Gold nanoparticles modified with nuclear localization peptides were synthesized and evaluated for their subcellular distribution in HeLa human cervical epithelium cells, 3T3/NIH murine fibroblastoma cells, and HepG2 human hepatocarcinoma cells. Video-enhanced color differential interference contrast microscopy and transmission electron microscopy indicated that transport of nanoparticles into the cytoplasm and nucleus depends on peptide sequence and cell line. Recently, the ability of certain peptides, called protein transduction domains (PTDs), to translocate cell and nuclear membranes in a receptor- and temperature-independent manner has been questioned (see for example, Lundberg, M.; Wikstrom, S.; Johansson, M. (2003) Mol. Ther. 8, 143–150). We have evaluated the cellular trajectory of gold nanoparticles carrying the PTD from HIV Tat protein. Our observations were that (1) the conjugates did not enter the nucleus of 3T3/NIH or HepG2 cells, and (2) cellular uptake of Tat PTD peptide–gold nanoparticle conjugates was temperature dependent, suggesting an endosomal pathway of uptake. Gold nanoparticles modified with the adenovirus nuclear localization signal and the integrin binding domain also entered cells via an energy-dependent mechanism, but in contrast to the Tat PTD, these signals triggered nuclear uptake of nanoparticles in HeLa and HepG2 cell lines.

INTRODUCTION

The diagnosis and treatment of disease at the cellular level would be greatly enhanced by the ability to deliver analyte probes and therapeutic agents into specific cells and cellular compartments. The nucleus is the desired target for cancer therapies that involve DNA–drug interactions, genes, short interfering RNA, and antisense strategies that target RNA splicing. These therapeutic agents are often prevented from reaching the cell nucleus because of size, charge, or, in the case of small molecules, because P-glycoproteins pump them out of the cell. Vectors that cross cell and nuclear membrane barriers efficiently and are unaffected by cellular pumping mechanisms could aid in the implementation of many novel disease treatments.

Targeted nuclear delivery is a challenging task, however, as any cell-specific nuclear probe must satisfy the following requirements: it must (i) be small enough to enter cells and cross the nuclear membrane (<100 nm for uptake by receptor-mediated endocytosis (RME) and <30 nm for import through nuclear pores), (ii) penetrate cellular membranes or bind to cell-specific plasma membrane receptors, (iii) bypass or escape endosomal/lysosomal pathways, (iv) penetrate nuclear membranes or access importins to pass through the nuclear pore complex, and (v) have low toxicity. Although viruses have evolved to deliver genes to cell nuclei, the design of synthetic systems (nonviral vectors) capable of performing all of these functions remains a challenge. Nonviral vectors such as dendrimers and liposomes currently appear to be safer and more cost-effective than viral vectors, but they are relatively less efficient at nuclear delivery (1). By combining membrane-translocation peptides from viruses with noninfectious materials, a potential route to safe and efficient nuclear targeting vectors may be afforded.

The present study demonstrates nuclear targeting by synthetic cellular targeting peptides complexed to a nanometer-sized gold particle scaffold (Figure 1). Gold makes an excellent intracellular targeting vector for three reasons. First, gold can be synthesized routinely in sizes varying continuously from 0.8 to 200 nm with ~10% size dispersity. Second, gold can be modified with a large collection of small molecules, peptides, proteins, DNA, and polymers. Moreover, all of these functional elements can be combined on a single particle simultaneously, often via simple one-pot procedures. Finally, gold par-
particles have enormous visible light extinction characteristics, (ε > 10^6 M^-1 cm^-1 at ~520 nm) (2), a behavior we have exploited in a modified form of video enhanced differential interference contrast light microscopy for tracking nanoparticle trajectories inside cells.

Gold particles modified with the nuclear localization signal (NLS) from SV-40 virus have been used extensively for investigating nuclear transport in the pioneering work of Feldherr and co-workers (3–8). The KKKRK sequence from the SV-40 virus has been shown to bind to importin-α, a nuclear transport protein that binds importin-β and enters the nucleus through the nuclear pore complex. However, in those studies the nanoparticle–peptide complex was injected directly into the cytoplasm near the nucleus, thus bypassing cellular membrane entry and endosomal/lysosomal pathways. The studies described here build on this established procedure to examine the ability of a number of viral targeting peptides to target the nucleus from outside intact cells.

The peptide sequence RKRRQRR from the Tat protein has been widely studied because of its role in initiation of transcription of the HIV genome. This sequence has also been found to promote uptake into cells across the plasma membrane (9, 10). For example, peptides containing this sequence have been used to shuttle nanoparticles (45 nm) into progenitor cells, allowing their detection by magnetic resonance imaging (11). Herein we describe experiments designed to assess the ability of peptide–gold nanoparticle complexes to cross the barriers of intact cells and reach the nucleus. It is shown that particle uptake and nuclear translocation depend strongly on the surface-bound peptide sequence and on the cell type for three cell lines studied, HeLa, 3T3/NIH, and HepG2. Although a single targeting peptide can traverse multiple cellular barriers (endosomal uptake, endosomal escape, and nuclear transport), as shown in HeLa cells, this is not a general feature. On the basis of this study, we conclude that combinations of peptides may be an effective strategy for targeting the nucleus of intact cells. Indeed, in previous work, we have shown that nanoparticles carrying both NLS and RME peptides are capable nuclear targeting vectors in HepG2 cells (12).

**Materials and Methods.**

**Materials.** Gold nanoparticles (20 nm diameter) were purchased from Ted Pella. Bovine serum albumin (BSA) was purchased from Roche (MW: 67000). 3-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) and dithiothreitol (DTT) were obtained from Pierce Chem., Co., Rockford, IL. Sephadex G10, G25, and G50 (for size exclusive liquid chromatograph) were purchased from Sigma. Centricon YM-30 and Microcon YM-100 were purchased from Millipore. 7.5% Tris-HCl prepacked gels (SDS–PAGE) (for electrophoresis) and protein assay kit (for protein concentration estimation) were purchased from Bio-Rad. Peptides were custom-made at UNC Microprotein Sequencing & Peptide Synthesis Facility (Chapel Hill, NC).

**Peptides.** A list of the four peptides that have been complexed to gold particles and incubated with the three cell lines is shown in Table 1. In this study, we focus on the peptide nuclear localization signal (NLS) derived from the Large T antigen of the SV40 virus (M1 in Table 1), a peptide derived from the HIV Tat protein (M2), and adenovirus fiber protein (M3), as well as a modified integrin binding domain peptide (M4). M1 is considered a classic NLS peptide and has been used as a targeting agent in cytochemical studies (6, 7). The CGGRKKRRQRRAP sequence in peptide M2 is an HIV NLS that is believed to employ an alternative nuclear transport pathway that involves only importin-β (13–18). The CGGFSTSLARKA sequence in peptide M3 is derived from the adenoviral fiber protein and is also a known NLS (19). While the NLS sequences of peptides M1, M2, and M3 are derived from viruses, peptide M4 is largely synthetic in nature, containing only the integrin binding domain and a segment of basic lysine residues. The integrins are a family of transmembrane glycoproteins exploited by a number of viruses (adenovirus, echovirus, and foot-and-mouth-disease virus), as well as bacterial intracellular pathogens, for cell entry (20). We expect that vectors carrying the integrin binding domain will have high affinity for integrins that will facilitate cell binding and internalization. The short stretch of positively charged lysine residues was added to mimic the cationic nature of many nuclear localization signals. Peptides M3 and M4 have not been used previously for intracellular nanoparticle delivery. Each peptide was prepared with a terminal cysteine to permit conjugation to the lysine residues on the surface of BSA (see below) and tested with PSORT software package, which predicts relative probability of subcellular localization of proteins based on their amino acid sequences using AAindex Analysis (21). Predictions for nuclear localization are shown in Table 1. The design criteria thus included both the targeting sequence and a structural component required for nanoparticle attachment and signal flexibility for receptor interaction. Addition of six lysine residues to peptide M4 increased the calculated prediction for nuclear delivery from ~21% to ~74%.

**Preparation of Gold Nanoparticle Vectors.** Peptide–gold particle complexes were assembled by conjugating peptides to bovine serum albumin (BSA) and then attaching BSA–peptide conjugates to gold nanoparticles (Figure 1). To prepare BSA–peptide conjugates, BSA was mixed with 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) in a 1:50 ratio (22). The number of MBS ligands bound to BSA was determined experimentally by a fluorescent assay using fluorescamine (22).

The succinimide ester group of MBS subsequently was reacted with a cysteine-terminated peptide using a BSA: peptide ratio of 1:20. After purification by gel filtration on Sephadex G50, the efficiency of peptide conjugation was estimated by mobility shift assay on SDS–PAGE (7.5%).
Cell Culture and Particle Delivery. HeLa, HepG2, and 3T3/NIH cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Minimum Essential Medium Eagle (EMEM) or Dulbecco’s Modified Eagles Medium (DMEM) growth media (BioWhittaker, Walkersville, MD) without addition of antibiotics at 37 °C in controlled CO2 atmosphere. For investigating the cellular localization of peptides, cells were plated on glass coverslips and grown to 75% confluency in 12-well plates and then incubated with nanoparticle conjugates (150 pM) for various times. It has been shown that the presence of serum in the culture medium inhibits cellular binding/uptake of cationic lipids and reduces their efficiency as nucleic acid carriers (23); therefore, most cellular delivery agents available today require serum-free media during delivery. The presence of BSA contributed greatly to the stability of nanoparticle conjugates in serum-containing media. All studies shown herein were conducted using media which contain 10% FBS. At indicated time points the coverslips were rinsed extensively with phosphate-buffered saline (PBS), and the cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and then rehydrated in PBS. Following fixation, the cover slip with cells were mounted onto glass slides with FluorSave (Calbiochem, San Diego, CA) mounting media and allowed to dry overnight prior to microscopy analysis.

The stability of protein–gold nanoparticle conjugates vs flocculation or protein exchange in EMEM growth media with 10% FBS was determined as follows. An aqueous suspension of gold nanoparticles (450 μL of 1.16 mM) was adjusted to pH 9 by adding 50 μL of 10 mM sodium carbonate buffer. Fluorescein isothiocyanate (FITC)-terminated M3 peptide was conjugated to BSA, and the BSA conjugate was slowly added to the gold sol. The final concentration of gold nanoparticles and M3–BSA conjugate was 0.98 nM and 1.78 μM, respectively. The mixture was incubated at room temperature for 15 min, and native BSA (20 μL of 2 mg/mL) was added to prevent flocculation. Six identical solutions were prepared using this protocol. In three of the solutions, any unbound FITC-labeled M3–BSA conjugates were removed by centrifugation. The fluorescence of the supernatant was measured to determine the concentration of unbound M3–BSA conjugate. The number of bound M3–BSA conjugates per gold nanoparticle was calculated by subtracting the number of unbound conjugates from the total number of conjugates added initially. The remaining three solutions were diluted 1:5 in EMEM growth medium without phenol-red (Cambrex Bio Science, Walkersville, MD). Visible absorption spectra of the solutions were acquired after 4 h, and no change of the gold nanoparticle plasmon absorption band was observed. The solutions were centrifuged, and the M3–BSA gold nanoparticle conjugates were then resuspended in 450 μL of sodium phosphate buffer (10 mM, pH 7). Dithiothreitol (DTT) (94 μL of 480 mM) was added and incubated for 24 h to exchange the bound M3–BSA conjugates on the gold nanoparticles. The solutions were centrifuged and the supernatants analyzed by fluorescence spectroscopy to determine the amount of bound M3–BSA conjugate on gold nanoparticles after exposure to growth medium. Since DTT may not remove all surface-bound proteins, this experiment yields an upper limit to the number of M3–BSA conjugates that are displaced in the growth medium.

Characterization of Particle Trajectories. The unique optical properties of colloidal gold enable their identification by relatively simple light microscopy techniques. Particles as small as 10 nm can be observed with video-enhanced microscopy techniques (albeit as an inflated diffraction image) (24, 25). However, high contrast microscopy techniques available currently rely on time-consuming postacquisition processing. In addition, high-density cellular granules can easily be mistaken for gold nanoparticles (26).

To improve the visibility of gold nanoparticles in cells, we combined the high resolution afforded by color video-enhanced microscopy with the high contrast and pseudo three-dimensional imaging capabilities of differential interference contrast microscopy (DIC). This hybrid technique (video-enhanced color differential interference contrast microscopy, VECDIC) easily distinguishes gold nanoparticles from cellular granules and organelles under polarized light conditions of DIC. Since DIC allows optical sectioning of the sample, the approximate location of nanoparticles inside cells was determined by varying the focal plane of the image. VECDIC allows cells and nanoparticles as small as 20 nm to be observed simultaneously without image overlays, albeit image resolution remains at the limits of optical microscopy ~ 200 nm (27). Thus, high throughput data analysis at the rate of several hundred slides per day is possible. VECDIC data were confirmed by TEM analysis. The colorimetric lactose dehydrogenase assay (LDH) was used to evaluate the cytotoxicity of peptide–BSA–gold nanoparticle conjugates (Cytotox 96, Promega).

Microscopy. Video-enhanced color DIC microscopy was performed on an upright Leica DMLB microscope equipped with a 0.9 numerical aperture (NA) condenser, differential interference contrast (DIC) optics, and 100× plan (NA 1.3) oil objective. The video system comprises a Nikon DMX-1200 color CCD digital camera, with software-based manual control of color balance and contrast enhancement on live and captured images.

TEM analysis was performed on a Philips CM-12 TEM (accelerating voltage was 100 kV) at the University of North Carolina Dental School Research Electron Microscopy Center. Cells were prepared for TEM by staining in 1% OsO4, dehydrating in ethanol gradient, immersing in LR white embedding resin, and curing under UV light. Microtomed sections were then placed on Formvar grids and poststained with uranyl acetate and lead acetate. A Malvern Zetasizer 1000HS was used to determine the hydrodynamic diameter of gold nanoparticle–BSA complexes.

RESULTS

Preparation of Gold Nanoparticle Vectors. Peptide–gold particle complexes were assembled by conjugating peptides to bovine serum albumin (BSA) and then attaching BSA–peptide conjugates to gold nanoparticles (Figure 1). Since the purity of BSA varies, it is important to determine the binding efficiency of the MBS and peptides to each batch of BSA. An initial ratio MBS:BSA of 50:1 resulted in 28 ± 3 MBS linkers per BSA molecule as measured by a fluorescencemass assay (22). The reaction of cysteine-terminated peptides with MBS–BSA conjugates was measured by gel shift assays and was found to be peptide dependent (Table 2).

BSA–peptide conjugates were complexed to 20 nm diameter gold particles in 25 mM pH 11 carbonate buffer to prevent colloidal gold aggregation caused by positively charged peptides. Dynamic light scattering and transmission electron microscopy revealed that BSA–peptide conjugates add ~4 nm to the hydrodynamic diameter of a 22 nm gold particle. Prior to complexation, gold particles and BSA had average hydrodynamic diameters
of 22 and 12 nm, respectively (Figure 2). The average hydrodynamic diameter of the BSA–gold-nanoparticle complex increased to 26 nm. BSA is an oblong molecule that can be approximated by a cylinder 3 nm in radius and 10 in length. Since the data show that BSA adds much less than 10 nm to the diameter of gold nanoparticles, it seems likely that BSA binds to the nanoparticle along its length. The fact that BSA does not add greatly to the size of the gold particle is important in its use in constructing intracellular targeting vectors because only particles with diameters below ca. 30 nm are capable of entering cell nuclei.

A surface coverage of 160 ± 8 BSA per 20 nm gold nanoparticle was determined previously by time-resolved fluorescence spectroscopy (22). This amount is difficult to rationalize if BSA adsorbs along its long axis; thus, we cannot rule out that some multilayer adsorption occurs on gold nanoparticles. Quantitative exchange studies performed in the presence of EMEM/10% FBS revealed that no more than 30% of M3–BSA conjugates were displaced from the gold nanoparticle surface in 4 h. This may represent exchange of more loosely bound multilayers. Irrespective of the location of the exchangeable protein (surface or multilayer), within the time scale of a typical delivery experiment (< 3 h), the data suggest that > 300 peptides are bound to each nanoparticle.

Cellular trajectories of peptide–gold particle complexes were characterized in the following three cell lines: HeLa, 3T3/NiH, and HepG2. These cell lines were chosen to illustrate cell-specific differences in nuclear targeting for each peptide investigated.

**Nanoparticle Delivery to HeLa Cells.** The fate of nanoparticles inside HeLa cells depended upon the targeting peptide complexed to the gold surface. In general, nanoparticle uptake followed one of three pathways: (i) cell entry followed by exocytosis; (ii) cell entry and transport up to the nuclear membrane; (iii) cell entry and nuclear translocation.

Nanoparticles modified with each of the peptides listed in Table 1 were observed inside HeLa cells by video-enhanced color differential interference contrast microscopy (Figure 3). After 1 h of incubation with HeLa cells, all four peptide–nanoparticle conjugates were found clustered together in cellular compartments that appear to be endosomes as observed by TEM (21). The behaviors of the peptide–nanoparticle complexes differed once they escaped the endosomal pathway.

Table 2. Number of Peptides Bound to BSA As Determined by 7.5% SDS–PAGE

<table>
<thead>
<tr>
<th>no.</th>
<th>peptide sequence</th>
<th>peptide/BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>CGGGPKKKRKVGG</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>M2</td>
<td>CGGRKKRRQRRRAP</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>M3</td>
<td>CGGFSTSLRARKA</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>M4</td>
<td>CKKKKKKGGRGDMFG</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

After 1 h of incubation with Hela cells, all four peptide–nanoparticle conjugates were found clustered together in cellular compartments that appear to be endosomes as observed by TEM (see M1 conjugates in Figures 3A and B). The behaviors of the peptide–nanoparticle complexes differed once they escaped the endosomal pathway.

After 1 h, nanoparticles carrying peptide M1 were able to escape the endosomal pathway and reach the nuclear membrane. At longer times (2–3 h), M1 nanoparticles were found clustered around the cytoplasmic side of the...
nuclear membrane with relatively few located inside the
nucleus (Figure 3C). Apparently, M1 nanoparticle com-
plexes were able to enter HeLa cells and escape the
endosomal pathway, but were unable to interact properly
with the nuclear pore complex.

Nanoparticles carrying peptide M2 were found in the
cytoplasm of HeLa cells after 1 h, and in 3 h only minimal
nuclear localization was observed (Figure 4A–C). The
peptide sequence from HIV Tat protein has been observed
previously to bypass endosomal pathways. This occurred
on a time scale of minutes, via mechanisms that are not
yet well understood (16). In contrast to those reports, we
observed slow uptake and primarily cytoplasmic uptake
of M2 nanoparticles.

Nanoparticles carrying peptide M3 accumulated around
the nuclear membrane after 2 h of incubation with HeLa
cells; after 3 h M3 nanoparticles were also found inside
the nucleus of over 70% of the observed cells (Figures
4D–F). Nuclear targeting was also observed during the
delivery of nanoparticles carrying peptide M4 (Figure
4G–I). In contrast to the other peptides studied, M4
peptides do not have any previously known NLS function,
carrying only the integrin binding domain plus six lysine
residues.

**Figure 4.** Incubation of HeLa cells with 20 nm diameter gold nanoparticles observed by VECDIC. Figure 4 (A–C) 0.5, 1.5, and 3 h incubation with nanoparticles carrying peptide M2; (D–F) 0.5, 1.5, and 3 h incubation with nanoparticles carrying peptide M3; (G–I) 0.5, 1.5, and 3 h incubation with nanoparticles carrying peptide M4.

**Nanoparticle Delivery to 3T3/NIH Cells.** 3T3/NIH
cells appear to be more selective than HeLa cells. While
M1, M3, and M4 nanoparticles were able to enter the
cytoplasm after 30 min of incubation with 3T3/NIH, none
of them were able to translocate the nuclear membrane
within 6 h (Figure 5). M2 nanoparticles were not found
inside the cytoplasm of 3T3/NIH cells by VECDIC
microscopy.

**Nanoparticle Delivery to HepG2 Cells.** Entry into
the cytoplasm of HepG2 cells was strongly dependent on
the peptide sequence attached to nanoparticles. M1 and
M2 nanoparticles were able to enter the cytoplasm of
HepG2 cells but were not found near the nuclear mem-
brane or inside the nucleus, which suggests that these
nanoparticles were unable to escape from endosomes.
While M3 nanoparticles were observed in the nucleus of
HeLa cells and the cytoplasm of 3T3/NIH cells, no
cellular uptake was observed when they were introduced
to HepG2 cells (Figure 6D–F). The most surprising
results were observed with M4 nanoparticles (Figure
6G–I). M4 nanoparticles not only entered the cytoplasm
of HepG2 cells, but also accumulated inside the nucleus
after 2 h of incubation time (Figure 6H,I).
Temperature Studies. BSA–peptide conjugates are polycationic proteins with pI values above 7.0 as determined by isoelectric focusing gel electrophoresis (not shown), and the cell membrane is a negatively charged complex aggregate of proteins, lipids, and carbohydrates. While xenobiotics are normally taken up by passive diffusion, considering the size of peptide–BSA–gold conjugates, passive diffusion is an unlikely translocation mechanism here. Pinocytosis and receptor-mediated endocytosis (RME) are the prime mechanisms for the uptake of large molecular structures (28). Cellular uptake of nanoparticles coated with peptide–BSA conjugates could occur by RME, or for the Tat RKKRRQRRR sequence, via the less understood peptide transduction domain mechanism (PTD). Endocytosis occurs within minutes to several hours and is energy dependent. Cell uptake by PTDs is reported to occur within minutes and is energy independent. The peptide–nanoparticle construct reported here did not enter cells in the first 30 min of delivery, suggesting either that the PTD mechanism is slowed by the nanoparticle or that it is not the mechanism of cell entry. To test the energy dependence of uptake, experiments were performed at 4 °C. At 4 °C sufficient energy is not available to support RME pathways (29). Internalization of all peptide–nanoparticle complexes was inhibited at low temperature suggesting an energy-dependent transport process. These results support the involvement of endocytosis as the major route for the internalization of all peptide-nanoparticle complexes studied here, including the M2 peptide.

**DISCUSSION**

Cellular trajectories of the NLS peptides studied here are summarized in Table 3. Notably, the data reveal a strong dependence of cellular targeting on the cell line and peptide studied. M1 nanoparticles were able to enter all cell lines studied, but there was little or no nuclear transport of these particles although M1 is a well-known NLS. Prior results have shown that when M1 nanoparticles are injected into the cytoplasm, thus bypassing the endosomal pathway, they will translocate the nuclear membrane (3–9). If M1 can trigger RME, and is capable of nuclear translocation once in the cytoplasm, why then can it not reach the nucleus from outside an intact cell? Two possible answers are that the M1 construct is trapped in endosomes or is somehow altered inside the...
endosome in a way that renders it incapable of interacting with importin-α. TEM and VECDIC show that M1 nanoparticles are evenly distributed throughout the cytoplasm, rather than clustered together. It is therefore likely that the physicochemical properties of M1 are altered during endosomal transport. Whether this involves a structural or chemical change is currently unknown.

In contrast, M3 nanoparticles were seemingly unaffected by endosomal transport of HeLa cell lines. This peptide was able to enter HeLa cells, escape endosomes, and pass through the nuclear pore complex. In 3T3/NIH cells, however, M3 nanoparticles were found clustered together in endosomes, apparently unable to escape and reach the nuclear membrane. Finally, there was no observed uptake of M3 nanoparticles in HepG2 cells. These differences were also reflected in the LDH cytotoxicity assay. M3–BSA–gold nanoparticle conjugates caused 20% cell death in HeLa cells after 3 h, a result that was traced solely to the M3–BSA construct. After 3 h incubation with the same construct, 3T3/NIH cells were 95% viable. Such large differences in cell viability are likely attributable to the strong nuclear delivery of the M3 conjugate in HeLa cells. We speculate that the highly cationic M3 peptide can interact strongly with DNA inside the HeLa nucleus.

The synthetic peptide M4, composed of the integrin binding domain (RGD) and a stretch of six lysine residues, was unable to reach the nucleus of 3T3/NIH cells but was the only peptide capable of targeting the nucleus of HepG2 cells. Integrin binding domains are known to induce cellular uptake by both RME and phagocytosis. However, they typically do not interact with the nuclear pore complex. Thus, it is likely that M4 nanoparticles enter the cell via the integrin binding domain and escape endosomes due to the presence of positively charged lysines, which are known to induce leakage of negatively charged vesicles and cells (30). The fragment of M4
consisting of six lysine residues could also be involved in nuclear transport, as it resembles the classic NLS sequences rich in lysines (e.g., large T NLS).

Perhaps the most surprising result of this study is the failure of M2 to target the nucleus of any cell line studied. M2 contains the arginine-rich sequence of the Tfr24 peptide from the HIV Tat protein. This sequence is known to bind the TAR RNA hairpin and is also one of a class of peptides known commonly as peptide transduction domains (PTDs). The observation of a PTD led to further refinement of the sequence to determine the region that was most active for transport through cell membranes. The minimal sequence for PTD activity has been determined to be the nine amino acids (RKKRRQRRR) (18). Most peptides that contain this sequence of nine amino acids appears to function as a PTD. PTDs have been reported to target nuclei of all cell lines using an energy-independent mechanism (10–12). Several recent studies have attributed the efficient nuclear targeting of PTDs to an artifact of cell fixation (31–33). According to these studies the positively charged peptides that contain the arginine-rich sequence bind tightly to the cell membrane. Once the cells are fixed, the membrane is disrupted and the peptides can diffuse into the cell. Once inside the cell there is a strong electrostatic attraction to DNA in the nucleus. Although similar protocols were used here for cell fixation, nuclear targeting was not observed for the M2 conjugated to gold nanoparticles. On the basis of these observations, we speculate that nanoparticle size may minimize the reported artifacts of cell fixation. This is logical since diffusion in the medium is greatly reduced for a particle of molar mass 50 MDa compared to a single small peptide.

CONCLUSIONS

Targeted nuclear delivery of diagnostic probes and therapeutics could improve disease detection and treatment. Accessing the nucleus from outside an intact cell is difficult, however, because of three membrane barriers: (1) outer cell membrane, (2) endosomal membrane, and (3) nuclear membrane. This paper highlighted the challenges associated with crossing these barriers. For example, the SV40 NLS is capable of entering HeLa cells via receptor-mediated endocytosis (this work). It is also able to cross the nuclear membrane if it is directly injected into the cytoplasm (3–9). Despite this, the SV40 NLS is not translocated from outside an intact HeLa cell to the nucleus because it cannot break free of endosomal compartments or is somehow altered as it travels through the endosomal pathway. These observations suggest that the most efficient nuclear targeting may result from a combination of targeting peptides attached to a single vector. This can be accomplished in many ways, e.g., using liposomes, dendrimers, or cyclodextrins. Our goals are to study further the ability of multipeptide gold nanoparticle conjugates to target cell nuclei.

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