

Intercellular Trafficking of Adenovirus-Delivered HSV VP22 from the Retinal Pigment Epithelium to the Photoreceptors—Implications for Gene Therapy

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Adenovirus (Ad)-mediated gene transfer is a promising technology for therapy of a wide variety of genetic disorders of the retina. The tropism of Ad vectors limits their utility to cells that express the coxsackie-adenovirus receptor. Upon ocular delivery, Ad vectors primarily infect the retinal pigment epithelium (RPE) and the Müller cells of the retina. However, the most frequent blinding diseases such as retinitis pigmentosa and age-related macular degeneration are associated with the expression of mutant proteins in the photoreceptors. In this study we demonstrate that adenovirus-delivered heterologous proteins fused to the herpes simplex virus tegument protein VP22 can translocate from infected cells to uninfected cells in culture and *in vivo*. We tested three different ocular cell lines, specifically Y79, RPE-J, and Chang C. We show that there is a 3.25-fold increase in the number of Y79 cells that take up GFP mediated by the intercellular trafficking properties of VP22. Our data are based on FACS analysis of *living* cells and there was no need for cell fixation for the effect to be observed. When adenovirus expressing a VP22-GFP fusion was injected into the subretinal space of adult mice, the VP22-GFP fusion peptides translocated from the RPE to all of the other layers of the retina, including the outer nuclear layer, which contains the photoreceptor cell bodies. Our study has significant implications for a wide variety of diseases of the retina and other organ systems.

Key Words: VP22; herpes simplex virus; adenovirus; retina; photoreceptor.

INTRODUCTION

Current estimates indicate visual impairment as one of the top 10 causes of disability in the United States, affecting approximately 14 million people [1]. Recent progress in genetic technologies has allowed us to elucidate the causes of a variety of diseases of the retina, including retinitis pigmentosa (RP), age-related macular degeneration (AMD), diabetic retinopathy, and glaucoma. These diseases collectively are the most common cause of blindness in the United States. In each of these diseases specific cell types of the retina degenerate, most often the photoreceptor cells. In many cases the degeneration begins with a loss of rod cells that is followed by a concomitant loss of the cone cells [2,3]. This happens even though the identified genetic cause often resides exclusively in the rod cells [reviewed in 4]. It is hypothesized that the rods release factors that are responsible for survival of the cones [5]. Hence, any proposed therapy will have signifi-

cantly more clinical benefit should it allow rescue of the majority of photoreceptor cells, ideally every photoreceptor cell in the retina.

Adeno-associated virus (AAV)-mediated gene transfer has shown promising results as a therapy for RP and allied disorders [6]. AAV vectors have a transgene capacity of approximately 4.8 kb [7]. However, many of the genes (cDNAs) involved in degeneration of the retina are particularly large. For example, the ABCR cDNA, which is associated with AMD, is 7 kb in length [8], or the dystrophin cDNA, which has been shown to be involved in visual dysfunction in Duchenne muscular dystrophy [9], is approximately 14 kb. Myosin VIIA, the gene responsible for Usher syndrome type III, a disease that associates congenital sensorineural deafness and RP, has a cDNA of approximately 6.6 kb [10]. Inclusion of these cDNAs in AAV vectors is not possible. Inclusion of large upstream gene regulatory elements for the smaller (<4.5 kb) cDNAs is also not possible. Inclusion of appropriate gene regula-

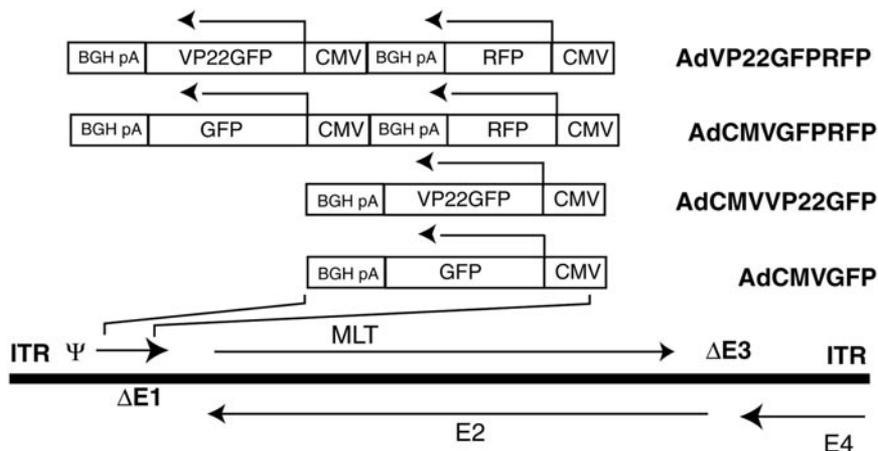


Fig. 1. Structure of viruses constructed for this study. All expression cassettes were placed in the antisense orientation (with respect to the E1 enhancer) into the E1 region of an E1/E3-deleted Ad5 virus. Each cDNA is regulated by the cytomegalovirus (CMV) promoter/enhancer. VP22, virion protein 22 of herpes simplex virus; RFP, red fluorescent protein; GFP, green fluorescent protein; BGH pA, bovine growth hormone polyadenylation signal; ITR, adenovirus inverted terminal repeat; Ψ , Ad packaging signals; MLT, major late transcription unit; E, early region.

tory elements is important in view of the observations that overexpression of normal gene products can lead to retinal degeneration both in the context of AAV-mediated gene transfer [11] and in transgenic mouse models of retinal degeneration [12]. Lentivirus has a transgene cloning capacity of approximately 7 kb and suffers from some of the same drawbacks as AAV [7] in terms of capacity for inclusion of large upstream regulatory elements. Moreover, HIV-based lentivirus vectors may need to pass higher standards in terms of safety in the context of treatment of nonlethal diseases. One promising solution to these issues is the use of "guttled" adenovirus vectors that have a cloning capacity of approximately 36 kb [13–16]. Using gutted adenovirus vectors we have previously demonstrated delivery of a 14-kb dystrophin cDNA to cells in culture [15]. More recently, using the same vector backbone, we have demonstrated partial correction of retinal degeneration in an animal model of RP [17]. However, adenovirus vectors do not infect photoreceptors very efficiently, presumably due to a lack of the coxsackie-adenovirus receptor [18] on the plasma membrane of photoreceptor cells. Instead, adenovirus vectors efficiently infect the adjacent retinal pigment epithelium (RPE), a supporting layer of cells in the retina that are in intimate contact with the photoreceptors.

In this study we postulate whether proteins synthesized in adenovirus-infected RPE cells can translocate across RPE cell membranes and enter other cell types of the retina, specifically the photoreceptor cells. Certain viral proteins such as the herpes simplex virus type 1 (HSV-1) VP22 protein, when fused or attached to heterologous proteins, allow those proteins to traverse cell membranes [19,20]. This ability to translocate has been shown to occur at 4°C and is hence not dependent on classical

endocytosis or energy [19]. This transport is extremely efficient *in vitro*, in which 100% of cells can be shown to take up heterologous proteins fused to VP22 [21]. However, there has been significant debate whether these observations are authentic or an artifact of fixation [22]. In this study we demonstrate intercellular trafficking of HSV VP22 in *living* ocular cells. We also demonstrate that the effect is observable *in vivo* in mouse retina. Hence, we provide supporting evidence that intercellular trafficking of VP22 is indeed real and observable under different experimental conditions. Our results have significant implications for gene transfer to photoreceptors and other cell types of the retina.

RESULTS

Adenovirus Serotype 5 Has Tropism for RPE and Müller Cells

Two commonly used methods for gene transfer to the retina include subretinal and intravitreal delivery of viral vectors. Subretinal injections involve delivery of virus into the region between the photoreceptors and the RPE by the creation of a temporary retinal detachment (bleb) that disappears after a few hours or days. Intravitreal injections involve the delivery of virus into the region between the retina and the lens. Several previous studies have examined the tropism of adenovirus (Ad) in the mouse eye; however, each has reached a slightly different conclusion [23–32], presumably due to slight variability in the injection technique, varying methods of virus purification and virus titer. To first identify which cell types of the retina are transduced by each type of injection in our hands, we constructed a first-generation Ad of serotype 5 expressing GFP regulated by a CMV promoter (Fig.

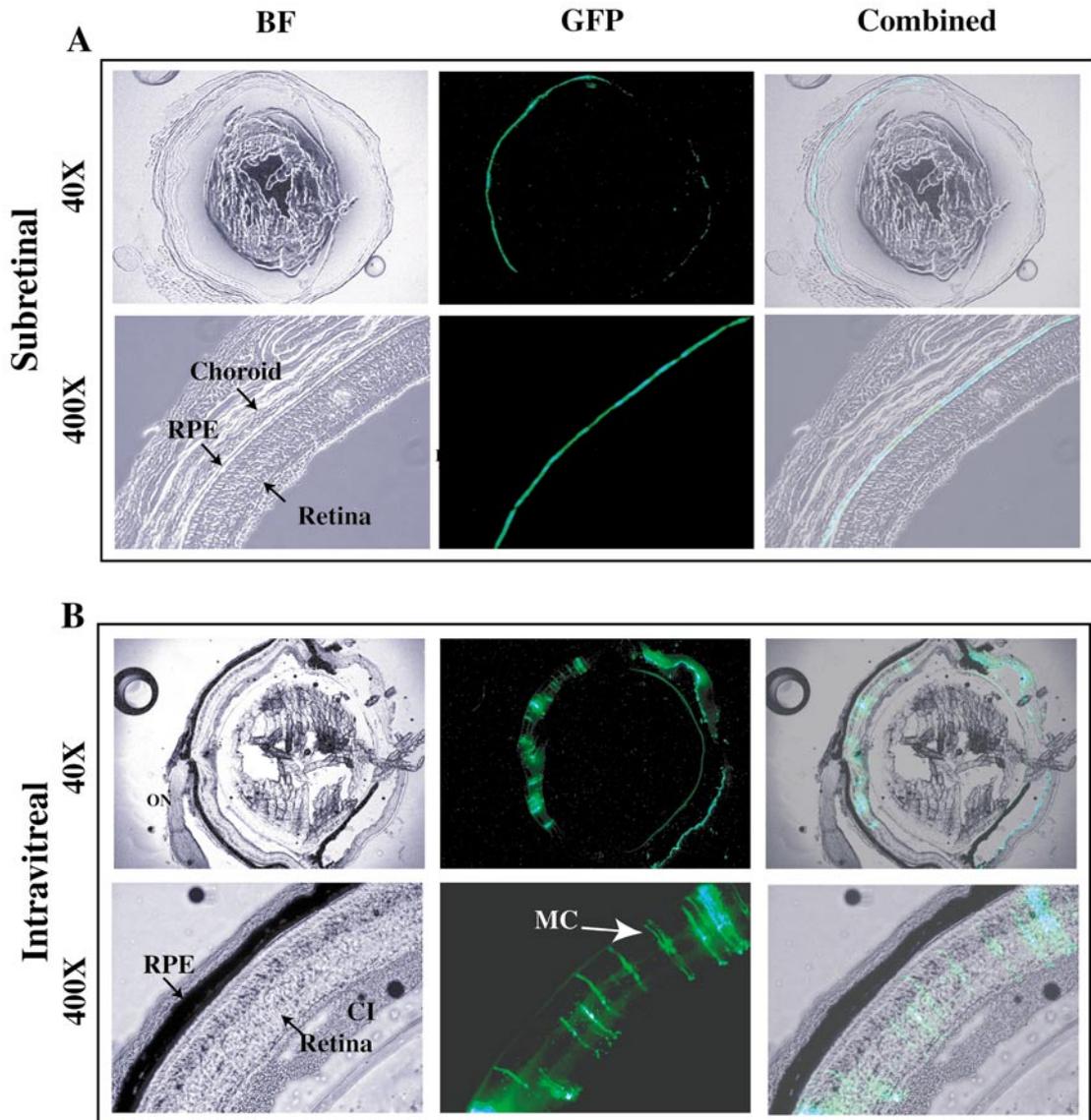


Fig. 2. Frozen sections of retinas injected with AdCMVGFP via the (A) subretinal route in 2-month-old C57BL/6J-Tyr^{c-2J/+} mouse. Approximately 80% of RPE cells are GFP-positive. (B) Intravitreal injection into C57BL/6J mouse of similar age, which allows gene delivery primarily to the Müller cells (MC), lens epithelium, corneal endothelium, and iris. Note the cellular infiltrate (CI) postinjection in this particular example. ON, optic nerve. Original magnifications 40 \times and 400 \times .

1). We injected 2 month-old C57BL/6J and C57BL/6J-Tyr^{c-2J/+} mice and determined that subretinal delivery of Ad leads to gene transfer primarily to the RPE. In fact, with a single injection as much as 80% of the RPE can be transduced (Fig. 2A). In contrast, intravitreal injection leads primarily to transduction of the Müller cells, lens epithelium, corneal endothelium, and iris (Fig. 2B). We observed these same patterns of expression in both C57BL/6J and in C57BL/6J-Tyr^{c-2J/+} mice. Transduction of the RPE is most easily observed in albino mice due to the absence of pigment.

Construction of a Fusion between VP22 and GFP

VP22 is a HSV tegument protein coded by the UL49 gene [33]. To characterize the intercellular spread of VP22 in retinal cells in culture and *in vivo*, we constructed an Ad (AdVP22GFPRFP) with a dual expression cassette (Fig. 1). One expression cassette consisted of a cDNA for VP22 fused to GFP and the second cassette expressed red fluorescent protein (RFP) without a VP22 fusion. Both transgenes were regulated by a CMV promoter and both cassettes were in the same (antisense) orientation with respect to the Ad E1 enhancer/promoter (Fig. 1). We de-

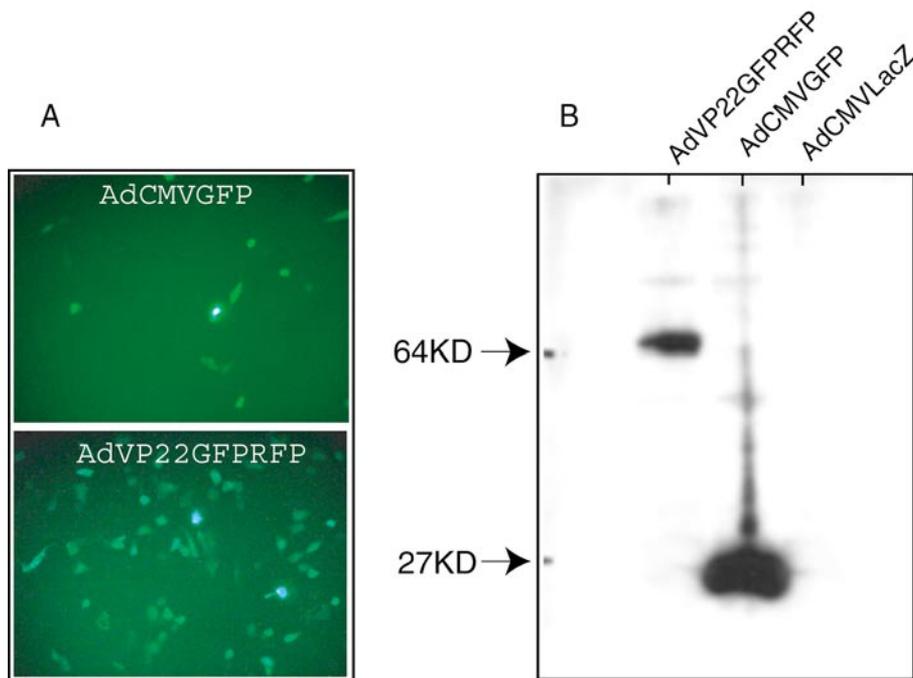


Fig. 3. GFP fluorescence from AdCMVGFP- or AdVP22GFPRFP-infected 911 cells in culture (A). Western analysis using GFP antibodies of protein lysates prepared from these cells indicates the expected product sizes of 64 (VP22–GFP) or 27 kDa (GFP).

signed the virus in this manner to take into consideration possible different expression levels from the two expression cassettes due to proximity with the E1 enhancer such that any differences in protein expression/localization could be attributed to VP22. First we identified whether the 33-kDa HSV VP22 successfully formed a fusion with the 27-kDa GFP by direct observation of fluorescence from virally infected 911 human embryonic retinoblast [34] cells (Fig. 3A). We then verified the expected product size of the VP22–GFP fusion by Western analysis (Fig. 3B) using antibodies reactive to GFP in AdVP22GFPRFP-infected 911 cell lysates. As expected, anti-GFP antibodies reacted specifically either to a 64-kDa fusion protein (VP22–GFP) or a 27-kDa GFP protein (Fig. 3). Unlike in some previous studies [35] we did not observe any significant degradation of the VP22–GFP fusion product.

***In Vitro* Studies**

To observe the possible intercellular spread of VP22 in culture, we tested three cell lines representative of ocular tissue—specifically Y79, RPE-J, and Chang C. Although the cellular origin of Y79 cells is not clearly defined, they are derived from the retina [36–38] and have previously been shown to express photoreceptor-specific genes [39]. These cells have been used in many studies to study photoreceptor biology [38]. RPE-J and Chang C are rat RPE [40] and human conjunctiva [41] cell lines, respectively. We examined intercellular transport of VP22–GFP

by infection of each ocular cell line with AdVP22GFPRFP compared with AdCMVGFP. We observed intercellular spread of VP22 both qualitatively by direct visualization of fluorescent cells (Fig. 4) and quantitatively by FACS analysis (Fig. 5). We observed that when Y79 cells were infected with AdVP22GFPRFP, approximately 22.79% of cells were RFP-positive (infected cells); however, 74.03% of cells were GFP-positive, representing a 225% increase (3.25-fold) in the number of cells that contained GFP but were *not* infected (Table 1). Similarly, for RPE-J and Chang C cells there was a 93% (1.93-fold) and 40% (1.40-fold) increase in the number of GFP-*only*-positive cells (Table 1). Since the number of GFP- and RFP-positive cells is approximately the same in AdCMVGFP-infected RPE-J and Chang C cells as determined by FACS (Fig. 5), it is concluded that the kinetics of the two expression cassettes in terms of transgene expression are comparable. Hence, the difference in number of GFP-positive cells may be attributed to an effect associated with the intercellular trafficking properties of VP22. However, we did note a slightly higher number of GFP-positive cells than RFP-positive cells in AdCMVGFP-infected Y79 cells that may contribute to slight overestimation of the effects of VP22 in these cells.

***In Vivo* Studies**

We next determined whether VP22 intercellular trafficking could be observed *in vivo* and whether the RPE cells of

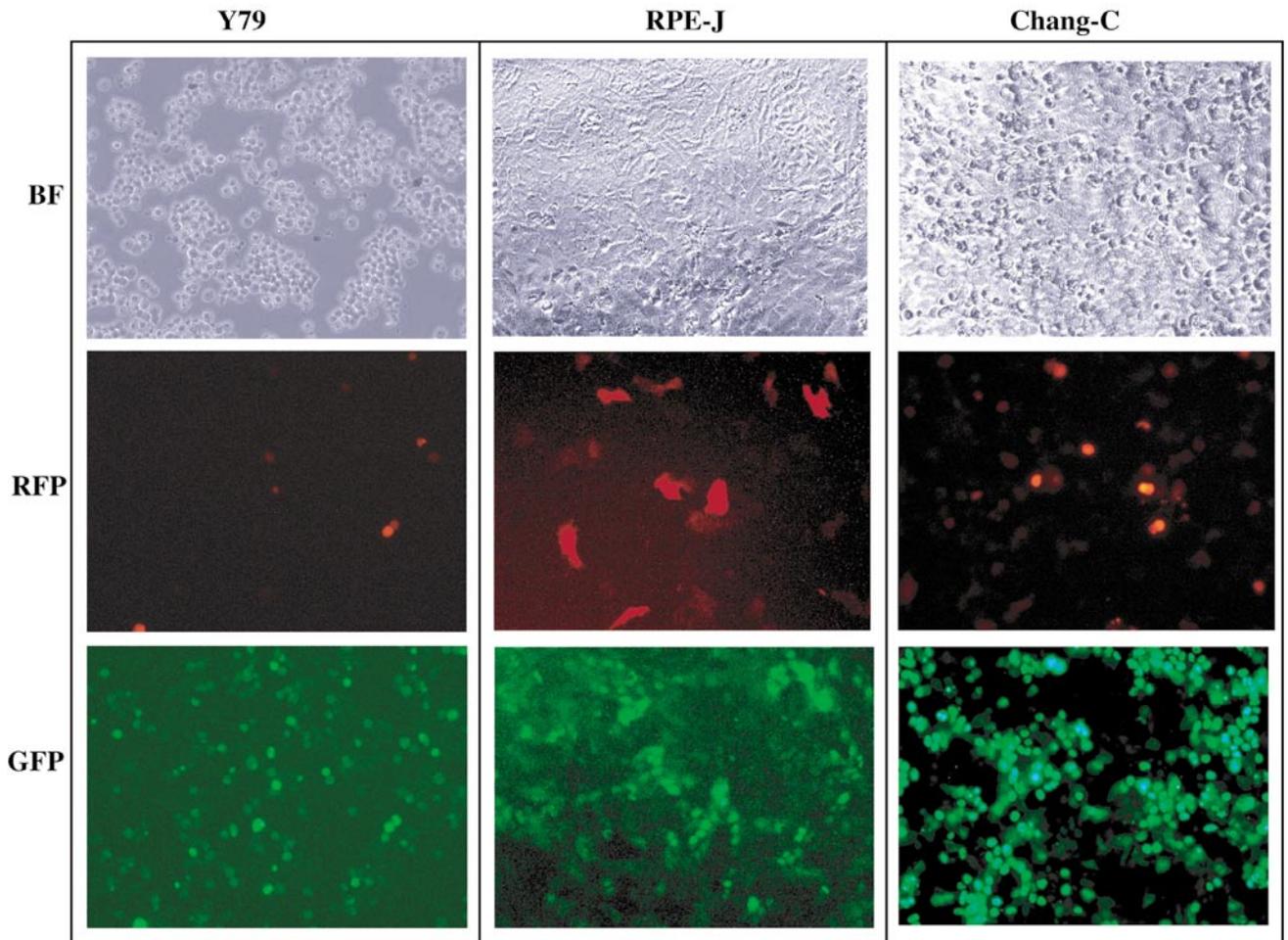


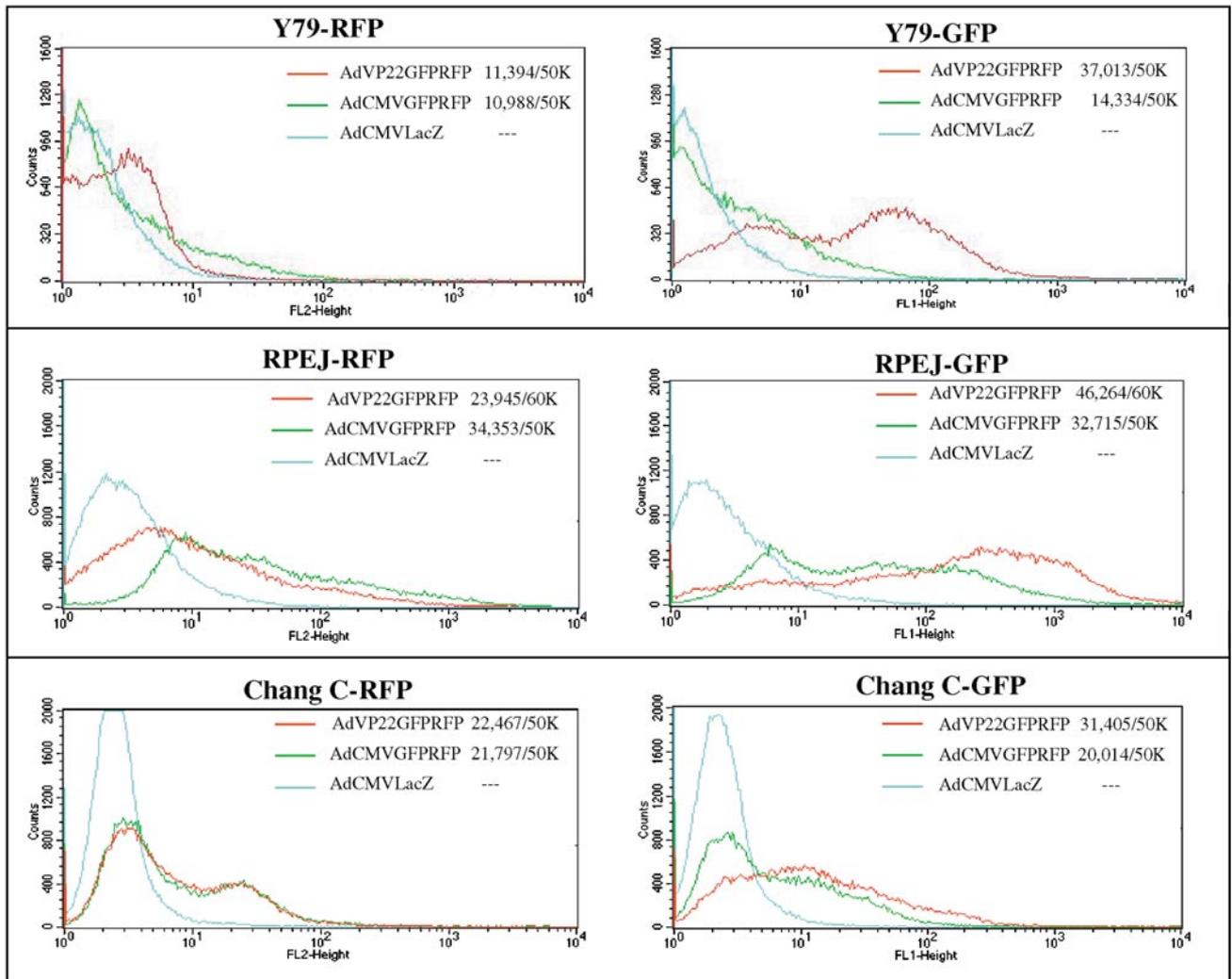
Fig. 4. Fluorescence microscopy of AdVP22GFPRFP-infected live ocular cells. BF, bright field. Original magnification $\times 100$.

the retina could be used as a source of protein for other cells. Subretinal injection of AdVP22GFPRFP indicates GFP-positive cells in the RPE as expected, but in contrast to results from AdCMVGFP (Fig. 2) or AdCMVGFRFP, cells in other layers of the retina (including the outer nuclear layer, which contains the photoreceptor cell bodies) were also GFP-positive (Fig. 6A). We found that the type of cells that contained trafficked GFP varied between injections. Closer examination of subretinally injected sections indicates that cells of each of the major layers in the retina contain trafficked GFP, including the inner and outer nuclear layers, the inner and outer plexiform layers, and the ganglion cell layer (Fig. 6B). Intravitreal injection also leads to an increase in the number of Müller cells that are GFP-positive (Fig. 6A). Interestingly, with intravitreal injections only Müller cells demonstrated uptake of trafficked GFP.

Localization of VP22 in Living Cells

Recently Lundberg and colleagues determined that VP22 localizes exclusively to the cell membrane and not to any other cellular structure prior to methanol fixation. Following fixation, VP22 was shown to translocate to the nucleus in those studies [22]. We have examined the localization of VP22 in living adenovirus-infected cells. We found that VP22-GFP may be seen on the cell membrane in addition to the nucleus and other undetermined cell structures (Fig. 7A). Clearly our results contrast to those of Lundberg. Although our viruses were plaque purified and quality tested postamplification, we considered the possibility of rearranged viruses (lacking RFP) affecting our experimental data. To address this possibility we constructed a virus expressing a fused VP22-GFP without an RFP cassette (Fig. 1). Infection of 911 cells then allows antibody probing for the Ad DNA binding protein (DBP)

A



B

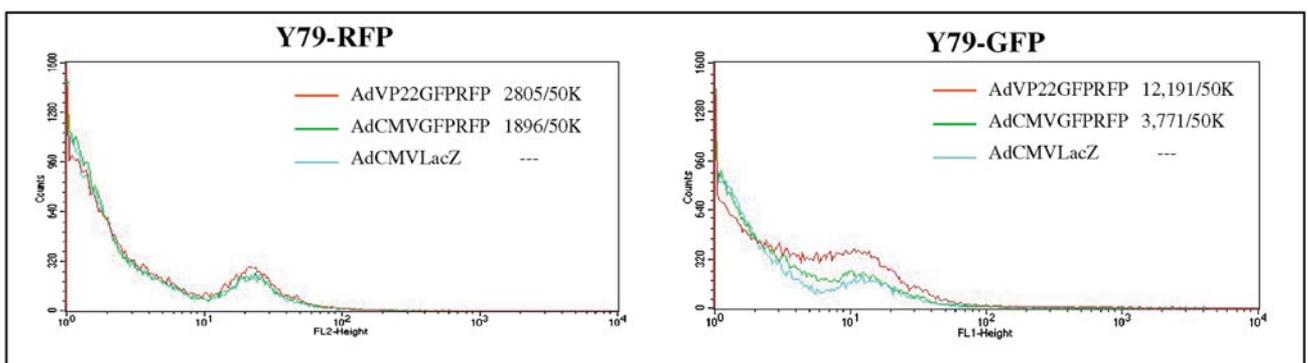


Fig. 5. Fluorescence-activated cell sorting (FACS) of Y79, RPE-J, and Chang C cells infected by AdVP22GFPRFP, AdCMVGFRFP, and AdCMVLacZ control (Ad expressing Lac Z) 48 h postinfection (A). Cell counts from AdCMVLacZ were subtracted from those of AdVP22GFPRFP and AdCMVGFRFP using BD CellQuest Pro software. GFP- (FL-1 Height) or RFP- (FL-2 Height) positive cells were counted for each virus and cell type. (B) FACS of Y79 cells infected with the same three viruses at 24 h postinfection.

TABLE 1 Summary of FACS data indicating the percentage of cells gated for GFP or RFP with each virus and cell type

Cell line/virus	RFP (%)	GFP (%)	Percentage/fold-increase (GFP)
Y79/AdVP22GFPRFP	22.79	74.03	225/3.25
Y79/AdCMVGFPRFP	21.98	28.67	30/1.30
RPE-J/AdVP22GFPRFP	39.91	77.10	93/1.93
RPE-J/AdCMVGFPRFP	68.71	65.43	—
Chang C/AdVP22GFPRFP	44.93	62.81	40/1.40
Chang C/AdCMVGFPRFP	43.59	40.02	—

The percentage/fold increase in GFP-only-positive cells for each experiment is indicated in the right most column.

that is present on the vector backbone. Since 911 cells allow replication of the viral genome, this is a sensitive method for detection of adenovirus within cells. Upon infection of 911 cells with AdVP22GFP followed by fixation and probing with anti-DBP, we found cells that are GFP-positive and rhodamine- (secondary antibody to DBP) negative (Fig. 7B). This experiment independently confirms some of our observations on living cells. Again, of note is that in fixed cells we found GFP staining to be mainly in the cytoplasm and not the nucleus as reported by Lundberg and colleagues [22]. In conclusion, the intercellular trafficking properties of HSV VP22 may be observed with or without cell fixation.

DISCUSSION

Recently several protein transduction domains (PTDs) that possess the unusual ability to traverse lipid bilayers in a receptor- and transporter-independent manner have been identified. PTDs that are chemically cross-linked to

heterologous proteins, antibodies, and enzymes have been shown to transduce those proteins across cell membranes [42,43]. Protein transduction was first reported a decade ago by Green and Frankel, who independently demonstrated that the Tat protein from HIV-1 was able to enter cells when added to the surrounding medium [44,45]. Subsequently, several other proteins with transducing capabilities have been identified, including the *Drosophila* homeotic transcription factor ANTP (encoded by the antennapedia gene) and the HSV-1 VP22 [19,46,47]. The domains responsible for transduction have been identified in each of these proteins [19,48–51].

Since the original reports of Elliot and O'Hare [19,20], intercellular trafficking of VP22 has been refuted in some studies. Hence, the authenticity of the effect has been called into question and the field remains controversial. The original observations were contradicted by Fang and colleagues [52]. These authors did not observe intercellular trafficking of VP22-GFP fusion proteins in cultured mammalian cells. In addition, Aints and colleagues [21] examined four cell lines that had been transfected with plasmids expressing VP22 fused to GFP. They found that translocated GFP-VP22 fusion proteins could be detected only after fixation. In fact, after fixation they observed GFP in 100% of cells in the monolayer. They did not detect functional GFP in live recipient cells. Recently, Lundberg and colleagues [22] examined intercellular trafficking of VP22 before and after methanol fixation. They concluded that VP22 is bound to the cell surface and methanol fixation results in permeabilization of the cell membrane and import of the VP22 occurs as a result of permeabilization. Falnes and colleagues [53] recently examined the ability of VP22 to translocate diphtheria toxin A fragment across cell membranes. Although import of only a few toxin molecules is sufficient to cause cell death,

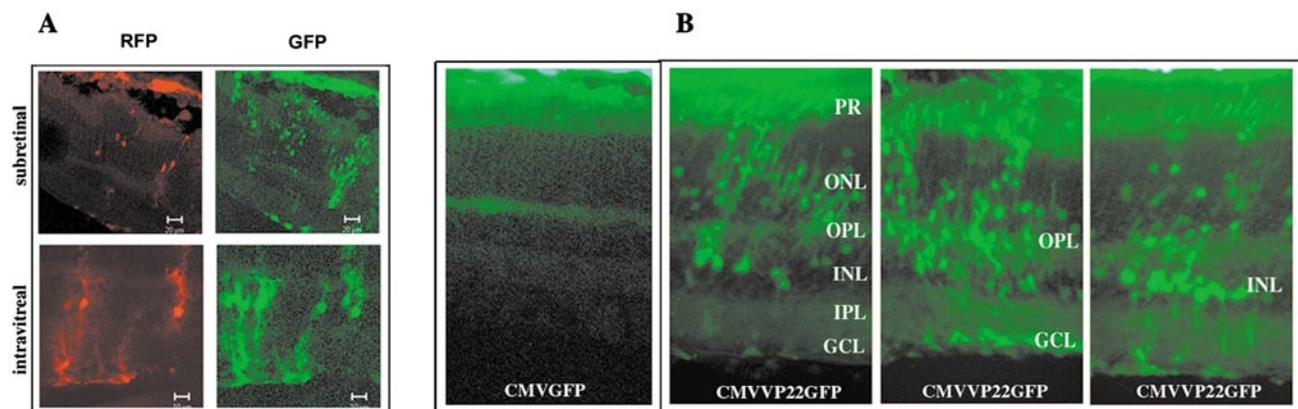


Fig. 6. Subretinal and intravitreal injection of AdVP22GFPRFP in adult (2 months) C57BL/6J retina 1 week postinjection (A). Closer examination of subretinal injections of various sections indicates GFP-positive cells in all of the major layers of the neural retina (B). GFP-positive cells in the inner retina are not as bright as those found in the RPE, and hence longer exposure times were required. PR, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

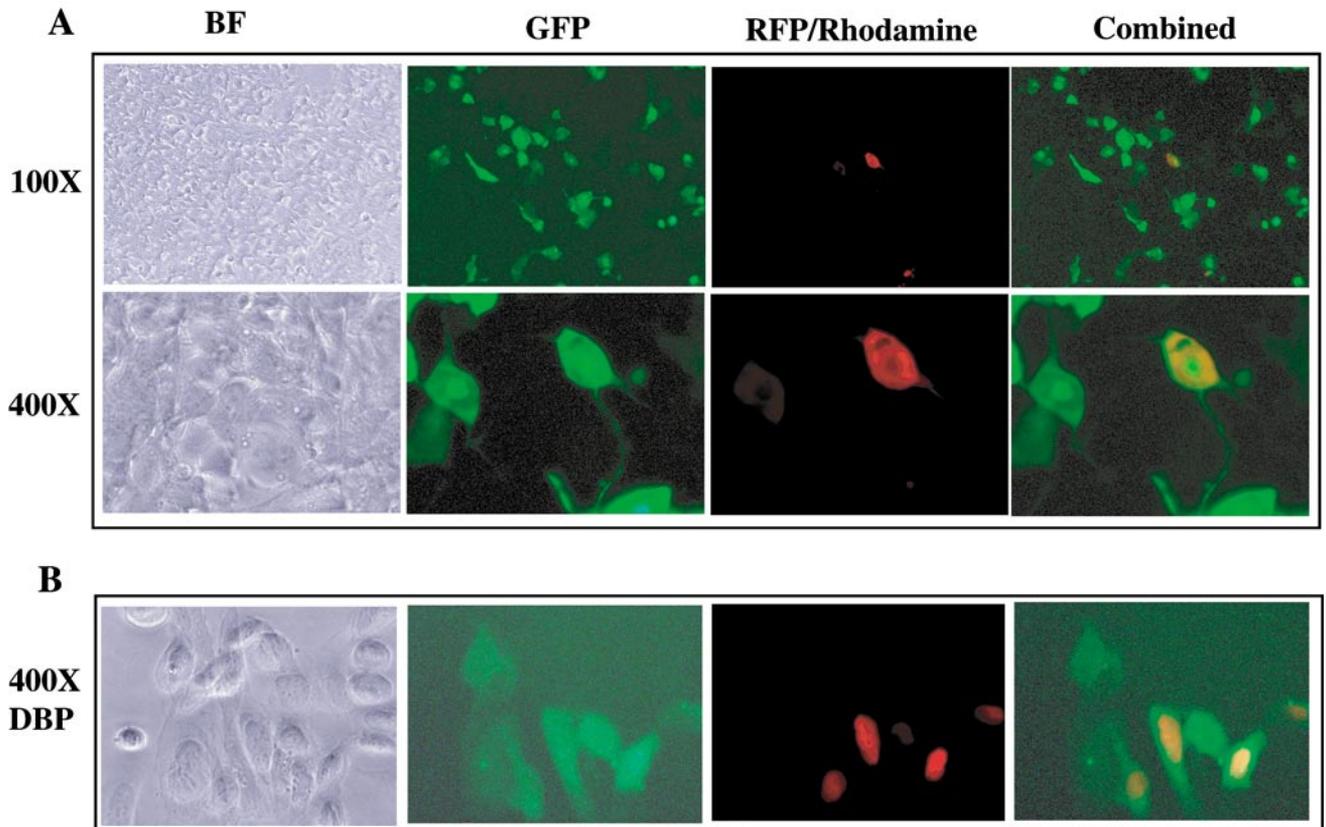


Fig. 7. GFP and RFP fluorescence of AdVP22GFP-RFP-infected live 911 cells (A) and GFP and rhodamine fluorescence from AdCMVVP22GFP-infected fixed 911 cells (B). Fixed cells were stained with primary antibody against adenovirus DNA binding protein (DBP). Note that in living cells there is GFP fluorescence in cellular structures other than the plasma membrane. GFP fluorescence is primarily cytoplasmic in fixed cells. Original magnifications 100 \times and 400 \times .

no toxicity was observed. These authors also concluded that the ability of VP22 to translocate across cell membranes is very inefficient.

In contrast, Wills and colleagues [54] demonstrated intercellular trafficking of a p53 tumor suppressor protein using a plasmid expressing full-length p53 fused in frame to full-length VP22. The p53-VP22 chimeric protein induced apoptosis both in transfected tumor cells and in neighboring cells, resulting in a widespread cytotoxic effect. VP22 has also now been shown to enhance intercellular trafficking of TK and amplify the TK/GCV killing effect [55].

One key difference between our study and those of others is our inclusion of a marker gene (RFP) in the same construct as that expressing the VP22-GFP fusion. This has allowed us to unambiguously differentiate cells that are expressing VP22-GFP from those that have taken it up from neighboring cells via intercellular transport. Unexpectedly, we found that there were many relatively bright cells that contained GFP but no RFP. This observation is in contrast to previous studies in which it was assumed that the recipient cells were the weakly fluorescent cells.

In this study we have demonstrated transport of the VP22-GFP fusion in three different ocular cell lines, both qualitatively (Fig. 4, fluorescence microscopy of live cells) and quantitatively (Fig. 5, FACS analysis of live cells). We had no difficulty in observing the VP22 effect directly in live cells. In fact, given previously published data, what was surprising to us was the presence and number of strongly GFP-positive cells (presumably VP22-GFP) that did not express RFP. We observed this for all three ocular cell types examined. The increase in VP22-mediated transduction of Y79 (3.25-fold) is comparable to or more often greater than that observed by Wybranietz and colleagues [35] in a variety of cell lines. This could reflect the context in which the fusion protein is expressed, i.e., adenovirus versus plasmid.

To augment our *in vitro* results, we have also shown protein transduction to many of the different cell layers of the retina, ranging from the RPE to the ganglion cells. While our main interests reside in the photoreceptor cells, other retinal cell types have been also shown to be involved in retinal disease. For example, X-linked retinopathy is characterized by microcystic-like changes of the

macular region and schisis within the inner retinal layers. The protein involved, retinoschisin, is released by photoreceptors and has functions within the inner retinal layers [56]. The ability of VP22-fused proteins to translocate from the RPE to these other cell layers makes these cells amenable to gene therapy.

A significantly surprising observation was the different cell types that demonstrated import/export of VP22-GFP *in vivo*. Whereas subretinal injection led to VP22-GFP trafficking to all the major cell layers in the retina (following primary infection of the RPE), intravitreal injection led to limited export/import of VP22 among Müller cells only. This limited export might parallel our observations in cell culture that demonstrated significant differences in efficiency between cell types that permit VP22-GFP trafficking. For example, Y79 cells permit significantly greater VP22-mediated intercellular trafficking than either Chang C or RPE-J cells (Table 1). A similar observation has been made by Wybranietz and colleagues [35] in various cell lines. Alternatively, one might explain this observation by assuming an activation of Müller cells in response to a viral infection and/or an immune response resulting from intravitreal injection (see cellular infiltrate in Fig. 2B) that leads to Müller cell division. Elliott and O'Hare have previously implicated cell division [57] and the cytoskeleton [19] in intra/intercellular trafficking of VP22. After retinal injury Müller cells undergo limited cell division and experience alterations in their cytoskeleton [58].

Despite our promising results some important questions remain. One problem that cannot be easily overcome is the potential deleterious effect of the presence of cell-specific proteins in the inappropriate cell type. For example, it is not clear what the effects of rod-specific β -phosphodiesterase (β PDE) would be in cone cells or some other cell type in the retina. In previous studies we have used the β PDE promoter to drive transgene expression specifically in rod cells [17]. In the context of VP22, it is not obvious how this shortcoming might be resolved. It is possible that the deleterious effects of an otherwise cell-specific protein will not outweigh the beneficial effects of rescue of photoreceptor degeneration by augmentation of normal gene products. This remains to be tested. Another concern might be the inappropriate functioning or folding of the heterologous peptide when fused to VP22. We included a flexible polyglycine linker between VP22 and GFP that had no significant effect on the function of GFP. We used a 300-aa VP22 peptide in our studies to demonstrate "proof of principle," whereas the PTD of VP22 has been mapped to within 33 amino acids, specifically residues 267–300 of VP22 [19]. Addition of a smaller number of amino acids may be advantageous. Several of the proteins in photoreceptor cells implicated in retinal degeneration are transmembrane proteins, such as rhodopsin and peripherin/RDS [59]. It is possible that fusion of VP22 to these proteins will affect the ability of these

proteins to localize in the photoreceptor disk membrane. However, since there are many examples in which small peptide fragments have been fused to proteins without significantly altering activity and/or function [60], it is quite likely that VP22-mediated intercellular trafficking will have application in some diseases of the retina.

In our study we demonstrated proof of principle using a first-generation Ad vector. Recent developments in Ad vector technology have enabled the construction of gutted Ads that allow "lifetime" correction of pathology in mouse models of inherited diseases [61]. Gutted vectors also evade the well-documented immune response associated with first-generation Ads, which leads to T-cell-mediated destruction of Ad-transduced cells. The ability of proteins to spread through retinal tissues opens up the possibility of a new range of diseases that might be amenable to treatment using Ad vectors.

MATERIALS AND METHODS

Recombinant adenovirus constructs. All expression cassettes were cloned in the antisense orientation with respect to the E1 enhancer/promoter in an E1/E3-deleted adenovirus backbone. AdVP22GFPRFP was constructed by first cloning the 1.8-kb *Bgl*II/*Eco*RI fragment from pVP22/*myc*-His (Invitrogen Life Technologies, Carlsbad, CA) into the *Bgl*II/*Eco*RI site of pQBI25-fa2 (Qbiogene, Carlsbad, CA). This fuses the VP22 open reading frame to the N terminus of GFP via a flexible polyglycine linker, generating pVP22GFP. The 3.3-kb *Bgl*II/*Xmn*I fragment from pVP22GFP was cloned into *Bgl*II/*Eco*RV-digested pShuttle [62] generating pShVP22GFP. An RFP expression cassette was added to this plasmid by cloning the 2.4-kb *Bgl*II/*Xmn*I fragment from pQBIRFP (see below) into *Bgl*II/*Nru*I-digested pShVP22GFP, generating pShVP22GFPRFP. This plasmid was digested with *Pme*I and recombined with pAdEasy-1 [62] by cotransformation into *Escherichia coli* BJ5183. The resulting plasmid, pAdVP22GFPRFP, was subsequently transformed into *E. coli* DH10B (Invitrogen Life Technologies) for large-scale plasmid amplification. pAdVP22GFPRFP was digested with *Pac*I and transfected into 911 cells (see below).

pQBIRFP was constructed by cloning the 0.7-kb *Sac*II/*Not*I fragment from pDsRed2-NI (Clontech, Palo Alto, CA) into *Sac*II/*Not*I-digested pQBI-tatGFP (Qbiogene). All other viruses were constructed as described above.

Production of adenovirus. pAdVP22GFPRFP (12.5 μ g) was digested with *Pac*I, extracted with phenol-chloroform, and precipitated with 100% ethanol for calcium phosphate transfection as described previously [15] into 3×10^6 911 cells [34] in a 60-mm plate. The transfection was allowed to proceed overnight in DMEM (Invitrogen Life Technologies) with 2% FBS (Invitrogen Life Technologies). Transfected cells were monitored in DMEM and 10% FBS for cytopathic effect (CPE), which was evident by approximately 6 to 10 days posttransfection. Cells were harvested and subjected to three cycles of freezing in dry-ice/ethanol bath and thawing in 37°C water bath to release the virus. Large-scale preparation of plaque-purified virus was performed by propagation of freeze-thaw lysate in 50 confluent 150-mm plates of 911 cells grown in DMEM and 2% FBS. Once the cells had reached complete CPE, they were harvested, pelleted, and resuspended in an equal volume of 10 mM Tris-Cl, pH 8.1. The suspension was freeze-thawed three times followed by centrifugation at 1200 rpm for 3 min. The supernatant was transferred to a fresh tube and the pellet resuspended in 2 ml 10 mM Tris-Cl, pH 8.1, 1 ml Freon (Sigma-Aldrich Corp., St. Louis, MO). The supernatant resulting from a second centrifugation of this suspension was pooled with the original supernatant and centrifuged through a 20-ml cesium chloride gradient (10 ml of a 1.45 g/ml CsCl solution in 10 mM Tris, pH 8.0; 10 ml of a 1.20 g/ml CsCl solution in 10 mM Tris, pH 8.0) in an SW28 rotor at 20,000 rpm for 2 h at 5°C. The virus band was collected with a 5-ml syringe and a 28-gauge needle and the virus

washed three times with 12 ml 1% sucrose in PBS by centrifugation through a 15-ml centrifugal filter device (50K NMWL; Millipore Corp., Bedford, MA). In this device, the virus was concentrated to a volume of 600 μ l with A_{260} of 0.4 to 1.0. Virus titers were determined by infection of 911 cells for 12 h and counting the number of RFP-positive cells per unit volume. This approach allowed precise normalization between different viral constructs.

Cell lines. Ocular cell lines were obtained from the American Type Culture Collection. Y79 cells were maintained in RPMI 1640 with 15% FBS. RPE-J cells were maintained in DMEM with 4% FBS and 0.1 mM nonessential amino acids. Chang C and 911 cells were maintained in DMEM with 10% FBS. For Chang C and 911 cells the medium was switched to DMEM with 2% FBS 1 h prior to infection. Media, FBS, and supplements were purchased from Invitrogen Life Technologies. Y79 cells were plated on 35-mm poly-D-lysine-coated plates (Becton-Dickinson, Bedford, MA) prior to infection.

Fluorescence microscopy (cells). Chang C and RPE-J cells were infected at a multiplicity of infection (m.o.i.) of 7×10^2 with either AdLacZ or AdVP22GFPRFP on glass chamber slides (Nalge Nunc International Corp., Rochester, NY). Y79 cells were infected at an m.o.i. of 7×10^3 . Infections were allowed to proceed at 37°C/5% CO₂ for 48 h. Cells were visualized with a Nikon Eclipse TS100 microscope with a short-arc mercury lamp and a filter for either RFP (excitation/emission maxima 558 nm/583 nm) or GFP (excitation/emission maxima 474 nm/509 nm). Images were captured using either CoolSnap or RS Image software.

Immunocytochemistry (cells). 911 cells were infected at an m.o.i. of 4×10^2 with either AdCMVVP22GFP or AdVP22GFPRFP. The infection was allowed to proceed for 16 h. The AdCMVVP22GFP-infected cells were fixed with 3.7% formaldehyde and stained for adenovirus DNA binding protein with monoclonal antibody 37-3 [63]. Secondary detection was performed using a rhodamine-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Flow cytometry. Y79, RPE-J, and Chang C cells were infected at an m.o.i. of 7×10^3 , 3×10^3 , and 3×10^2 , respectively. Infections were allowed to proceed for 48 h at 37°C/5% CO₂. Infected cells were harvested by incubation with 1 \times trypsin-EDTA, rinsed with 1 \times PBS, and resuspended in 1 \times PBS to a concentration of 1×10^6 cells/ml. Cells with GFP fluorescence were detected using a FACScan (Becton-Dickinson) with a 15-MW argon laser (488 nm) activation source and a 530 \pm 15 nm filter. RFP fluorescence was detected using a 585 \pm 21 nm filter. Analysis was performed using CellQuest Pro software (Becton-Dickinson).

Isolation of protein for Western analyses. 911 cells were infected at an m.o.i. of 250. Cells were harvested 36 h later by scraping, rinsed twice with PBS, and homogenized in PBS containing leupeptin (10 μ g/ml), aprotinin (10 μ g/ml), and PMSF (0.1 mM). Ten micrograms of protein was loaded on a 12% denaturing gel (BMA, Rockland, ME). VP22-GFP and GFP were detected using the monoclonal GFP antibody 11E5 (Qbiogene). Secondary detection was performed using an HRP-conjugated anti-mouse antibody (Jackson ImmunoResearch) and ECL kit (Bio-Rad, Hercules, CA). Chemiluminescent signal was detected by exposure to Kodak BioMax ML film.

Intravitreal/subretinal injections. All animals were cared for in accordance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research and in compliance with federal, state, and local regulations. C57BL/6J and C57BL/6J-Tyr^{c-2J/+} mice were bred in our vivarium and reared under 12-h daily cycles of light and darkness. Mice were anesthetized with ip injection of 2.5% Avertin (0.015 ml/g body weight). Subretinal and intravitreal injections were performed with a transscleral transchoroidal approach [64] with a 30.5-gauge needle attached to a micromanipulator. A total of 4×10^9 particles of the relevant virus were injected by either approach. AdCMVlacZ-injected or PBS-injected contralateral eyes served as controls. Eyes were analyzed 7 days after injection.

Histology/immunofluorescence. Seven days postinjection, mice were sacrificed by CO₂ inhalation. Eyes were enucleated, fixed for 30 min in 4%

paraformaldehyde, embedded in tissue freezing medium (Triangle Bio-medical Sciences, Durham, NC), and sectioned to 14 μ m thickness. The tissues were viewed and images captured either with a Nikon Eclipse TS100 (as for cells in culture) or with a Zeiss LSM 510 laser scanning confocal microscope using LSM 510 software, version 2.8.

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