Clathrin-mediated Endocytosis and Subsequent Endo-Lysosomal Trafficking of Adeno-associated Virus/Phage*§

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Background: Uptake and intracellular trafficking mechanisms involved in Adeno-Associated Virus/Phage (AAVP)-mediated gene delivery are unknown.

Results: Endocytosis is dynamin and clathrin-dependent, and gene transfer is restricted by accumulation in the endolysosomal degradative pathway.

Conclusion: The lysosome presents a major barrier to efficient gene delivery by this vector.

Significance: Incorporation of lysosome-disruption strategies will enhance gene delivery by future bacteriophage-based vectors.

Adeno-associated virus/phage (AAVP) is a gene delivery vector constructed as a hybrid between adeno-associated virus and filamentous phage. Tumor targeting following systemic administration has previously been demonstrated in several in vivo cancer models, with tumor specificity achieved through display of an αv integrin-targeting ligand on the capsid. However, high titers of AAVP are required for transduction of large numbers of mammalian cells. This study is the first to investigate the mechanisms involved in entry and intracellular trafficking of AAVP. Using a combination of flow cytometry, confocal, and electron microscopy techniques, together with pharmacological agents, RNAi and dominant negative mutants, we have demonstrated that targeted AAVP endocytosis is both dynamin and clathrin-dependent. Following entry, the majority of AAVP particles are sequestered by the endosomal-lysosomal degradative pathway. Finally, we have demonstrated that disruption of this pathway leads to improved transgene expression by AAVP, thus demonstrating that escape from the late endosomes/lysosomes is a critical step for improving gene delivery by AAVP. These findings have important implications for the rational design of improved AAVP and RGD-targeted vectors.

Successful viral gene therapy requires the generation of tissue-targeted gene delivery vectors, capable of producing sustained transgene expression efficiently but with limited toxicity. Most progress in viral gene therapy has involved eukaryotic viruses such as Adenovirus, adeno-associated virus (AAV), and retroviruses (e.g. lentivirus), which have been shown to provide higher levels of transgene delivery compared with non-viral vectors (1). Unfortunately, systemic therapy using these eukaryotic viruses has had limited success due to undesired uptake by the liver and reticulo-endothelial system, insertional mutagenesis, immunogenicity arising from reactions with the complement system or pre-existing antibodies, and broad tropism for mammalian cells (2). Viral tropism may be modified by the addition of tissue-specific ligands to viral capsid proteins to mediate a ligand-receptor interaction on the target tissue. However, addition of these ligands to eukaryotic viruses can alter the structure of the viral capsid, which can reduce efficacy and diminish targeting properties of the peptides themselves (3).

Bacteriophage (phage) have been proposed as safe vectors for targeted delivery of transgenes as they have no intrinsic tropism for mammalian cell receptors but can be modified to display tissue-specific ligands on the capsidic proteins without disruption of virus structure (4–8). However, despite some apparent advantages over eukaryotic viruses, tissue-targeted phage vectors have shown limited efficacy as bacteriophage has evolved to infect bacteria only and has no optimized strategy to express transgenes upon entry into eukaryotic cells (5). To overcome this limitation, a new generation of hybrid prokaryotic-eukaryotic viral vectors was recently reported (9). AAV/phage or AAVP was generated as a chimera between two single-stranded DNA viruses; AAV and a derivative of filamentous M13 bacteriophage

**This study was funded by Grant G0701159 from the UK Medical Research Council (MRC), and Research Fellowship RF/2/RFG/2009/0114 from The Leverhulme Trust.

This article contains supplemental Figs. S1–S4.

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§1 The abbreviations used are: AAV, adeno-associated virus; AAVP, adeno-associated virus/phage; ITR, inverted terminal repeat; RGD, arginine-glycine-aspartate; TNFα, tumor necrosis factor α; PFA, paraformaldehyde; LMA-1, early endosome antigen 1; LAMP-1, lysosomal-associated membrane protein-1; TEM, transmission electron microscopy; DAB, diaminobenzidine; LUC, luciferase; ANOVA, analysis of variance; EE, early endosome; MVB, multivesicular body; LYS, lysosome.
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riophage. In the targeted AAVP vector, a mammalian transgene cassette flanked by inverted terminal repeat (ITR) sequences from an fd-tet (10) bacteriophage clone displaying a double-cyclic arginine-glycine-aspartate (RGD) ligand on the minor pIII coat protein. This RGD peptide is a well-established targeting motif for \( \alpha_v \) integrins, which are overexpressed in tumor vascular endothelium and tumor cells but absent or expressed at low levels in normal endothelial cells (11, 12). The introduction of AAV ITRs into the phage vector increased transduction efficiency over conventional phage-based vectors, and tumor targeting and therapy were demonstrated in several pre-clinical in vivo models. More recently, targeted RGD-AAVP was used to deliver the anti-vascular agent tumor necrosis factor \( \alpha \) (TNFa) to dogs with inoperable spontaneous soft-tissue sarcomas, with total tumor eradication reported in some of these animals (13).

Despite the apparent success of this novel vector in vivo, high titers of AAVP are required for mammalian cell transduction. In vitro experiments have shown that while 100% of cells internalize targeted RGD-AAVP, only up to 10% express the transgene (9, 14). Little is known about the mechanisms of RGD-AAVP endocytosis and intracellular trafficking, understanding these mechanisms could have important implications for achieving efficient gene delivery.

Eukaryotic viruses have evolved to bind cell surface receptors, which determine cellular entry via numerous biochemically and morphologically distinct endocytic pathways, including clathrin-mediated endocytosis, caveolae formation, macropinocytosis, and other non-clathrin, non-caveolar pathways (15). Integrins are commonly used receptors (or co-receptors) for entry of eukaryotic viruses, several integrin heterodimers recognize RGD sequences displayed on the exposed loops of viral capsid proteins, including several adenovirus serotypes, foot and mouth disease virus, and coxsackievirus A9 (16–18). Endocytosis following binding to integrin heterodimers can occur by several endocytic pathways, the most intensively studied of which is clathrin-mediated endocytosis (19). Clathrin-mediated endocytosis is a process by which ligand-receptor binding at the plasma membrane results in receptor clustering and coated pit formation. The clathrin-coated pits then invaginate and pinch off from the plasma membrane to form intracellular clathrin-coated vesicles. The vesicles then shed their coats to form tubulo-vesicular compartments termed early-endosomes, from which membrane-associated cargo can be recycled to the plasma membrane or soluble cargo can be sorted into late endosomes and further to lysosomes (15). Several viruses have adapted to utilize this degradative endocytic pathway for cell entry and intracellular trafficking. In many cases, endosomal escape is achieved through fusion or penetration of the endosomal membrane either by pH-induced conformational changes to capsid proteins, or by proteolytic cleavage of viral proteins by acid-dependent endosomal proteases (20).

Here we present the findings of the most detailed study of the entry and intracellular trafficking of the RGD-AAVP vector. We have confirmed that endocytosis of RGD-AAVP is mediated by \( \alpha_v \) integrins and that endocytosis is at least in part dependent on dynamin and clathrin. We have developed electron microscopic and confocal imaging methods to follow entry and trafficking of RGD-AAVP, and we have observed sequestration of the majority of viral particles by the endo-lysosomal degradative pathway. By determining the intracellular fate of AAVP, we were able to significantly increase transgene expression through disruption of the lysosomes using the lysosomal agent chloroquine, and also achieve transgene expression in previously non-permissive cell lines. These findings have important implications for the rational design of the next generation of AAVP, as well as for other bacteriophage-based gene therapy vectors.

EXPERIMENTAL PROCEDURES

Cell Lines, AAVP Production, and Reagents—The Human Embryonic Kidney (HEK293) and human cervical cancer (HeLa) cell lines were purchased from American Type Culture Collection (ATCC). The Human Glioblastoma U87 cell line was a gift from Cancer Research UK. The Human Melanoma M21 and M21L cell lines were a kind gift from Professor David Chereshe (University of California, La Jolla). The DU145 prostate cancer cell line was a kind gift from Dr Paul Mintz (Imperial College London). HEK293, U87, DU145, M21 and M21L cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) and HeLa cells were maintained in Eagle’s Minimal Essential Medium (MEM, Sigma). All media were supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin (100 units/ml, Sigma), streptomycin (100 \( \mu \)g/ml, Sigma), and l-glutamine (2 mm, Sigma). Cells were cultured in a humidified 5% \( \text{CO}_2 \) atmosphere at 37 °C and passaged every 2–3 days. Targeted (RGD) and non-targeted (fd) AAVP viral particles were prepared following the previously published protocol (14). Stock virus preparations were sterile-filtered and titrated by plaque assay. Titer is expressed as bacterial transducing units measured per \( \mu l \) (TU/\( \mu l \)).

Sucrose, dynasore, chloroquine, and bafilomycin A1 were obtained from Sigma. Stock solutions were either prepared in DMSO (dynasore, bafilomycin) or sterile tissue culture-grade water (succrose, chloroquine).

The GFP-tagged dominant negative mutant Dynamin II-K44A plasmid was a kind gift from Professor Ari Helenius and Dr Jason Mercer (ETH-Zurich) and was obtained with the permission of Dr. Mark McNiven (Mayo Clinic, Rochester). HEK293 or HeLa cells were transiently transfected with dynamin II-K44A using FuGENE 6 transfection reagent (Promega) according to the manufacturer’s instructions.

Human clathrin heavy chain (CHC17)-targeted siRNAs siCHC1 (targeted to sequence AACGAAATGAGCTGTCTTT-GAAGA) (21) and siCHC2 (Hs_CLTC_10HP) and non-targeted “allstars” negative control siRNA were purchased from Qiagen. HEK293 cells were transfected using Lipofectamine RNAiMax (Invitrogen), according to the manufacturer’s instructions.

Rabbit anti-fd phage and horseradish peroxidase (HRP) conjugated goat anti-mouse antibodies were obtained from Sigma and mouse anti-early endosome antigen 1 (EEA-1) from BD Biosciences. Rabbit anti-\( \alpha_v \) integrin and mouse anti-lysosomal-associated membrane protein 1 (LAMP-1) were obtained from
Abcam. Mouse anti-CHC17 and mouse anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology. AlexaFluor-labeled human transferrin, HRP-transferrin, and goat anti-rabbit/mouse AlexaFluor-labeled secondary antibodies were obtained from Invitrogen.

**Flow Cytometry**—Cells were seeded in 24-well plates and grown until 70–80% confluent. The cells were serum-starved for 30 min prior to addition of RGD-AAVP or fd-AAVP (1 × 10^5 TU/cell) and incubated for 2 h at 37 °C. The cells were cooled on ice to halt endocytosis and washed three times in cold PBS to remove non-bound phage. The cells were trypsinized for 5 min at 37 °C (to remove surface-bound phage) and pelleted by centrifugation at 2000 rpm for 5 min before fixation in 4% paraformaldehyde (PFA, pH 7.2) for 10 min at room temperature (RT).

For internalization experiments involving endocytic inhibitors, HEK293 cells were either pretreated with 80 μM dynasore or 0.4 μM sorcein in serum-free medium for 30 min, transfected with dynamin-II K44A for 24 h, or transfected with siRNAs for 72 h prior to addition of phage. The cells were incubated with RGD-AAVP, fd-AAVP for 2 h, or AlexaFluor-647 transferrin (5 μg/ml) for 30 min before washing and fixation. Cells which received AAVP were washed and fixed as previously stated. Samples treated with transferrin were cooled on ice and washed three times in acidic glycine buffer (0.1 M glycine, 0.1 M sodium chloride, pH 3) and once with PBS before trypsinization and fixation.

Cells were stained for internalized AAVP as follows; the cells were blocked for 30 min in 0.1% saponin in 2% BSA-PBS prior to staining in rabbit anti-fd-phage (diluted 1:1000) in 0.1% saponin in 1% BSA-PBS for 1 h at room temperature. The cells were washed and pelleted three times in 0.1% saponin in 1% BSA-PBS before incubation with goat anti-rabbit AlexaFluor-647 (diluted 1:500) for 1 h at room temperature (in darkness). The cells were washed twice with 0.1% saponin-PBS and resuspended in PBS prior to analysis.

**FACs analysis** was performed using a BD FACscalibur Flow Cytometer (BD Biosciences) equipped with an argon-ion laser (488 nm) and red-diode laser (635 nm). The mean fluorescence intensity was measured for the GFP (FL1)-positive cell population. Results were analyzed using FloJo (TreeStar) software.

**Confocal Microscopy**—Cells were seeded on poly-d-lysine coated coverslips in 12-well plates and grown until 60–70% confluent. Cells to be stained for α5 integrins were washed and fixed in 4% PFA, 250 mM HEPES buffer for 15 min at room temperature. To investigate RGD-AAVP trafficking and colocalization with endocytic markers, the HEK293, HeLa, U87, and DU145 cells were serum-starved in serum-free DMEM or MEM for 30 min before cooling on ice. RGD-AAVP was added to the cells at 1 × 10^6 TU/cell and incubated on ice for 30 min, non-bound RGD-AAVP was then removed by washing with cold PBS before warm serum-free medium was added to the cells. After incubation at 37 °C for the indicated times, the cells were cooled on ice, washed three times with ice-cold PBS and fixed in 4% PFA in 250 mM HEPES for 10 min on ice, then 8% PFA for 20 min at room temperature. For confocal experiments involving transferrin uptake, 10 μg/ml AlexaFluor-647-labeled transferrin was added to the serum-free medium during incubation on ice. After 30 min, the cells were washed to remove non-bound RGD-AAVP or transferrin and warmed to 37 °C for indicated times before washing and fixation as above. Cells were washed three times in PBS, quenched with 50 mM ammonium chloride, washed, and permeabilized with 0.2% Triton X-100 (in PBS) for 5 min. The cells were washed three times in PBS and blocked for 30 min in 2% BSA-PBS. The cells were incubated with primary antibodies; rabbit anti-α5 integrin primary antibody (diluted 1:50), rabbit anti-fd phage (1:1000), mouse anti-EEA-1 (1:100), or mouse anti-LAMP1 (1:200) diluted in 1% BSA-PBS for 1 h, washed three times in 1% BSA-PBS and incubated in secondary AlexaFluor-conjugated antibodies (diluted 1:750) with/without DAPI (diluted 1:2000, Sigma) for 1 h. Cells were washed three times in PBS and twice in distilled water, allowed to air-dry, and mounted on microscope slides with mowiol mounting medium (prepared in-house).

For double-immunofluorescent labeling of AAVP, HEK293 or HeLa cells transfected with dynamin II-K44A were incubated with either RGD-AAVP or AlexaFluor-647 transferrin for 2 h before fixation. The cells were stained for phage using a two-step process to distinguish between external and internal particles. The cells were first fixed and blocked in 2% BSA before staining with rabbit anti-fd-phage antibody (1:1000) and goat anti-rabbit AlexaFluor-594 (1:750, to mark external phage). The cells were washed three times in PBS before permeabilization with 0.2% Triton X-100. The cells were blocked again in 2% BSA-PBS before staining with rabbit anti-fd phage primary antibody (1:1000) and goat anti-rabbit AlexaFluor-647 (1:750). The cells were washed and mounted as previously stated.

Confocal images were acquired using a Leica SP5 confocal microscope or a Zeiss Pascal confocal microscope using ×63 oil objectives. Images were acquired as full z-stacks and presented as either single section images or projections of full z-stacks (indicated in figure legends). Images were processed using ImageJ and Adobe Photoshop (Adobe Systems).

The number of internalized phage particles was quantified in transfected cells by counting from confocal image stacks; n number indicates number of cells in which phage particles were counted. The average number of internalized phage particles per cell is presented as a percentage of internalized phage particles in non-transfected controls.

**Immunoblotting**—Cell monolayers were washed 3 times in ice-cold PBS and lysed in extraction buffer (250 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.25% Triton-X100) supplemented with protease inhibitor mixture (Roche Applied Science). Extracts were clarified by centrifugation (12,000 × g for 5 min), and protein content determined by Bradford assay. 20 μg of protein was denatured in SDS sample buffer for 5 min at 100 °C. Proteins present in the extracts were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked in 5% milk in TBS-0.1% Tween before immunoblotting using mouse anti-clathrin (diluted 1:500) and mouse anti-GAPDH (1:5000) antibodies for 2 h, fol-
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lowed by goat anti-mouse-HRP (1:5000) for 1 h. Proteins were detected using supersignal west pico chemiluminescent substrate (Thermo Scientific). Rainbow molecular weight markers (GE Healthcare) were used to assess protein sizes. The expected band sizes were 37 kDa (GAPDH) and 192 kDa (clathrin heavy chain).

Transmission Electron Microscopy (TEM)—For negative staining of RGD-AAVP particles, phage stock solution was diluted in sterile double-distilled water and 400 mesh formvar-coated grids applied to 5-μl drops of sample for 2 min. The grids were then washed in water and stained using 1% uranyl acetate for 30 s, after which the excess stain was removed and grids air-dried.

Immunogold labeling of RGD-AAVP was characterized on virus particles bound to EM grids. 5-μl drops of diluted phage were applied to formvar-coated grids. The grids were blocked in 5% FCS prior to addition of anti-fd antibody diluted 1:100 in 5% FCS for 60 min at room temperature. The grids were washed in PBS and incubated with protein-A-gold (9 nm) diluted 1:100 in PBS for 45 min. The grids were washed in PBS, then in water before incubation with 1% uranyl acetate for 30 s, and air-dried before imaging.

Time course experiments of entry and trafficking of immunogold-labeled RGD-AAVP were performed as follows. HEK293 cells were seeded in poly-D-lysine coated 35-mm dishes and grown until 70% confluent. The cells were serum-starved for 30 min prior to cooling and incubation with 1 × 10^12 RGD-AAVP particles for 30 min on ice. The cells were washed three times with ice-cold 1% BSA-PBS before addition of rabbit anti-fd antibody, diluted 1:100 in BSA-PBS. The cells were incubated on ice for 30 min, washed with 1% BSA-PBS and subsequently incubated with protein-A-gold (9 nm particles) diluted 1:100 in BSA-PBS. The cells were washed three times with 1% BSA-PBS and before addition of warm medium containing 10 mg/ml horseradish peroxidase (HRP, Serva) or 5 μg/ml HRP-Transferrin. The cells were fixed on ice between 0–4 h post warm-up, the cells were first cooled on ice to halt endocytosis, washed in cold PBS and fixed in 0.5% glutaraldehyde (TAAB) in 200 mM sodium cacodylate. The cells were embedded in epon resin following pre-tissue sections were cut parallel to the surface of the dish using a Leica ultramicrotome. The sections were collected onto 50 mesh formvar-coated grids and stained for 30 s in Reynold’s lead citrate, washed with water and air-dried before analysis.

Samples were viewed by using an FEI Tecnai G² electron microscope with a Soft Imaging System Megaview III CCD camera. Images were collected at 1376 × 1032 × 16 pixels using AnalysSIS version Docu software (Olympus Soft Imaging Solutions).

Luciferase Reporter Gene Expression—Cells were seeded in 48-well plates and allowed to proliferate until 70–80% confluent. The cells were incubated for 4 h with RGD-AAVP or fd-AAVP encoding the luciferase (LLIC) reporter gene at 1 × 10^6 transducing units per cell (TU/cell) in serum-free medium containing chloroquine or bafilomycin. For experiments involving chloroquine, an equal volume of complete medium was added to the wells after 4 h, medium was changed after overnight incubation. For experiments involving bafilomycin, the serum-free medium and phage were removed and replaced with complete medium. Luciferase assays were performed on day 7 post-transduction, using Promega Steady-glo® luciferite kits. Luciferase expression was normalized to protein concentration as measured by Bradford assay (Sigma) and data presented as relative luminescence units (RLU) per 100 μg of protein. Luciferase expression and protein concentration were measured using a Promega Glomax plate reader.

Statistics—Statistical analyses were performed by using GraphPad Prism 5.0 software. Error bars represent standard error of the mean (S.E.), p values were generated by Student’s t test or ANOVA with Bonferroni post-hoc analysis and denoted as follows: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

RESULTS

Cell Entry of Targeted AAVP Requires αv Integrins—The double-cyclic RGD ligand displayed on the targeted AAVP vector enables binding to tumor vascular endothelial cells and tumor cells overexpressing αv integrins (9). We used αv integrin positive (M21) and αv integrin negative (M21L) melanoma cell lines (23) to confirm that binding to these integrins mediates endocytosis of the vector, and to establish a protocol for flow cytomeric analysis of AAVP entry. M21 or M21L cells were incubated with the targeted vector (RGD-AAVP) or non-targeted vector (fd-AAVP) for 2 h, after which the cells were fixed and immunofluorescence-stained for phage. RGD-AAVP internalization in αv integrin-deficient M21L melanoma cells was significantly reduced when compared with αv integrin-positive M21 melanoma cells. Uptake of the targeted vector did not differ significantly from uptake of non-targeted control fd-AAVP in M21L cells (Fig. 1A).

The study was extended to investigate endocytosis of RGD and fd-AAVP in a further four cell lines: HEK293 cells, which have been previously used as a model for transduction by RGD-AAVP in vitro, and three human cancer cell lines; HeLa (cervical cancer), U87 (glioblastoma), and DU145 (prostate cancer). RGD-AAVP uptake was significantly higher than fd-AAVP in all four cell lines (Fig. 1B). The cell lines were confirmed as positive for αv integrin expression using immunofluorescence staining and confocal microscopy (Fig. 1C).

RGD-AAVP Uptake Requires Dynamin and Clathrin—To elucidate the mechanisms involved in endocytosis of RGD-
AAVP, we first chose to investigate the effect of dynamin inhibition on entry of the vector. Dynamin is a cytosolic GTPase which is required for clathrin-mediated endocytosis as well as formation of caveolae but is not required for macropinocytosis (24–26). To determine whether RGD-AAVP entry requires dynamin, we first investigated the effect of dynasore, a pharmacological agent which functions as an acute inhibitor of dynamin 1 and dynamin 2 GTPase activity in vitro and blocks endocytic functions previously shown to require dynamin (27). HEK293 cells were treated with dynasore prior to, and during incubation with, RGD-AAVP, fd-AAVP or fluorescently-labeled transferrin, which served as a control for clathrin-mediated endocytosis. The cells were immunofluorescence-stained for internalized AAVP particles and phage-associated fluorescence determined by flow cytometry. Flow cytometric analysis revealed a marked decrease in the uptake of both transferrin and RGD-AAVP, while the vehicle control DMSO had little or no effect on endocytosis (Fig. 2A).

To confirm the involvement of dynamin in RGD-AAVP entry, we next studied the effect of expression of the GFP-tagged dominant negative mutant dynamin II-K44A, which is defective in GTP binding and hydrolysis (24). HEK293 cells were transfected 24 h prior to addition of RGD-AAVP, fd-AAVP, or transferrin control. Internalized phage/transferin-associated fluorescence was determined by flow cytometry. HEK293 cells expressing dynamin II-K44A showed a significant decrease in endocytosis of both vector and transferrin compared with non-transfected controls (Fig. 2B).

Inhibition of RGD-AAVP and transferrin uptake by dynamin II-K44A was confirmed by confocal microscopy of HEK293 and HeLa cells. Cells were transiently transfected 24 h prior to addition of RGD-AAVP or transferrin. Internal and external phage were differentiated through a double-immunofluorescence-staining protocol; external phage particles were first stained before permeabilization of the cells and staining for internal phage. Representative z-stack projections of HEK293 cells are shown in Fig. 2C. Internalized RGD-AAVP (top panels) was observed as punctate spherical structures in the cytoplasm, whereas the majority of external phage appears filamentous. Lower numbers of internalized particles observed in the GFP-positive cells compared with non-transfected cells. Transferrin was used as a positive control (lower panels), uptake of which was strongly inhibited by expression of dynamin II-K44A-GFP.

Having established a role for dynamin in cellular entry of RGD-AAVP, we chose to investigate whether entry of the vector requires clathrin, as dynamin is essential for clathrin-mediated endocytosis. To investigate the potential involvement of clathrin in AAVP endocytosis, we first determined the ability of sucrose to inhibit RGD-AAVP entry. High concentrations of sucrose have been shown to lead to the formation of clathrin microcages on the inner surface of the plasma membrane which depletes the cytoplasmic pools of clathrin necessary for normal coated pit formation (28). HEK293 cells treated with sucrose prior to, and during incubation with, RGD or fd-AAVP showed a significant decrease in uptake of the targeted vector (Fig. 2E). The ability of sucrose to inhibit clathrin-mediated endocytosis was again determined by measuring uptake of transferrin, treatment with 0.4 M sucrose significantly decreased uptake of transferrin compared with non-treated cells.

Clathrin-mediated endocytosis of AAVP was confirmed through the use of siRNAs targeted against clathrin heavy chain. HEK293 cells transfected with two siRNAs (siCHC1, siCHC2) showed significantly diminished internalization of targeted AAVP and transferrin (Fig. 2F). Off-target effects of transfection were minimal, as there was little effect from trans-
fection with non-targeted control siRNA. siRNA-mediated reduction of CHC expression was confirmed by Western blotting. As shown in Fig. 2G, transfection with negative control siRNA had no apparent effect on clathrin expression, whereas cells receiving siCHC1 and siCHC2 were depleted of clathrin heavy chain. siCHC2-mediated clathrin knockdown was less efficient than siCHC1, this observation is reflected in the flow cytometry data (Fig. 2F).
Microscopic Imaging of RGD-AAVP and Colocalization with Transferrin—Endocytosis of RGD-AAVP was investigated further by developing TEM methods of detection. RGD-AAVP particles were first characterized by negative staining (Fig. 3A). As anticipated, the particles were observed to have similar morphology to that of wild-type M13 filamentous phage, with the exception that particles were measured at ~1.4 μm long, compared with ~900 nm reported for the wild-type M13 virus. This is consistent with previous observations that the filamentous phage-derived particles can grow in length to accommodate DNA from inserted transgenes (10). Fig. 3B shows the appearance of immunofluorescence-stained RGD-AAVP, which also appears filamentous when bound to the cell surface.

Our initial attempts to investigate endocytosis of RGD-AAVP in HEK293 cells by transmission electron microscopy were hindered as the virions could not be detected in epon resin-embedded samples. A pre-embedding immunogold-labeling protocol was therefore developed in order to visualize gold-labeled phage in epon sections. Negative-stained RGD-AAVP was first compared with RGD-AAVP labeled with an anti-phage polyclonal antibody and 9 nm protein-A-gold, bound to formvar-coated EM grids (Fig. 3, C and D). RGD-AAVP was then cold-bound to HEK293 cells before labeling with anti-fd phage antibody and protein-A-gold. The cells were embedded in epon and visualized, gold-labeled RGD-AAVP is shown in Fig. 3E, while the 9 nm colloidal gold particles can be clearly visualized, no or little contrast was observed from the AAVP particles.

The pre-embedding immunogold labeling protocol was used to investigate intracellular trafficking of the vector in HEK293 cells by electron microscopy. The cells were incubated with HRP-transferrin and RGD-AAVP on ice, to allow binding but not endocytosis, before immunogold labeling the RGD-AAVP (while on ice). The cells were warmed to 37 °C for different times before fixation. Gold particles were observed in transferrin-positive early endosomes (EE, Fig. 3F) after 10 min, and transferrin-positive multivesicular bodies after 20 min incubation at 37 °C (MVB, Fig. 3G). Colocalization with transferrin was also confirmed by confocal microscopy. RGD-AAVP and transferrin were cold-bound to HEK293 or HeLa cells before warming and fixation. Immunofluorescence-stained RGD-AAVP particles were observed in transferrin-positive vesicles between 10 and 30 min post-warming in both cell lines, representative images of both cell lines after 30 min incubation at 37 °C are shown in Fig. 3H.

RGD-AAVP Enters the Endosomal-Lysosomal Degradative Pathway—Having observed RGD-AAVP colocalization with transferrin in early endosomes and multivesicular bodies, we reasoned that RGD-AAVP may further traffic to the late endosomal-lysosomal degradative pathway which eukaryotic viruses are able to escape or avoid. RGD-AAVP endocytosis was investigated in HEK293 cells by transmission electron microscopy, using the fluid-phase marker HRP, which is an established marker of early/late endosomes and lysosomes. AAVP-associated gold particles were observed in early entry vesicles which were in close proximity to clathrin-coated pits after 10 min (indicated by arrows, Fig. 4A). Gold particles were also observed in HRP-positive early endosomes after 20 min (Fig. 4B) and...
multivesicular bodies after 30 min (Fig. 4C), confirming our previous TEM observations using HRP-transferrin. Gold particles were seen to accumulate over time in larger multivesicular bodies after 1 h (Fig. 4D) and in dense multilamellar lysosomes 4 h post-warming (LYS, Fig. 4E).

Immunofluorescence experiments were carried out in order to confirm RGD-AAVP trafficking to the endosomal-lysosomal degradative pathway. Time-course experiments of AAVP internalization were performed in HEK293, HeLa, U87, and DU145 cells. AAVP was first cold-bound to the cells before warming and fixation after different time-points. Transient colocalization with EEA-1, a marker for early endosomes, was observed in all cells lines between 10 and 60 min post-warming. Fig. 5 shows representative images of all four cell lines after 30 min at 37 °C (arrows indicate colocalized fluorescence). Representative single optical sections are shown, scale bars, 10 μm.

**Chloroquine Increases RGD-AAVP-mediated Luciferase Expression**—We first performed time-course experiments in HEK293, HeLa, U87, and DU145 cells to establish the levels of AAVP-mediated luciferase reporter gene expression over time, and to establish a time of peak transgene expression in vitro. Luciferase expression was detected in HEK293 and U87 cells between 24 and 48 h post-transduction, consistent with previously published data (9). RGD-AAVP-mediated luciferase expression was observed to climb over 7 days post-transduction, after which the cells could no longer be maintained in culture. Importantly, no luciferase expression was observed in HeLa or DU145 cells at any time, relative luminescence units were determined as background by comparison to non-treated cells (Fig. 7A).

Having previously demonstrated that RGD-AAVP is endocytosed in all four cell lines, it was surprising that no transgene expression was detected in HeLa or DU145 cells. We therefore chose to investigate whether transduction could be established or increased through the use of pharmacological agents that interfere with the endosomal/lysosomal system. Bafilomycin A1 is a potent inhibitor of vacuolar ATPases which prevents acidification of endosomes (29) while chloroquine neutralizes

![FIGURE 4. RGD-AAVP colocalizes with the fluid-phase marker HRP.](image)

![FIGURE 5. RGD-AAVP transiently colocalizes with EEA-1.](image)
acidic organelles such as late endosomes (30). RGD-AAVP-LUC and fd-AAVP-LUC were added to HEK293, HeLa, U87, or DU145 cells in the presence of varying concentrations of chloroquine or bafilomycin and luciferase expression was determined at day 7 post-transduction. The results show that while no significant change in luciferase expression was observed in cells treated with bafilomycin (supplemental Fig. S3), there was a dose-dependent increase in RGD-AAVP-mediated luciferase expression with increasing chloroquine concentrations in all cell lines tested (Fig. 7B). Treatment with 200 μM chloroquine for 4 h resulted in a 2.8-fold increase in RGD-AAVP-mediated luciferase expression over non-treated cells in HEK293 cells, and 9.2-fold increase in U87 cells. Interestingly, HeLa and DU145 cells again showed no expression of luciferase in cells treated with RGD-AAVP-LUC alone but expression rose to detectable levels following chloroquine treatment. Non-targeted fd-AAVP-mediated luciferase expression remained significantly lower than that mediated by RGD-AAVP in all cell lines. Alteration of the endo-lysosomal pathway under the same conditions was investigated by electron microscopy of HeLa cells (supplemental Fig. S4). The fluid-phase marker HRP was used to label the degradative pathway prior to addition of bafilomycin or chloroquine. The HRP-positive late endosomes/lysosomes of chloroquine-treated cells appeared dilated with associated disturbance of internal membrane structures (supplemental Fig. S4C). Non-treated and bafilomycin-treated HeLa cells contained HRP-positive late endosomes and lysosomes with typically compact structures (supplemental Fig. S4, A and B).
Entry and Intracellular Trafficking of AAVP

DISCUSSION

Prokaryotic virus-based gene therapy vectors have some potential advantages over eukaryotic vectors in terms of cost, ease of capsid manipulation, and large transgene cargo potential. However, these viruses are considered poor gene delivery vehicles due to inefficient transgene expression. Larocca et al. described that filamentous phage displaying FGF was able to deliver GFP to COS-1 cells with 0.4% transduction efficiency (7). More recently, a filamentous phage displaying the full length adenovirus penton base was shown to transduce up to 4% of HeLa cells despite observations that 100% of cells internalized the virus (5). The hybrid vector AAVP has shown improved transgene expression over conventional phage-based vectors, and tumor targeting and therapy have been demonstrated in several pre-clinical in vivo cancer models. However, high titers of AAVP are still required for transduction of large numbers of cells in culture. In vivo tumor therapy is therefore likely to be influenced by other factors, such as the heterotypic “bystander effect” which facilitates transport of cytotoxic metabolites between the transduced and neighboring non-transduced tumor cells, thus enhancing therapeutic effect (31).

In this study, we have investigated the mechanisms involved in uptake and intracellular trafficking of AAVP to identify key barriers to transduction. We first confirmed that αv integrins are required for endocytosis of RGD-AAVP through comparison of uptake in M21 and M21L cells, and demonstrated targeted vector uptake in four other αv integrin-positive cell lines. Following αv integrin binding, our data suggest that endocytosis requires both dynamin and clathrin, and that the majority of RGD-AAVP subsequently enters the endosomal/lysosomal degradative pathway, which is restrictive to transgene expression.

The role of dynamin in RGD-AAVP endocytosis was established using two complementary inhibitors. Uptake in HEK293 cells either transiently transfected with the dominant negative mutant dynamin II-K44A or treated with the chemical agent dynasore, resulted in significant reduction of uptake of both RGD-AAVP and the transferrin control. The effect of transient expression of dynamin II-K44A could also be seen by confocal microscopy, where uptake of RGD-AAVP was significantly inhibited in HEK293 and HeLa cells expressing the mutant plasmid. These results indicate that functional dynamin is required for endocytosis of RGD-AAVP. As dynamin controls clathrin and caveolea-mediated endocytosis, but not macropinocytosis, we chose to investigate the effect of hypertonic treatment on vector endocytosis. Incubation with high concentrations of sucrose significantly inhibited uptake of RGD-AAVP, these data were confirmed through inhibition of AAVP internalization using siRNAs directed against clathrin heavy chain. These findings indicate that clathrin is also required for RGD-AAVP endocytosis. Further evidence for the involvement of clathrin was demonstrated by confocal and electron microscopy, as RGD-AAVP was observed to enter the same endocytic vesicles as HRP-transferrin.

The potential role of clathrin in uptake of RGD-AAVP may be surprising, given that traditionally, clathrin pits are restricted to cargoes of up to 200 nm in size (32), whereas electron microscopy of RGD-AAVP particles revealed filamentous virions of ~1.4 μm in length. However, recent publications have suggested that clathrin pits can accommodate much larger cargoes such as listeria (33) and ebola virus (34). Furthermore, our studies do not exclude the involvement of other entry pathways in RGD-AAVP uptake. While receptor-mediated endocytic pathways such as the dynamin-dependent clathrin and caveolar pathways are well characterized as viral entry portals, many viruses are able to utilize multiple uptake pathways. Virus binding to and/or cross-linking their specific receptors can lead to activation of downstream signaling events which increase endocytic uptake (35, 36). For example, the binding of αv integrins by RGD motifs on the adenovirus penton base has been shown to promote secondary uptake through macropinocytosis (37). It is possible that integrin binding by RGD-AAVP may also stimulate uptake through alternative endocytic routes.

Having observed RGD-AAVP colocalization with transferrin in early endosomes and multivesicular bodies, we reasoned that RGD-AAVP may further traffic through the late endosomal/lysosomal degradative pathway which eukaryotic viruses have adapted to escape or avoid. Electron microscopic analysis of RGD-AAVP uptake in the presence of the fluid-phase marker HRP revealed vector accumulation in late endosomes and lysosomes. Association of RGD-AAVP with this endosomal/lysosomal degradative pathway was confirmed by confocal time-course experiments. In the four cell lines tested, transient association with EEA-1 was observed, while association with LAMP-1 increased over time. Colocalization with LAMP-1 was also observed 24 h after addition of the vector, suggesting that the vector remains associated with the lysosomes. Treatment with chloroquine increased the transduction efficiency of RGD-AAVP in HEK293 and U87 cells, and importantly, induced expression in the previously non-permissive HeLa and DU145 cell lines. These data demonstrate that endosomal/lysosomal accumulation presents a barrier to gene delivery by AAVP. Our findings are supported by those of Ivanenkov et al., who observed that treatment with chloroquine increased the titer of filamentous phage that could be recovered after internalization in mammalian cells, demonstrating that phage are degraded in acidic compartments (4). No change in transgene expression was observed when cells were transduced in the presence of bafilomycin, presumably because while bafilomycin is known to neutralize acidic compartments without vesicle swelling (29), chloroquine has been reported to cause swelling and destabilization of late endosomes and lysosomes at high concentrations (38). This effect was observed in HeLa cells treated with the lysosomotropic agents. While late endosomes and lysosomes of bafilomycin-treated cells were of similar morphology to non-treated cells, these structures were noticeably disturbed in chloroquine-treated cells. Given this evidence, we suggest that chloroquine-induced lysosomal perturbation facilitates AAVP escape from the degradative pathway, which leads to improved gene delivery.

In conclusion, we have demonstrated that by investigating the endocytosis and intracellular trafficking of AAVP, we have identified the endosomal-lysosomal pathway as a major barrier to efficient transduction. Importantly, we have shown that disturbing this pathway is a viable strategy for improving transgene...
expression. These findings will be invaluable for the rational design of the next generation of AAVP gene transfer vectors.

Acknowledgments—We thank the following researchers for contributions of reagents: Professors Renata Pasqualini and Wadid Arap for the bacteriophages and Escherichia coli (K91) reagents, Professor Ari Helenius and Dr. Jason Mercer (ETH-Zurich) for the GFP-tagged dominant negative mutant dynamin II-K44A plasmid, Professor David Cheresh (University of California, La Jolla) for the M21 and M21L melanoma cell lines, Dr. Paul Mints (Imperial College London) for the DU145 prostate cancer cell line and Professor Nicholas Mazarakis (Imperial College London) for the use of equipment. We would also like to thank Dr. James Hislop (Imperial College London) for advice regarding experiments and for editorial assistance.

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