Identification of Adenovirus Serotype 5 Hexon Regions that Interact with Scavenger Receptors

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Abstract

Most of an intravenous dose of species C adenovirus serotype 5 (Ad5) is destroyed by liver Kupffer cells. In contrast, another species C virus, Ad6, evades these cells to mediate more efficient liver gene delivery. Given that this difference in Kupffer cell interaction is mediated by the hypervariable (HVR) loops of the virus hexon protein, we genetically modified each of the seven HVRs of Ad5 with a cysteine residue to enable conditional blocking of these sites with polyethylene glycol (PEG). We show that these modifications do not affect in vitro virus transduction. In contrast, after intravenous injection, targeted PEGylation at HVRs 1, 2, 5 and 7 increased viral liver transduction up to 20 fold. Elimination or saturation of liver Kupffer cells did not significantly affect this increase in the liver transduction. In vitro, PEGylation blocked uptake of viruses via the Kupffer cell scavenger receptor SRA-II. These data suggest that HVR1, 2, 5, and 7 of Ad5 may be involved in Kupffer cell recognition and subsequent destruction. These data also demonstrate that this conditional genetic-chemical mutation strategy is a useful tool to investigate the interactions of viruses with host tissues.
**Introduction**

Adenovirus serotype 5 (Ad5) has proven to be one of the most potent *in vivo* gene delivery vectors for liver-directed gene therapy. As much as 95 to 98% of an intravenously (i.v.) injected dose of Ad5 is trafficked to the liver (12). The adenovirus capsid is comprised of three major proteins: hexon, penton base, and fiber (reviewed in (7)). Based on numerous *in vitro* studies, Ad fiber and penton base have been shown to be cellular attachment proteins. The fiber of Ad serotype 5 (Ad5) binds coxsackie and adenovirus receptor (CAR) (3), which triggers binding of penton base to αv integrins via an RGD motif (35, 36) and results in viral cell internalization. Although necessary for providing specificity of the virus *in vitro*, modifications to the fiber have little effect on vector tropism *in vivo* (25). Instead, increasing evidence suggests that hexon plays a large role in the natural liver tropism of Ad5.

Hexon is the most abundant viral capsid protein with 720 monomers per virion. Hexon organizes into trimers so that three hexon monomers and their loops wrap tightly around each other to create a tower-like structure with a central depression (20, 28). Each hexon monomer has seven flexible, serotype-specific loops, named hypervariable regions (HVR) (23) that are predicted to be located on the surface of the hexon trimer and the virion (29). This location allows the HVRs of hexon to interact with neutralizing antibodies, receptors, proteins, and cells. Considering there are 5,040 (720 x 7 = 5040) HVRs per virion, these represent a complex, exposed surface for many interactions.

After intravenous (i.v.) injection, Ad5 exhibits the greatest transduction within liver hepatocytes (12). Despite this robust *in vivo* gene delivery, approximately 90% of the injected dose is sequestered and destroyed by resident liver macrophages called...
Kupffer cells (1). These antigen-presenting cells not only destroy the virus, but are themselves destroyed. This cellular necrosis plays a role in inflammation and innate and adaptive immune responses to the virus (21). Previous studies indicate that Kupffer cells take up Ad5 via scavenger receptors (13, 37). It is hypothesized that scavenger receptors on these cells recognize the virus by interacting with the highly charged HVRs on Ad5 (1, 37). In particular, HVR1 is thought to be a good ligand for scavenger receptors (1), since it is the largest HVR and also has a number of negatively charged amino acids (18)). Results shown here indicate that hexon and its HVRs are important surfaces involved in Kupffer cell recognition. By evading such cells, adenoviruses should be able to more readily transduce downstream sites.

After evading the reticuloendothelial system (which includes both Kupffer cells and liver sinusoidal endothelial cells), Ad5 enters the liver parenchyma through fenestrations in the vessel walls (32). If Ad can enter the parenchyma, robust hepatocyte infection is dependent not only on cell binding ligands evolved by the virus, but also on host blood proteins. Evidence shows that Ad5 hexon binds to vitamin K dependent coagulation factors, such as factor X (FX), with high affinity, and this interaction markedly increases hepatocyte infection (26, 31). Structural and mutational analysis has revealed that FX binds to the top of the hexon trimer depression, with predicted interactions with HVR5, HVR7, and possibly HVR3 (16, 33). These data implicate the HVRs of the Ad5 hexon in two pharmacologic bottlenecks of the virus after i.v. injection: 1) binding or evasion of Kupffer cells and 2) binding of FX and retargeting to hepatocytes.
We recently compared the biology of Ad5 with another species C adenovirus, Ad6 (18, 34). In this work, we found that native Ad6 mediates higher liver transduction than Ad5 after i.v. injection (34). To identify the underlying molecular basis for this difference, the HVRs of Ad5 were replaced with those of Ad6 producing a virus called Ad5/6 (Fig. 1A and (18)). When Ad5 and Ad5/6 were compared, the Ad5/6 virus mediated 10-fold increases in hepatocyte transduction after i.v. injection. This effect appeared to be due to reduced interactions of Ad5/6 with macrophages and Kupffer cells.

These data suggest that particular surfaces of the Ad5 hexon are involved in Kupffer cell recognition. Given this and the pivotal role of Kupffer cell depletion during systemic therapy, we evaluated the roles of each of the seven HVRs by conditionally mutating them using genetic and chemical engineering techniques (18, 19). Single cysteine residues were inserted into each of the seven HVRs of Ad5 hexon individually. These “silent” mutations were then “activated” by specifically modifying the site by reaction with cysteine-reactive polyethylene glycol (PEG) to conditionally block interactions with each HVR. By this approach, we probed the role of each HVR in Kupffer cell recognition.
**Materials and Methods**

**Cell Culture.** Human embryonic kidney cells (293) were purchased from Microbix. A549 Human Lung Carcinoma cells (ATCC CCL-185), Chinese Hamster Ovary (ATCC CCL-61), and RAW 264.7 murine monocyte-macrophage cells (ATCC CRL-2278) were purchased from the American Type Culture Collection (ATCC). All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal calf serum (Gibco) and penicillin/streptomycin at 100 U/ml (Gibco). CHO-CAR cells were developed by selecting stable transfectants of CMV-CAR plasmids with G418. CHO-SREC and CHO-SRAII cells were kindly provided by Dr. Brent Berwin (Dartmouth, NH) and Dr. Monty Krieger (MIT, MA), respectively. The latter was cultured in Ham's-12 media supplemented with 10% lipid reduced FBS (Hyclone), penicillin/streptomycin at 100 U/ml, 250 μM mevalonic acid, 3 μg/ml acLDL, 40 μM mevinolin and 300 μg/ml G418. Normal media was used during comparative experiments.

**Adenoviruses.** Ad unmodified control: First-generation, replication-defective (E1 and E3 deleted) Ad5 vector carrying enhanced green fluorescent protein and luciferase fusion gene (GL) was produced with the AdEasy system (Qbiogene/MP Biomedicals) in 293 cells as described in Mok et al. (2005). Cysteine-modified: Ad5 HVR sequence segments with cysteine insertions were purchased from Genscript. Using flanking Apal and AvrII restriction sites, each HVR segment was cloned into a shuttle plasmid and recombined into the Ad backbone with Red Recombinase cells. Viruses were purified...
on two consecutive CsCl gradient centrifugations; in order to prevent disulfide bond formation, the first gradient was supplemented with 10 mM DTT and the second was supplemented with 1 mM according to a previously published protocol. Vectors were desalted using Econopac 10-DG chromatography columns (Bio-Rad), resuspended in degassed 0.5M Sucrose in KPBS buffer and stored at -80°C. Viral particle (vp) concentration was determined by $A_{260}$ measurements. Infectious unit titers were determined by TCID$_{50}$, where viruses were serially diluted on 293 cells in a 96-well plate. Wells were scored for cytopathic effect after 2 weeks.

**PEGylation.** Linear, heterobifunctional polyethylene glycol (PEG) with biotin and maleimide (mal-PEG-biotin), or methyl and maleimide (PEG without biotin cap) linkers was purchased in 5, 20, and 40 kDa lengths (JenKem Technology). 0.5kDa Peg2 — biotin was purchased from Thermo Scientific and 5 kDa methyl and succinimide heterobifunctional PEG was purchased from Sunbright. Upon use, all PEGs were overlaid with nonreactive nitrogen gas and stored at -20°C in desiccant. For PEGylation of cysteine-modified vectors, 1 mg PEG was added per $1 \times 10^{11}$ vp and rotated at room temp for 1.5 h. After incubation, the virus mixtures were dialyzed at room temperature into degassed and N$_2$ overlaid 0.5M sucrose/1X KPBS buffer for 2 h, and then in fresh buffer at 4°C overnight. After dialysis, vector suspensions were injected into mice within 3 h. In some cases, genome copies after dialysis were determined by real-time PCR to confirm amounts of virus being injected.
Quantification of PEGylation. Equivalent amounts of cys-modified and control viruses were PEGylated. Excess PEG was removed by three sequential buffer exchanges each into 0.5M sucrose buffer using Bio-Spin 30 Tris Columns (Biorad). In 3 wells, 1 x 10^9 vp of each virus were bound in a 96-well high-binding ELISA plate. A protein standard of fully biotinylated MBP-AviTag protein (Avidity) was also plated. After 2 h at room temp, the wells were blocked with 3% BSA overnight. Avidin-hrp (1:1000 dilution) was used as a probe for 1 h at room temp. TMB substrate (100 μl/well) was used for detection, followed by 25 μl of 2M sulfuric acid after 15 minutes to stop the reaction. The plate was read at 405nm using a Beckman Coulter DTX 880 Multimode Detector system. An inverse assay to quantify unconjugated cysteines was also used to assess the number of PEGylation events per virion. Cys-modified virus was mock treated or PEGylated at room temp for 1 h. Excess IR800-maleimide (LICOR) was added to each virus suspension for 1 h at room temp. Mock or PEGylated vectors (1 x 10^10 vp) were run on a 7.5% polyacrylamide gel. The Kodak Image Station In Vivo FX was used to detect fluorescence for 80 min (755 nm excitation, 830 nm emission).

Western blot. PEGylated (biotin-5kDaPEG-mal) or mock PEGylated vectors were loaded at 1 x 10^10 vp onto a 7.5% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and blocked overnight in 5% milk. The membrane was probed with 1:2000 dilution of neutravidin-hrp. SuperSignal West Pico Chemiluminescent substrate (Pierce) and Kodak Image Station In Vivo FX were used for detection.
Animals. Female BALB/c mice (6-8 weeks) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and were housed in the Mayo Clinic Animal Facility. All animal experiments were performed according to the policies and procedures of Mayo Clinic.

Ad injections. All viruses were diluted in PBS and mice were injected via the tail vein (i.v.) with a total viral dose of $1 \times 10^{10}$ vp in 100 µl PBS, except when otherwise specified. For Ad predosing, mice were injected i.v. with $3 \times 10^{10}$ vp of Ad5dsRed in 100 µl of PBS 4 hours prior to i.v. injection of vectors. For in vivo scavenger receptor experiments, mice were injected i.v. with 100 µg of polyinosinic acid, or poly (I), in a total of 100 µl PBS 5 minutes prior to Ad vector injection.

Bioluminescence imaging in vivo. Mice were imaged 24h after Ad injection. All mice were anesthetized with ketamine/xylazine and injected intraperitoneally (i.p.) with 100 µl of D-luciferin (20mg/ml; Molecular Imaging Products) and were imaged on the Lumazone Imaging System (Photometrics, Roper Scientific) for 1-10 min with 1x1 or 2x2 binning using no filters or photomultiplication. Lumazone imaging software was used to determine total light intensity per mouse (photons) for data analysis.

Luciferase assay in vitro. Cell lines were plated to ~80-90% confluency in 96-well plates and infected with Ad vectors for 24 hours at 37°C. For Factor X (FX) experiments, FX was added at 10 µg/ml, Cells were lysed with 1x passive lysis buffer (Promega) and 50 µl of luciferase assay reagent was added to each well. Relative luminescence units (RLU) was determined with a Beckman Coulter DTX 880 Multimode Detector system.
Quantitative Real-time PCR. Concentration of DNA samples was determined by Abs$_{260}$ and then diluted to 20ng/µl. Real-time PCR was performed on the DNA using the Applied Biosystems Prism 7900HT sequence detection system with SDS 2.3 software. Each well contained 10 µl Sybr Green (Applied Biosystems), 1 µl H$_2$O, 2 µl of 3 µM F Primer, 2 µl of 3 µM R Primer, and 5 µl sample (i.e. 100 ng DNA/well). The parameters used were 1 cycle of 95°C for 15 min, and 40 cycles of 95°C for 15 s, 55°C for 1 min, and 72°C for 30 sec. To normalize data, PCR was done for both the cellular housekeeping gene GAPDH (using previously published F primer 5’- CGTGTTCCTACCCCCAATGT -3’ and R primer 5’- TGTCATCATACTGCGAGTTTCT-3’)) and viral CMV gene (F primer 5’- CAAGTGTATCATATGCCAAGTACGCCCC-3’ and R primer 5’- CCCCGTGAGTCAAACCGCTATCCACGCC-3’). Viral DNA was normalized to cellular DNA by dividing CMV genomes by GAPDH genomes.

Data analysis. Graphs and statistical analyses were performed using Prism Graphical software. Analysis between two groups (such as virus alone or virus with PEG) was determined by Unpaired t-test. Analysis between 3 or more groups was done by one-way ANOVA (Bonferroni post-test). Graphs show Means + SD.

Results
Ad5/6 mediates increased liver transduction and evades scavenger receptors.

The Ad5/6 virus mediates 10-fold increases in hepatocyte transduction after i.v. injection (Fig. 1A and (18)). This effect appeared to be due to reduced interactions of Ad6 HVRs with macrophages and Kupffer cells (18).

Previous data suggests that Ad5 is phagocytosed by Kupffer cells via scavenger receptors (14). Broadly specific scavenger receptors recognize negative charge clusters such as those that are present in Ad HVRs (Fig. 2A). To compare virus interactions with these receptors, Ad5 and Ad5/6 were incubated with CHO cells that were modified to express CAR (the cognate receptor for Ad5), SRA-II (the Kupffer cell scavenger receptor), or SREC (the endothelial cell scavenger receptor) (2, 4, 18).

CHO cells normally express low levels of CAR and are relatively non-permissive for Ad5 infection ((3, 22) and Fig. 1B). Addition of CAR to CHO cells increased transduction 10-fold for both Ad5 and Ad5/6 (Fig. 1B). When CHO cells expressed SRA-II in the absence of CAR, transduction by Ad5 was also increased. In contrast, SRA-II expression caused no increase in transfection by Ad5/6. On CHO-SRA-II transduction by Ad5 was 20-fold higher than by Ad5/6 (p < 0.0001 by two-tailed T-test).

SREC increased Ad5 transduction only slightly, but this was higher than transduction for Ad5/6 (p < 0.01 by two-tailed T-test).

Insertion of cysteine residues into single HVRs for targeted PEGylation. The data in Figure 1 suggested that Ad5 hexon interacts more strongly with the Kupffer cell scavenger receptor than the Ad6 hexon. Given that Ad5 and Ad5/6 differ only in their...
HVRs, these data suggested that the interaction of Ad5 with SRA-II occurs at the surface of one or more of its HVRs.

To investigate which Ad5 HVRs are critical for these interactions, single cysteines were inserted into each of the seven HVRs of Ad5 by red recombination (Fig. 2A). These modifications were applied in the context of a replication-defective Ad5 viral genome expressing the GFP-Luciferase (GFPLuc) fusion transgene. Each virus was rescued in 293 cells and amplified in cell factories. Virus particles were purified by double CsCl banding in the presence of the reducing agent dithiothreitol (DTT) in order to maintain the inserted cysteines in their non-oxidized form, thereby preventing disulfide formation and virion clumping (19). All viruses grew to similar titers of 2 - 6 x $10^{13}$ virus particles (vp) except HVR1, which produced ~10-fold fewer viral (repeated 4 times). When the viruses were assayed for their 50% tissue culture infectious dose (TCID$_{50}$), most of the vectors had vp/infectious unit (vp/iu) units within 2-18 fold of the unmodified virus (Table 1). In contrast, the HVR1 virus had vp/iu 50-fold higher than Ad5. While infectious unit measures were somewhat reduced, the transduction efficiency by the modified viruses was largely unchanged when compared to unmodified Ad5 (Fig. 2C and Table 1).

To conditionally block the HVRs, each of the cysteine-modified vectors was reacted with maleimide-activated linear 5 kDa polyethylene glycol (PEG), capped without or with a terminal biotin (for detection). Vectors were suspended in a 0.5 M sucrose buffer to maintain virion activity during these reactions (8). Excess PEG was removed by dialysis against 0.5 M sucrose. Mock PEGylated viruses were treated in parallel without the addition of PEG.
Attachment of PEG to the virion was determined by detecting the biotin head group on each PEG with streptavidin-horseradish peroxidase (HRP) by western blot (Fig. 2B). Faint background staining was shown for control Ad while all PEGylated cysteine-modified viruses were strongly detected by streptavidin-HRP, indicating that each had been conjugated with 5 kDa PEG. To quantify the number of cysteine residues that were conjugated by PEGylation, unmodified Ad and a representative cysteine-modified virus, HVR5, were PEGylated with biotin-5kDa PEG-maleimide. Their biotins were quantified by ELISA. This method demonstrated that there were approximately 540 PEG molecules added to the HVR5 virus (data not shown). As a reciprocal assay to detect unreacted cysteines, unmodified and PEGylated HVR5 was reacted with the small fluorophore IR800-maleimide and labeling was analyzed by fluorescence after SDS-PAGE. By this assay, 67% of available cysteines were protected by PEG (data not shown). These assays suggest that approximately 2/3 of inserted cysteines are being protected by 5 kDa PEG. This modification fraction translates to approximately 2 PEG molecules per hexon trimer. Given that PEGylation levels were similar by western blot for all of the HVR-modified viruses (Fig. 2B), these data suggest that they all may have similar numbers of PEGs as HVR5.

During recombination, HVR6 modified Ad lost its GFPLuc transgene. As HVR6 is relatively buried and has not been implicated in Ad5 interactions, analysis of this HVR modification was not deemed to be crucial and testing proceeded without this vector.

Targeted PEGylation of hexon preserves viral infectivity in vitro. We and others have previously shown that Ad5 can be non-specifically PEGylated with 5 kDa N-
hydroxy-succinimide-activated PEG (NHS-PEG). This reagent reacts with up to 15,000 lysines on the surface of the virus and is useful for protecting the virus from pre-existing neutralizing antibodies (9) and reducing side effects (15, 24). While NHS-PEG is potent for shielding, when it is applied under saturating conditions it inactivates the virus in vitro, but not in vivo (10, 24).

We hypothesized that targeting PEGylation to specific sites on the virion would only inactivate virus functions specific to that site. Therefore, we predicted that the PEGylated cysteine-modified viruses should retain in vitro activity unless a critical HVR was involved in virus entry events. Previous data targeting PEGylation to HVR5 of Ad5 have shown that viral activity is retained (27), supporting the concept that targeted modification of all seven HVRs may similarly retain in vitro activity.

To investigate the in vitro activity of specifically PEGylated vectors, A549 cells were infected with 1000 virus particles (vp)/cell of control and cysteine-modified vectors, with or without conjugation to PEG. Their transduction activity was compared 24 hours later by luciferase assay (Fig. 2C). There was no significant difference in expression between the cysteine-modified vectors and the unmodified control virus with or without PEG modification (Table 1). To contrast this with NHS-PEGylation, the HVR5 modified virus was non-specifically PEGylated with NHS-PEG or was specifically PEGylated at its targeted cysteine with PEG-maleimide. Consistent with previous results, NHS-PEG reduced transduction more than 100-fold compared to both mock treated and specifically PEGylated adenovirus (Fig. 2D, p < 0.01 by one way ANOVA). In contrast, targeting PEGylation to HVR5 preserved in vitro virus activity. When each of the
viruses was tested for FX-mediated transduction, FX increased in vitro transduction of
all of the viruses, plus or minus PEG (Supplemental Fig. 1).

PEGylation of select HVRs increases liver transduction. Since targeted PEGylation
did not affect in vitro transduction, 1 x 10^{10} vp of each PEGylated and mock PEGylated
vector was injected i.v. into groups of female BALB/c mice to determine whether these
modifications altered in vivo transduction. After 24 h, liver transduction was assessed
by luciferase imaging (Fig. 3). HVRwt control virus mediated robust and similar liver
transduction whether mock or treated with PEG. The transduction levels of mock
PEGylated cysteine-modified vectors were similar or slightly lower than HVRwt. Viruses
that were PEGylated on HVR1, 2, 5, and 7 mediated up to 10-40-fold increases in liver
transduction over HVRwt expression (p < 0.01 by one way ANOVA). In contrast,
PEGylation of HVR3 and HVR4 mediated smaller increases in transduction that were
not statistically-significant. These data suggest that HVR1, 2, 5, and 7 may play a role
in virus sequestration in vivo.

Kupffer cell depletion does not affect transduction by vectors after HVR-targeted
PEGylation. Eliminating Kupffer cells by predosing increases liver transduction by
unmodified Ad5 10 to 40-fold (18). As found previously with Ad5/6, we hypothesized
that HVR PEGylation might reduce uptake of Ad by liver Kupffer cells. If so, PEGylated
vectors should be largely unaffected by the presence or absence of liver Kupffer cells.
To test this, Kupffer cells were eliminated by predosing mice with irrelevant Ad5-dsRed
4 hours before HVR vector injection as in (18). Luciferase imaging 24 hours later
showed that predosing increased unmodified Ad5 transduction 10-fold as expected (Fig. 4A). HVR3-PEGylated vector transduction was also significantly increased by predosing (p < 0.01). In contrast, predosing did not increase transduction by PEGylated HVR1, 4, or 5 suggesting that Kupffer cell elimination and shielding these HVRs impact the same pathways.

High dose injection increase liver transduction more for unmodified Ad5 than for PEGylated Ad5. Kupffer cells are a major viral sink for i.v. injected Ad at doses below its "threshold dose" (6). At doses of Ad5 that saturate and exceed this threshold, transduction becomes linear with dose. If HVR-PEGylated vectors avoid Kupffer cells, they should also avoid this threshold effect.

To test this, unmodified Ad5 or HVR5 virus were PEGylated with PEG-maleimide and mice were injected i.v. with 30-fold higher doses (3 x 10^{11} vp) than before. Under these conditions, increasing the Ad5 dose 30-fold increased liver transduction 10-fold (Fig. 4B). In contrast, Ad-HVR5 only increased its expression 2.5-fold. These data suggest that PEGylation of HVR5 avoids the threshold effect that normally impacts Ad5.

Blocking scavenger receptor in vivo does affect transduction by PEGylated HVR1. Negatively charged polyinosinic acid (poly (I)) is a ligand for scavenger receptors and has been shown to block Kupffer phagocytosis of Ad5 in vivo (14). Mice were injected with poly (I) five minutes prior to i.v. injection of 1 x 10^{10} vp of Ad5 or Ad5/6 and transgene expression was determined 24 hours later by luciferase imaging.
Injection of poly(I) before Ad5 increased Ad5 transduction more than 10-fold, but had little effect on Ad5/6 (Fig. 1A).

HVR1 has the largest number of charged amino acids (Fig. 2A) and has been implicated as a potential scavenger receptor domain (1) so this vector was used to test the effect of poly(I) on PEGylated Ads. Injection of poly (I) prior to injection of unmodified Ad5 again boosted transduction 10-fold (p < 0.01 by two-tailed T-test) (Fig. 5). Poly(I) also increased transduction of unPEGylated Ad-HVR1 to a lesser degree. In contrast, poly(I) did not increase transduction of PEGylated Ad-HVR1, but surprisingly decreased transduction by the modified virus (p < 0.01 by two-tailed T-test).

PEGylated HVR vectors are inefficiently recognized by scavenger receptors in vitro. These data suggested that PEGylated-HVR1 virus bypasses Kupffer cell scavenger receptor uptake. To test this more specifically, CHO cells expressing CAR or the scavenger receptors SRA-II or SREC were incubated with selected HVR-modified Ads at 1000 vg/cell with and without PEGylation and transduction was measured 36 hours later (Fig. 6). Addition of CAR to the CHO cells increased transduction by the unmodified and PEGylated vectors ~10-fold. Transduction by unPEGylated HVR vectors was increased by expression of SRA-II. In contrast, PEGylation of all of the tested HVR-modified vectors reduced this SRA-II-mediated transduction. SREC on CHO cells did not mediate increased transduction vector expression, but PEGylation of HVR7 and HVR3 reduced background transduction on these cells.
Ad5 has potential for use as a vector for liver gene transfer due to its natural tropism for hepatocytes. However, sequestration of most of the injected dose of this virus by liver Kupffer cells necessitates the use of extremely large doses of vector that can trigger widespread toxicity. To maximize the effective dose of therapeutic Ads for liver gene therapy, optimal vectors for liver gene delivery must retain their ability to bind blood factors (i.e. retain liver tropism) while minimize cellular sequestration (i.e. evade Kupffer cells) (18). In contrast, increasing Ad delivery beyond the liver will likely require evading Kupffer cells and hepatocytes.

Previous work implicated the hexon protein of Ad5 as a target for Kupffer cell sequestration (1). In this study, we have probed the Ad5 hexon in an effort to detect regions involved in its interactions with these cells. This work builds on our recent observations that Ad5 and Ad6, two highly related species C adenoviruses, have profoundly different interactions with liver Kupffer cells (18). We previously demonstrated that the Ad5 hexon is strongly recognized by Kupffer cells and macrophages, whereas Ad5 displaying the Ad6 hexon was not (18). Given that the only molecular differences between Ad5 and Ad5/6 are the HVRs of their hexons, we hypothesized that it was these hypervariable loops that are the key surfaces for interaction with Kupffer cells. We demonstrate here that the Ad5 HVR domain is indeed recognized by the Kupffer cell scavenger receptor SRA-II whereas the Ad6 HVR domain is not.

To probe HVR-specific interactions with SRA-II and Kupffer cells, we applied targeted PEGylation to all seven of the Ad5 HVRs using the approach that previously
was used to target PEG to HVR5 (11, 27). By this systematic approach, targeted
PEGylation of HVR1, 2, 5, and 7 produced marked increases in liver hepatocyte
transduction in vivo after intravenous injection. In contrast, PEGylation of HVR3 and 4
produced weak effects that did not reach statistical significance. These data implicate
HVR1, 2, 5, and 7 as important regions, which are mostly exposed on the virus surface
that affect Ad5 pharmacology in vivo.

As shown previously with Ad5/6, the increased in vivo transduction by the
PEGylated Ads was not further increased by eliminating Kupffer cells prior to injection of
these vectors. Nor was it increased by blocking scavenger receptors in vivo with poly
(I). These PEGylated vectors also did not suffer the same threshold effect as
unmodified Ad5. These data suggest that the in vivo liver transduction increases that
were observed for HVR1, 2, 5, and 7 are mediated at least in part by protecting the virus
from Kupffer cell sequestration.

Kupffer cells likely play a predominant role in the observed in vivo effects of
Ad5/6 and HVR-PEGylated Ad5. However, we cannot exclude that some of these
effects may occur by reducing interactions with other cells and proteins. For example,
interactions with blood cells, endothelial cells, and blood proteins may be also be
reduced by PEGylation (reviewed in (17)). In the context of Ad5 (which suffers from
strong Kupffer cell depletion), targeted PEGylation may affect this by modulating
sequestration. If this bottleneck is blunted, other downstream interactions may be
secondarily reduced by PEGylation. For other viruses that may natively avoid Kupffer
cells, targeted HVR PEGylation may probe only downstream events. This study begins
probing these interactions at this first bottleneck and lays the foundation for probing

other downstream events.

Since HVR1 is the most negatively charged HVR (with a net negative charge of -13 amino acids (18)), it is reasonable to implicate HVR1 as a ligand for scavenger receptor recognition (1). Consistent with this, targeted PEGylation of HVR1 markedly increased in vivo transduction and reduced in vitro interactions with SRA-II as expected. However, targeted PEGylation of HVRs 2, 5, and 7 also reduced these interactions and appeared to modulate interactions with Kupffer cells. HVR3 PEGylation also displays reduced interactions with SRA-II in vitro, but showed weaker effects in vivo.

The x-ray structure of the hexon trimer shows that HVRs 1, 2, 5, and 7 (i.e. the four sites at which PEGylation causes >10-fold increased liver expression) are clustered on the surface of the hexon trimer (Fig. 7). In the context of the entire virion, two regions that may contribute to binding to Kupffer cells are: 1) the depression at the center of each hexon trimer and 2) a depression at the center of three hexon trimers. Given that PEGylation of HVRs 1, 2, 5, and 7 have the strongest effects on Kupffer cell interactions in vivo, that either of these depressions or the rim of either depression may be a binding surface for SRA-II and scavenger receptors. (Fig. 7B).

The binding site of FX has been localized by cryo-electron microscopy and mutagenesis studies to the center depression of the hexon trimer (indicated by a yellow dashed circle, Fig. 7B)(5). Several pieces of data suggest that FX and scavenger receptor may not bind the same surface on hexon. First, FX binding does not preclude Kupffer cells phagocytosis. Second, PEGylation of the HVRs inhibited SRA-II binding, but did not inhibit interactions with FX in vitro (Supplemental Fig. 1). Nor did this
PEGylation affect liver transduction in vivo. Indeed, hepatocyte transduction was markedly enhanced by shielding the HVRs an effect opposite to what one would predict if FX binding was at the same sites. These data are consistent with the fact that the sites of PEGylation line the edge of the depression at the center of the hexon trimer, rather than in the depression where FX binds.

This suggests that the scavenger receptor binding site may be located between three hexon trimers (white circle, Fig. 7B) rather than in the center of each hexon trimer where FX binds (yellow circle, Fig. 7B). Interestingly, HVR4 is situated on the bottom surface of this cavity between hexon trimers and HVR3 projects more into this depression than into the depression within each trimer (Fig. 7B). Their close proximity to this depression may explain why PEGylation of HVR3 and HVR4 mediated detectable, but weaker effects on liver expression or SRA-II binding than HVRs 1, 2, 5, and 7, which are more accessible on the surface of the virion.

If this depression between trimers or the rim of this depression is involved in scavenger receptor interactions, this could also occur at different locations on the virion. The twelve hexon trimers on each facet of the virion essentially all face the same direction on this surface. Therefore, the depressions between trimers within the facet are chemically identically with exception of where minor coat proteins IIIa and pIX protrude (28). In addition, a wider depression between trimers is also present at the edge of each facet. Therefore, it is possible that scavenger receptor binding may also occur on the face of the facet, at the facet's edge, or at sites where minor capsid proteins are localized.
Both FX and SRA-II have an affinity for anionic surfaces. FX binds anions via chelated calcium on its GLA domain (30) and SRA-II binds polyanions through its scavenger receptor cysteine-rich domain (18). It is interesting that adenoviruses that do not appear to be taken up by Kupffer cells (species B Ad11 and 35, species D Ad26 and 48 (11, 27) and unpublished observations) are not thought to bind FX (4). However, Ad6 (of species C) breaks this concordance. The Ad6 hexon appears to have reduced interactions with SRA-II and Kupffer cells, but still binds FX albeit at 10-fold lower affinity than Ad5 hexon (18). This suggests that FX and scavenger receptor binding are separable, but more direct studies are needed to tease out this biology.

In summary, targeted PEGylation of each HVR domain appears to be a useful strategy to investigate the specific surfaces on hexon that mediate interactions with a variety of host components. This conditional shielding approach may have utility to probe the interactions of HVRs and other viral surfaces with other proteins in and outside of cells. Beyond its use in probing virus biology, the targeted PEGylation approach may also have practical value for liver gene transfer. In this case, targeted PEGylation of HVRs 1, 2, 5, or 7 generated viruses with 10 to 40-fold enhanced in vivo hepatocyte transduction. Unlike previous efforts to randomly PEGylate Ad with NHS-PEG, this approach and previous targeted modification of HVR5 (27) preserves virus binding to CAR and FX and retains full virus function in vitro. Therefore, targeted PEGylation may provide insight into the interactions of Ad with the host and may be useful in optimizing vectors for purposes such as liver gene transfer and beyond.
Acknowledgments

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Table 1. Bioactivity of HVR-modified Vectors

<table>
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<tr>
<th>Virus</th>
<th>VP/Infectious Units - PEG</th>
<th>VP/Luciferase - PEG</th>
<th>VP/Luciferase + PEG</th>
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<tr>
<td></td>
<td>VP/IU</td>
<td>VP/RLU</td>
<td>VP/RLU</td>
</tr>
<tr>
<td></td>
<td>VP/ IU wt</td>
<td>VP/RLU wt</td>
<td>VP/RLU wt</td>
</tr>
<tr>
<td>HVRwt</td>
<td>32</td>
<td>178</td>
<td>177</td>
</tr>
<tr>
<td>HVR1</td>
<td>1778</td>
<td>221</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>1.2</td>
<td>1.1</td>
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<tr>
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<td>160</td>
<td>164</td>
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<td>0.9</td>
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<td>0.8</td>
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</table>
Figure Legends

Figure 1. The Ad5 hexon hypervariable (HVR) domain is critical for scavenger receptor recognition. (A) BALB/c mice were predosed i.v. with PBS or 100 µg of poly(I). They were injected i.v. 5 min later with 1 x 10^{10} vp of each vector. After 24 h, mice were imaged with a Lumazone imager for 3 min, 2x2 binning, and luciferase expression was quantified. (B) CHO cells expressing the coxsackie and adenovirus receptor (CAR) or scavenger receptors (SRA-II and SREC) were infected with 1000 vp/cell of Ad5 or a previously created Ad5 vector containing the Ad6 HVR cassette (Ad5/6) for 36 hours and then assessed by luciferase assay. N = 10. *p < 0.05; **p < 0.0001; ns = not significant.

Figure 2. Cysteine-modified vectors can be specifically PEGylated and are still infectious in vitro. (A) Gene sequence alignment of Ad5 and Ad6 hexon. Boxes indicate hypervariable (HVR) domains. Yellow: HVR1; Green: HVR2; Light blue: HVR3, Orange: HVR4; Red: HVR5; Brown: HVR6; Blue: HVR7. C’s indicate position of cysteine (cys) insertions at amino acid 151, 191, 215, 254, 275, 310, 435. Natural cys occurs at position 132, 236, 420, 523. Black circles: potentially critical Factor X binding residues. (B) Mock treated (control) or cysteine-modified vectors were conjugated to 5 kDa maleimide-PEG-biotin (PEG) or were not conjugated. Samples were subjected to SDS-PAGE and the PEG-biotin was detected by Western blot with streptavidin-HRP. (C) A549 cells were transduced with control or PEGylated cysteine-modified vectors at an MOI of 1000 vp/cell. After 24 h, cells were lysed and luciferase transgene
expression was determined by spectrophotometer. N=3. (D) Luciferase expression was also measured in A549 cells transduced with Ad-HVR5 PEGylated with either 5kDa succinimide-PEG (NHS-PEG) or PEG at an MOI of 1000 vp/cell. *p < 0.05; **p < 0.01.

**Figure 3.** PEGylation at HVR 1, 2, 5, and 7 results in dramatically increased liver expression. BALB/c mice were injected i.v. with 1 x 10^{10} vp of mock treated or PEGylated virus. (A) They were imaged 24 hours later using a Lumazone imager for 3 min 1x1 or 2x2 binning. Shown are representative white light images overlaid with luciferase expression in pseudo-color. (B) Quantification of luciferase expression from multiple experimental repeats. Sample sizes (N): HVRwt=21; HVR1=20; HVR2, 7=10; HVR3=9; HVR4=8; HVR5=18. **p < 0.01.

**Figure 4.** Saturation of Kupffer cells does not boost expression of select cysteine-modified vectors. (A) Mice were predosed i.v. with 3 x 10^{10} vp in 100 µL of PBS. After 4 h, the mice were injected i.v. with 1 x 10^{10} vp in PBS of PEGylated control or HVR-modified vectors. N = 3 to 8. Mice were imaged 24 hours later for 3 min, 1x1 binning (B) Mice were injected i.v. with a high dose of 3 x 10^{11} vp in 100ul of PBS and then imaged after 24 hours for 10 sec, 1x1 binning. **p < 0.05; ***p < 0.01.

**Figure 5.** Poly (I) predose does not boost expression of PEGylated Ad-HVR1. BALB/c mice were injected i.v. with PBS or 100 µg of Poly (I). After 5 min, 1 x 10^{10} vp of Ad or Ad-HVR1 with or without PEG were injected i.v. Mice were imaged for luciferase expression after 24 hours for 3 min at 2x2 binning. Shown are (A) white light images.
overlaid with luciferase expression in pseudo-color from 800 to 3500 gray values and (B) quantification of luciferase expression. N = 3. **p < 0.01. ns = not significant.

**Figure 6.** Targeted PEGylation decreases scavenger receptor mediated viral transduction. Control (None), Coxsackie and adenovirus receptor (CAR), scavenger receptor-AII (SR-AII) or scavenger receptor from endothelial cells (SREC) were stably expressed on CHO cells. These cells were infected with 1000 vg/cell of HVRwt, 1, 2, 3, 5, and 7 vectors that had been mock treated or PEGylated. Expression was determined 36 hours later by luciferase assay. N = 4. *p < 0.05; **p < 0.01; ***p<0.001; ns = not significant.

**Figure 7.** Model of hypervariable (HVR) domain interactions with host components. A hexon trimer of trimers, based on the recent X-ray structure of adenovirus, with structured HVR domains in color. Yellow: HVR1; Green: HVR2; Light blue: HVR3, Orange: HVR4; Red: HVR5; Brown: HVR6; Blue: HVR7. (A and B) Top down view of hexon nonamer (trimer of trimers) with HVRs highlighted. (B) Potential FX and scavenger receptor binding surfaces. Light yellow dashed circle = hexon trimer central depression and putative Factor X (FX) binding region. White dashed circle = hexon nonamer central depression and alternate recognition surfaces. (C) Side view, with a simulation of unstructured residues. Speculated locations for the unstructured HVR1 and HVR5 loops are shown as dashed loops of dashed spheres.
References


**Khare Figure 1**

**Panel A**

- **Y-axis**: Luminescence (Photons)
- **X-axis**: Vector (Ad5GL, Ad5/6GL)
- **Legend**: PBS, Poly (I)
- **Significance**: * (p < 0.05), ns (not significant)

**Panel B**

- **Y-axis**: Luminescence (RLU)
- **X-axis**: CHO cells (None, CAR, SRA-II, SREC)
- **Legend**: Ad5GL, Ad5/6GL
- **Significance**: ns (not significant), ** (p < 0.01), * (p < 0.05)
- **Background**: Dashed line

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*Downloaded from [jvi.asm.org](http://jvi.asm.org) on October 22, 2017 by guest*
Khare Figure 3

A

HVR<sup>wt</sup>  HVR<sub>1</sub>  HVR<sub>2</sub>  HVR<sub>3</sub>  HVR<sub>4</sub>  HVR<sub>5</sub>  HVR<sub>7</sub>

- PEG

+ PEG

B

![Bar chart showing luminescence (photons) for different vectors with and without PEG.](chart.png)

**Vector**

**Luminescence (Photons)**

- PEG  + PEG

HVR<sup>wt</sup>  HVR<sub>1</sub>  HVR<sub>2</sub>  HVR<sub>3</sub>  HVR<sub>4</sub>  HVR<sub>5</sub>  HVR<sub>7</sub>
Khare Figure 4

A

B

Ad vector

Ad vector

Luminescence (Photons)

10^13

10^12

10^11

10^10

10^9

10^8

10^7

10^6

10^5

10^4

10^3

10^2

10^1

10^0

Adpeg

HVR1peg

HVR3peg

HVR4peg

HVR5peg

AdRed

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Khare Figure 6

HVR<sub>wt</sub>

- PEG | + PEG
---|---
None | ns
CAR | ns
SRA-II | ns
SREC | ns

HVR<sub>1</sub>

- PEG | + PEG
---|---
None | ns
CAR | *
SRA-II | **
SREC | ns

HVR<sub>2</sub>

- PEG | + PEG
---|---
None | *
CAR | ns
SRA-II | ***
SREC | ns

HVR<sub>3</sub>

- PEG | + PEG
---|---
None | ns
CAR | *
SRA-II | *
SREC | ****

HVR<sub>5</sub>

- PEG | + PEG
---|---
None | ns
CAR | ns
SRA-II | ns
SREC | ns

HVR<sub>7</sub>

- PEG | + PEG
---|---
None | *
CAR | ns
SRA-II | **
SREC | *