluorescence microscopy. To study the effect of low temperature on the cellular uptake of C105Y, HuH7 cells were preincubated at 4 °C for 1 h in serum-free medium. The cells were then coincubated with AF-594-C105Y and AF-488-transferrin for 30 min at 4 °C. The cell were then washed with PBS and either fixed in 2% paraformaldehyde or further incubated in serum-free medium for 30 min at 37 °C. For colocalization studies, the cells were preincubated with fluorescent live cell markers in serum-free medium at 37 °C for 15 min followed by addition of fluorescently labeled peptide for 5 min in the same medium at room temperature. Subsequently the cells were fixed and analyzed by fluorescence microscopy.

Fluorescence Microscopy—For analysis of the subcellular localization of C105Y in live cells, HuH7 cells were cultured on coverslips and mounted into a diffusion chamber. FITC-C105Y in PBS was continuously perfused into the chamber for 1 min followed by PBS. The cells were imaged by wide field fluorescent microscopy over a period of 20 min starting with peptide addition. At 20 min, time lapse acquisition was stopped, and a z-stack was acquired to determine peptide location. Alternatively, the subcellular localization of C105Y was evaluated in fixed cells. The cells to be processed for immunofluorescence were washed with PBS and fixed for 5 min at room temperature with 2% paraformaldehyde in PBS. All of the samples were examined in dual channels using a Zeiss Axiovert 200 utilizing wide field microscopy with Autoquant deconvolution software for analysis, because of the superior signal to noise ratio of wide field microscopy images in comparison with laser scanning confocal microscopy (24). Acquired z1-sections were processed using Hygens confocal deconvolution software.

Antibody Staining—FITC-C105Y (1 μM) was added to four-well glass slides containing HuH7 cells plated 2 days prior. After 5 min of incubation of cells with FITC-C105Y peptide at 21 °C, the cells were washed and fixed with 2% paraformaldehyde. The cells were then permeabilized with 0.1% Nonidet P-40 for 30 min before blocking with 1% bovine
TABLE 1
Primary structures of peptides used in this study

| Name      | Amino Acid sequence | Size  |
|-----------|---------------------|-------|
| α1-AT Partial Amino | ...SIPEVKFNKPFVFLMIEQNTK... | 55 kDa |
| C105Y     | CSIPPEVKFRKPFVYLI   | 2363 Da |
| PFVYLI    | ...                       | 1190 Da |
| SCRAM     | ...NKPLILVFY           | 1351 Da |
| ΔFVYLI    | ...SIPEVKFN            | 1515 Da |
| D-C105Y   | d-CSIPPEVFKNKPFVYLI   | 2353 Da |

serum albumin in PBS and the addition of primary antibody in 1% bovine serum albumin for 1 h. Following this, the cells were washed and incubated with Texas Red-labeled secondary antibody.

**RESULTS**

The peptides used in this study are shown in Table 1. C105Y is a fluorescein-labeled, 17-amino acid sequence based on residues 346-374 of sec-R. PFVYLI is a synthetic peptide that represents the C-terminal portion of C105Y and includes the proposed binding sequence to α1-AT. SCRAM is a peptide with the scrambled binding sequence of C105Y (ILVYF). ΔFVYLI is a C-terminal truncation mutant designed to eliminate the proposed binding sequence of C105Y. D-C105Y is the D-isomer of C105Y, which was synthesized with D-amino acids to determine the specific structural requirements of C105Y uptake. C105Y Peptide Localizes to the Nucleus and Nucleolus in Fixed HuH7 Cells and Other Cell Types—To determine whether C105Y peptide alone can enter different cell types, the peptide was fluorescein-labeled and added to cultured human hepatoma (HuH7), mouse fibroblast (3T3), and primary HTE cells. Surprisingly, after only 5 min of incubation, C105Y peptide (Fig. 1, A–C) was not only present in the cytoplasm but showed enrichment of fluorescent signal in the nucleus and nucleolus. This pattern was more pronounced in HuH7 cells compared with 3T3 and HTE cells. To locate C105Y in the nucleolus, we stained HuH7 cells with an antibody against the nucleolar protein nucleolin (Fig. 1E), which is normally present in the nucleolus and sometimes in the cytoplasm of cells. The orange color in the merged image (Fig. 1F) indicates that C105Y colocalizes with the nucleolin present in the nucleolus. These data, taken together, suggest that C105Y can enter the nucleus and nucleolus in several different cell types very rapidly.

C105Y Peptide Is Rapidly Internalized by Live HuH7 Cells—The data in fixed cells suggest that C105Y can rapidly enter cells, a characteristic of most CPPs. However, recent evidence suggests that cell fixation, even under mild conditions, can lead to an artificial uptake or redistribution of these peptides (25). To confirm that C105Y can rapidly enter cells and that our previous observation was not due to an artifact of fixation, FL-C105Y was added to live HuH7 cells. The cells were visualized by live cell fluorescence microscopy, and images were taken every 20 s for the first 5 min and every 2 min thereafter (Fig. 2). These time lapse images revealed that C105Y peptide is internalized by HuH7 cells within a few seconds and reaches the nucleus and nucleolus as rapidly as 3 min.

Therefore, the ability of C105Y to penetrate cells and localize to the nucleus and nucleolus is not an artifact of fixation, because C105Y can enter living cells.

**Sequence-dependent Uptake of C105Y Peptide**—Although cells internalize C105Y peptide, it was unknown which part of the C105Y sequence was responsible for its internalization and nuclear localization. Previous work by others indicates that a pentapeptide sequence in the C-terminal portion of α1-AT (FVFLM) was necessary to bind the putative sec-R (15). A synthetic analog of this peptide with sequence FVYLI could block binding and internalization of α1-AT complexes. When the amino acid sequence of these synthetic peptides were altered by mutating specific residues or scrambling amino acid sequences, they were unable to block the binding and internalization of α1-AT complexes. These data suggested that the binding of α1-AT complexes to the sec-R was sequence-specific (16). Therefore, we hypothesized that because the synthetic peptide PFVYLI could bind the putative sec-R and block the internalization of α1-AT complexes, it would also be possible to enter cells similarly to C105Y. Additionally, we wanted to determine whether altering this putative binding sequence would abolish the ability of C105Y to enter cells. Consequently, we engineered a scrambled mutant (SCRAM) and a truncated peptide (ΔFVYLI) that lacks the proposed binding sequence to determine whether these peptides could be internalized and trafficked to the nucleus. Amino acid sequences for the above-mentioned peptide can be found in Table 1.

The previously reported pentapeptide-binding sequence PFVYLI (Fig. 3A) and the scrambled mutant SCRAM (Fig. 3D) were internalized by HuH7 cells and displayed a staining pattern identical to C105Y (Fig. 1A). However, the peptide lacking the pentapeptide-binding sequence (ΔFVYLI) was not taken up by HuH7 cells (Fig. 3G). Therefore, it appears that the C-terminal amino acids of C105Y (PFVYLI) alone can mediate cellular uptake and nuclear targeting of C105Y. Interestingly neither cellular uptake nor nuclear localization of the scrambled mutant is impaired, which suggests that the order of amino acids within the C-terminal region does not influence cell penetration and nuclear localization. This is in contrast to prior data for putative sec-R ligands and suggests that this binding site may not be accessed in this model or at least does not account for most of the uptake.
Uptake of the C105Y D-isomer—To further test whether C105Y accessed HuH7 cells via a receptor mediated pathway or as a cell-penetrating peptide, we synthesized the D-isomer of C105Y. Previously published data suggest that D-isomers of the TAT CPP were taken up as efficiently as the native peptide (26), indicating that TAT entered cells in a receptor-independent fashion. FL-D-C105Y accumulated in the cytoplasm of HuH7 cells as outlined by FM4–64 (Fig. 4B), a marker for endocytic vesicles, but did not enter the nucleus or nucleolus (Fig. 4A).

However, although cytoplasmic accumulation of D-C105Y occurs, its pattern differs from the distribution pattern of C105Y, SCRAM, and PFVYLI in the cytoplasm. These data indicate that no specific spatial orientation of the amino acids comprising the hydrophobic C terminus is needed for cell penetration. This provides further evidence that cellular uptake is not a receptor-mediated event. However, nuclear and nucleolar targeting appears to be stereo-selective.

C105Y Nucleolar Localization, but Not Internalization, Is an Energy-dependent Process—We wanted to elucidate the mechanism by which C105Y enters cells and determine whether uptake is energy-dependent.

Receptor-mediated endocytosis through clathrin-coated pits is an energy-dependent process that can be inhibited by incubating cells at 4 °C. To determine whether the import of C105Y into cells is energy-dependent, FL-C105Y peptide was incubated with HuH7 cells at 4 °C. As a control, AF-488-transferrin, which is internalized through clathrin-coated pits, was incubated with HuH7 cells at 4 °C.
rafts,” are small structures that freely diffuse on the cell surface. These domains on the plasma membrane, generally referred to as “lipid rafts,” are particularly enriched in cholesterol and glycosylphosphatidyl inositol-anchored proteins. It has been shown that ligands or receptors which bind to these domains are targeted to early endosomes and subsequently transported to the perinuclear region of the cell (22). A distinct pathway for the selective uptake of molecules by cells is via clathrin-coated pits, whereby proteins bind to specific receptors on the cell surface, and receptor complexes cluster and are internalized in an energy-dependent manner (27). Known inhibitors of clathrin-mediated endocytosis, methyl-β-cyclodextrin (MβCD), chlorpromazine, and cytochalasin D, are known to inhibit clathrin-mediated endocytosis (21). We therefore tested the effect of these drugs with transferrin, a marker of clathrin-coated peptides, and C105Y peptide (Fig. 8, A–C). Taken together, these results confirm that C105Y uptake is not inhibited by chlorpromazine, cytochalasin D, and nocodazole (Fig. 8, A–D). Control experiments performed to address the effects of these drugs with transferrin, a marker of clathrin-mediated endocytosis, suggested that C105Y uptake is not inhibited by chlorpromazine, cytochalasin D, and nocodazole (Fig. 8, E–G). Taken together, these results confirm that endocytic pathways are not involved in C105Y peptide uptake but may involve lipid rafts or other endocytic pathways that are inhibited by MβCD for nucleolar targeting.

C105Y Peptide Is Internalized by a Clathrin-independent Pathway—Endocytosis can occur through a variety of mechanisms, each providing a distinct pathway for the selective uptake of molecules by cells. These distinct pathways include clathrin-mediated, caveolae/lipid raft-mediated, and clathrin- and caveoleal-independent endocytosis. It has been described that entry of specific proteins into cells is via clathrin-coated pits, whereby proteins bind to specific receptors on the cell surface, and these ligand-receptor complexes enter cells through an endocytic pathway (27). To examine the role of clathrin-coated pits in C105Y uptake, stable HuH7-GFP and HuH7-ΔEps15 cell lines were created expressing either EGFP or EGFP-DIII, respectively (Fig. 6, B and E). The EGFP-DIII plasmid construct was created by Benmerah et al. (23) and encodes a partial Eps15 sequence that is normally required for the early steps of clathrin-dependent endocytosis. The EGFP-DIII construct has a deletion in the C-terminal domain of Eps15, which contains AP-2-binding regions important for targeting AP-2 to the clathrin-coated pits. It was shown that transfection of this construct into HeLa cells (23) and HuH7 cells (data not shown) inhibited transferrin uptake and thus interfered with clathrin-coated pit formation. When fluorescently labeled C105Y was added to either of these stable cell lines, it was rapidly internalized and targeted to the nucleus and nucleolus (Fig. 6, A and D), suggesting that clathrin-coated pits are not involved in C105Y uptake.

C105Y Internalization Occurs Independent of Caveolae—Cholesterol-rich domains on the plasma membrane, generally referred to as “lipid rafts,” are small structures that freely diffuse on the cell surface. These lipid rafts are subdivided into two distinct categories: caveolin-containing lipid rafts, called caveolae, and clathrin- and caveolin-independent lipid rafts. Caveolae are flask-shaped, noncoated invaginations on the plasma membrane, which contain many diverse signaling molecules and membrane transporters. Clathrin- and caveolin-independent lipid rafts are similar to caveolae in structure but lack the protein caveolin. Additionally, macropinocytosis is a rapid lipid raft-dependent and receptor-independent form of endocytosis. Each of these aforementioned structures can enter cells through an endocytic mechanism. Human hepatoma cells express very low levels of caveolin-1 (Cav-1) and Cav-2 yet rapidly take up C105Y, suggesting a role of noncaveolar raft domains in C105Y uptake and/or trafficking (28). To confirm this, HuH7 cell lysates were immunoblotted with anti-caveolin-1 and anti-caveolin-2 antibodies and compared with cell lysates from C6 glioma cells (Fig. 7). Additionally, we tested C6-Cav-1 glioma cells, which exhibit reduced but not absent levels of Cav-1 and Cav-2 compared with wild type C6 cells. The C6-Cav-1 knockdown cells line was created by Bhatnagar et al. (22) to stably knock down Cav-1 expression utilizing a DNA vector-based small interfering RNA. When FL-C105Y was added to C6 and C6-Cav-1 cells, it was internalized rapidly, localizing predominantly to the nuclei and nucleoli of these cells (Fig. 7), with an identical staining pattern to HuH7 cells (Fig. 1). These data, taken together, indicate that C105Y uptake and trafficking occur independent of caveoleae.

Endocytosis Is Not Involved in Uptake of C105Y—To investigate the possible involvement of different endocytic pathways in the cellular uptake of C105Y peptide, we tested the effect of chlorpromazine (a known inhibitor of clathrin-mediated endocytosis), methyl-β-cyclodextrin (MβCD) which depletes membrane cholesterol, inhibiting pathways dependent on lipid rafts), cytochalasin D (an inhibitor of F-actin elongation required for macropinocytosis and caveoleal endocytosis), and nocodazole (which inhibits microtubule formation). Among all these tested drugs only methyl-β-cyclodextrin (MβCD) had an inhibitory effect on C105Y localization to the nucleolus (Fig. 8D). For all other drugs C105Y peptide (Fig. 8, A–C) exhibited an identical staining pattern to the control (data not shown). Control experiments performed to address the effects of these drugs with transferrin, a marker of clathrin-mediated endocytosis, suggested that C105Y uptake is not inhibited by chlorpromazine, cytochalasin D, and nocodazole (Fig. 8, E–G). Taken together, these results confirm that endocytic pathways are not involved in C105Y peptide uptake but may involve lipid rafts or other endocytic pathways that are inhibited by MβCD for nucleolar targeting.
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Intracellular Trafficking of C105Y Occurs by an Endocytic Mechanism—Pinocytosis (including macropinocytosis), the uptake of fluid and solutes by cells, can be measured by the intracellular accumulation of tracer molecules. This process is conserved in all eukaryotic cells and is required for diverse cellular functions. We wanted to determine whether the intracellular trafficking of C105Y might be facilitated through endocytic vesicles or lipid rafts, as suggested by the effect of MβCD on C105Y nuclear localization. Consequently, HuH7 cells were coincubated with C105Y (Fig. 9A) and a fluid phase marker of endocytosis: FM1–43X (Fig. 9B). The merged image (Fig. 9C) shows that there is overlap between the cytoplasmic structures stained by C105Y and the cytoplasmic membrane-bound vesicles stained by the FM1–43X in the cytoplasm. Furthermore, colocalization of C105Y with FM1–43X was also apparent in C6 and C6-Cav-1 cells (Fig. 7, E and F). This suggests that C105Y peptide may utilize endocytic vesicles stained by FM1–43X to traffic inside the cell.

DISCUSSION

In this study we report that C105Y is a novel cell-penetrating peptide, based on its ability to enter the cytoplasm, nucleus, and nucleolus of live cells very rapidly. Although the mechanism of cell penetration by CPPs is not fully understood, in general these peptides enter cells rapidly in a receptor-independent fashion. Most of the previously identified CPPs share one of several motifs: either multiple positive charges, hydrophobicity, or α-helical structure. Many CPPs, such as TAT peptide, penetrate cell membranes via positively charged residues, such as arginines, but others, such as penetratin, translocate into cells via its central hydrophobic core (26, 29). It appears that C105Y enters cells through its hydrophobic C-terminal sequence (PFVYLI), because this six-amino acid sequence itself can enter cells and localize to the nucleus in an identical fashion to full-length C105Y, and the peptide lacking this sequence (ΔFVYLI) fails to internalize into cells. Furthermore, the order of the amino acids present in the hydrophobic core is not critical, because the scrambled C105Y mutant PILFVY behaves similarly to the authentic C105Y and PFVYLI. These data further support the notion that uptake of C105Y is not via a specific receptor, depending only on the biochemical properties of their sequences and not the order of their amino acid residues.

Earlier studies on cellular translocation of CPPs suggested that none of the classical receptor-, transporter-, or endocytosis-mediated processes are involved in their uptake. Instead it was proposed that the uptake of CPPs might occur through either direct membrane penetration or through the formation of inverted micelles (30). However, other recent evidence suggests that some endocytic process can be involved in the uptake of CPPs. For example the TAT-derived peptide is first endocytosed into a vesicular compartment before release into the cytoplasm (8). Several endocytic pathways exist that may mediate the internalization and intracellular trafficking of CPPs. These include endocytosis via clathrin-coated pits or endocytosis via lipid rafts. C105Y uptake and internalization does not occur through known endocytic pathways. It was particularly important to investigate clathrin-mediated endocytosis for C105Y, because it was originally regarded as a high affinity ligand for such a receptor, the sec-R. Low temperature, chlorpromazine, cytochalasin D, nocodazole, stable cell lines expressing EGFP-DIII, or use of the D-isomer did not interfere with C105Y peptide uptake, indicating no participation of receptor- and clathrin-mediated endocytosis. Furthermore, C105Y peptide can enter cells with or without caveolin-1 and
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FIGURE 8. C105Y uptake is not impaired by inhibitor drugs targeting different endocytic pathways. HuH7 cells were incubated with chlorpromazine (C, G, and K), methyl-β-cyclodextrin (D, H, and I), cytochalasin D (A, E, and I), and nocodazole (B, F, and J) 30 min prior to the addition of both AF-596-labeled C105Y (A–D) and FITC-labeled transferrin (E–H) for 5 min at 21 °C. The cells were then washed, fixed, and analyzed by fluorescence wide field microscopy with deconvolution software. The merged images of transferrin (green) and C105Y (red) are shown in F–L.

TABLE 2
Summary of data on C105Y uptake and intracellular localization

| C105Y peptides | Cell entry | Nuclear entry | Nucleolar entry |
|----------------|------------|---------------|-----------------|
| C105Y          | +          | +             | +               |
| PVFYLI         | +          | +             | +               |
| SCRAM           | +          | +             | +               |
| ΔFVYLI         | −          | −             | −               |
| D-C105Y        | +          | −             | −               |

| Cell types      |            |               |                 |
|-----------------|------------|---------------|-----------------|
| HuH7            | +          | +             | +               |
| HuH7-GFP        | +          | +             | +               |
| HuH7-ΔEps15     | +          | +             | +               |
| C6              | +          | +             | +               |
| C6-Cav-1        | +          | +             | +               |
| 3T3             | +          | +             | +               |
| HTE             | +          | +             | +               |

| Inhibition      |            |               |                 |
|-----------------|------------|---------------|-----------------|
| 4 °C            | +          | +             | −               |
| Cytochalasin D  | +          | +             | +               |
| Nocodazole      | +          | +             | +               |
| Chlorpromazine  | +          | +             | +               |
| Methyl-β-cyclodextrin | +          | +             | −               |

result from the ability of C105Y to enter cells via a pathway independent of clathrin and caveolae and its ability to be transported to the nucleus and nucleolus by nondegradative pathways.

Interestingly, C105Y localizes to the nucleolus, a plurifunctional organelle responsible for ribosome biogenesis, RNA processing, viral replication, and tumor suppression. Thus C105Y is a good candidate to deliver potentially therapeutic cargoes (e.g. antivirals and tumor suppressors) to this site (33, 34). Because the pathway by which nontargeted complexes enter cells is not well understood, it is also possible that caveolin-2, so caveolin-mediated endocytosis is not involved. The aforementioned data are summarized in Table 2.

However, C105Y peptide did colocalize with intracellular vesicles stained by FM1–43X, a fluid phase endocytic marker. This suggests that these membrane-bound vesicles might facilitate intracellular C105Y peptide trafficking. However, the mechanism of membrane translocation of C105Y needs to be further investigated and might occur similarly to penetratin, which enters cells via a nonendocytic and receptor- and transporter-independent pathway. Instead penetratin, which needs the hydrophobic residue tryptophan for internalization, has been shown to condense plasmid DNA by electrostatic interactions. In vitro these C105Y-polyK-coupled DNA complexes, gave up to 100-fold increase in gene expression compared with polyK/DNA complexes also did not contain C105Y peptide. These C105Y-polyK-DNA complexes also increased gene expression in vivo. In many instances expression from nonpeptide containing complexes is not detectable, but strong expression is observed from C105Y containing nanoparticles (18, 19). The results obtained by Wu et al. (31) and Patel et al. (32), which used minor variants on the C105Y sequence, but both containing the FVYLI core sequence to target AAV viruses and other gene delivery systems, respectively, show that the addition of this ligand markedly increases transfection efficiency of virus particles into airway cells and enhanced gene expression when linked to other gene delivery systems. Our data now suggest that enhanced gene expression from sec-R-targeted complexes...
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C105Y enhances gene expression simply by facilitating nuclear entry. These results suggest a potential role for using C105Y to deliver other therapeutic cargoes to cells.

The nuclear targeting of C105Y is likely sequence-dependent. A pentapeptide with the sequence VPMLK, derived from the Ku70 protein, has similar amino acid composition to PTVYLL. This peptide, VPMLK, can penetrate cells, can localize to the nucleus, and is one of the shortest CPPs described to date (35, 36). The Lamond laboratory, in an effort to characterize proteins within the nucleolus, determined that some short peptide motifs show specific enrichment in the nucleolar proteome (33). This nucleolar binding motif is very similar to the VPMLK sequence found within C105Y and the VPMLK sequence derived from the Ku70 protein. Therefore, it may not be coincidence that these two peptides localize to the nucleus and/or nucleolus.

In conclusion, we identified C105Y to be a novel cell-penetrating peptide that is rapidly internalized by live cells by an energy-independent process via caveolin- and clathrin-independent lipid rafts. Moreover, it traffics rapidly to the nucleus and nucleolus. These properties probably account for the ability of this peptide to increase gene transfer and gene expression both in vitro and in vivo, when conjugated to polyK-DNA complexes or included in the capsid of AAV. Consequently, C105Y may be a useful carrier of other molecular cargos as well.

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