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# Mutation in the platelet-derived growth factor receptor alpha inhibits adeno-associated virus type 5 transduction

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ABSTRACT

Due to its non-pathogenic lifecycle, little is known about the cellular determinants of infection by adeno-associated virus (AAV). To identify these critical cellular factors, we took advantage of the gene transfer abilities of AAV in combination with a forward genetic selection to identify proteins critical for transduction by this virus. AAV serotype 5 (AAV5) vectors encoding the furin gene were used to transduce furin-deficient cells followed by selection with furin-dependent toxins. A population of cells specifically resistant to AAV5 transduction was identified and sequence analysis suggested all had a single amino acid mutation in the leader sequence of the platelet-derived growth factor receptor alpha (PDGFRα) gene. Characterization of this mutation suggested it inhibited PDGFRα trafficking resulting in limited expression on the plasma membrane. Mutagenesis and transfection experiments confirmed the effect of this mutation on PDGFRα trafficking, and the AAV5 resistant phenotype could be rescued by transfection with wild type PDGFRα.

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## Introduction

The entry process of a virus into a cell is not strictly determined by the virus but is very much dependent upon the proteins that are expressed by the cell and the interactions that occur between the virus and these proteins. Identification of these proteins has not only resulted in development of antiviral therapies but has also advanced our understanding of cell biology. Identification of the receptor(s) or intracellular proteins associated with viral infection can be a formidable task. In addition to the use of engineered cell lines and biochemical assays, several powerful genomic techniques have been used to accomplish this task. These include a radiation hybrid approach that identified the receptor for jaagsiekte virus entry (Rai et al., 2001) and a bioinformatics-based approach termed comparative genome analysis (CGA) developed in our laboratory which identified a receptor for adeno-associated virus type 5 (AAV5) and AAV6 and several genes important to the infection of Ebola virus (Brindley et al., 2011; Di Pasquale et al., 2003; Kondratowicz et al., 2011; Quinn et al., 2009; Weller et al., 2010). While these approaches have been successful, each has limitations. Genetic selection is a powerful approach for understanding complex biological processes. While reverse genetics is a common approach for understanding the function of a known gene by deleting it, forward genetics uses selective pressure on hemizygous cells to identify genes conferring a specific phenotype. Genetic screens using gene knockout techniques or complementation with cDNA libraries followed by selection have been used to identify genes involved in apoptosis or cytokine response pathways (Kumar et al., 1997; Velazquez et al., 1992).

In this study we investigated whether forward genetic selection could help to understand critical steps in virus infection processes. As a model virus, we chose AAV5. While AAV5 is not associated with any disease, vectors based on this virus have utility for gene transfer to the eye and lung as well as for delivering a genetic vaccination against viral agents (Alexander et al., 2007; Kuck et al., 2006; Zabner et al., 2000). Efficient transduction with AAV5 is known to require both N-linked α2–3 sialic acid as an attachment receptor as well as expression of the platelet-derived growth factor receptors (PDGFRα) for internalization (Di Pasquale et al., 2003; Kaludov et al., 2001). However, other proteins are likely to be important in this process. Following selection of a spontaneously mutated cell population for variants

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that resisted transduction, we confirmed the importance of PDGFRα in AAV5 transduction and identified a region in the leader sequence of PDGFRα which is responsible for the proper trafficking of this protein. Transfection experiments demonstrated that the AAV5-resistant phenotype could be rescued by wild type PDGFRα.

## Experimental procedures

### *Construction of AAV2F, AAV5F, AAV2B and AAV5B and vector production*

A HindIII and BlpI fragment containing the furin cDNA was isolated from the pAGEFur plasmid (Hatsuzawa et al., 1990) and ligated into HindIII- and BlpI-digested AAV2rnLacZ and AAV5rnLacZ plasmids to create AAV2F and AAV5F plasmids, respectively. AAV2B and AAV5B encoding the beta galactosidase gene were previously described (Kaludov et al., 2001). Recombinant particles were produced in 293T cells as previously described (Alisky et al., 2000).

### *Cloning of cDNA*

RNA was isolated from CHO cells by extraction of the cells with Triazol (Invitrogen, Carlsbad, CA). DNA was synthesized using the Smart Race kit (Clontech, Mountain View, CA) and sequenced using a series of primers based on regions conserved across the mouse, rat, and human cDNAs of PDGFRα. Plasmids expressing either human or CHO PDGFRα were assembled and expressed in the DualGC vector (Stratagene) using the CMV promoter (phPDGFRα or pcPDGFRα respectively).

### *Cell culture*

CHO-K1 (ATCC CCL-61) and CHO FD11 cells (Gordon et al., 1995), were cultured at 37 °C, 5% $CO_2$ in alpha minimal essential medium (AMEM) (Biofluids) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. A549 cells were maintained at 37 °C, 5% $CO_2$ in HAMs F12 and D10 medium (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Madin–Darby Canine Kidney (MDCK) cells were a gift from Bob Weller. Cells were cultured at 37 °C, 5% $CO_2$ in Dulbecco's modified essential medium (DMEM) (Biofluids) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μ/ml streptomycin.

### *Toxin screen*

Sensitivity to *Pseudomonas* exotoxin A (PE) toxin was determined by plating $1 \times 10^4$ CHO-K1 and CHO FD11 cells and then incubating them with increasing amounts of PE (List Biologics) for 48 h. Two days post-selection, surviving cells were counted using a microscope and a calibrated ocular. Sensitivity to anthrax toxins was determined using protective antigen (PA, List Biologics) from *Bacillus anthracis* in combination with fusion protein FP59 (a fusion of anthrax lethal factor with the catalytic domain of PE (Gordon et al., 1995; Klimpel et al., 1992)). A modified form of PA (PA-RAAR, having amino acids 164-RKKR-167 changed to RAAR) was also used in the screen to improve the specificity for cleavage by furin. CHO-K1 and CHO FD11 cells were incubated with increasing amounts of PA for 48 h in concert with 20 ng/ml FP59. Two days post-selection surviving cells were counted using a microscope and a calibrated ocular.

### *Confocal immunofluorescence*

Parental and mutant CHO cells were fixed with 1% paraformaldehyde for 15 min at room temperature prior to permeabilizing with 0.2% Triton X-100 in PBS and blocking. Cell surface PDGFRα expression was evaluated by incubating non-permeablized cells with primary mouse monoclonal anti-PDGFRα antibody (1:500) (Santa Cruz Biotechnology, CA) and secondary antibody (Alexa Fluor 488 anti-mouse, Molecular Probes) (1:1000).

## Results

### *Design of AAV-based genetic selection*

A recessive screen involves the identification of a change in phenotype because of the loss of gene expression. An example of this technique is the use of selection in a polyclonal population in which mutagenesis or loss of a gene results in a block in gene expression and alters the cell phenotype. Hemizygotic cells are an advantage in recessive screens and we chose to use the hemizygous furin-deficient Chinese Hamster Ovary cell mutant CHO FD11 (Gordon et al., 1995), which has been successfully used in recessive screens. Although the original FD11 isolate was clonal following the initial selection, subsequent passages and expansions can result in drift and spontaneous mutations to accumulate at other hemizygous loci, so that the resulting population of cells can be useful in subsequent screenings. Thus, the strategy to identify genes associated with AAV transduction involved transducing this population of CHO FD11 cells with AAV vectors encoding furin (AAVF) and then selecting resistant cells with either PE or PA toxin. To determine which serotypes could be screened using this strategy, CHO FD11 cells were transduced with three different serotypes (AAV2, AAV4, AAV5) of AAV encoding the $\beta$-gal gene and their transduction efficiencies compared (Fig. 1a). CHO FD11 cells were permissive for both AAV2 and AAV5 but resistant to AAV4 transduction. Because AAV5 exhibited a higher transduction efficiency, it was chosen for the recessive screen.

### *CHO FD11 transduction and PE selection*

To determine the optimal toxin concentration, cell killing experiments were first done using the furin expressing cells (CHO-K1) and the furin deficient and toxin-resistant CHO FD11 cell line. Concentrations of 0.3 μg/ml PE and 0.03 μg/ml PA were chosen for use in the screening. All PA selections were carried out in concert with addition of 20 ng/ml FP59 (Gordon et al., 1995). An MOI of 500 AAV5F virions/cell led to a 99% kill of the cells subsequently treated with PE (Fig. 1b), while at an MOI of 1000 no cells survived. However, even at an MOI of 1000, transduction with AAV4F did not affect cell viability, confirming the previous data in Fig. 1a regarding the different cell tropisms of AAV serotypes. From this data, we concluded that AAV5 transduction (i.e., rAAV5F) could complement furin deficiency in CHO FD11 cells.

### *Selection of CHO FD11 cells resistant to AAV5 transduction*

To conduct the selection, four rounds of transduction were carried out with increasing amounts of vector on pooled cells. As AAV vectors tend to remain episomal, they will eventually be lost in a dividing tissue culture cell population allowing for subsequent rounds of selection. To minimize the potential of false positives that might occur due to resistance to PE selection, the cells were challenged with PA in the second round of selection instead of PE and then challenged with PE in subsequent rounds.