Role of the putative heparan sulfate glycosaminoglycan-binding site of the adenovirus type 5 fiber shaft on liver detargeting and knob-mediated retargeting

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Liver tropism hampers systemic administration of adenovirus in gene therapy and virotherapy. In consequence, tumour targeting requires the combination of capsid modifications that abrogate liver transduction and redirect adenoviral vectors to tumour cells. Coxsackievirus and adenovirus receptor (CAR), integrins and heparan sulfate glycosaminoglycans (HSG) are receptors involved in adenovirus type 5 (Ad5) entry into cells. The in vitro and in vivo properties of Ad5 vectors unable to bind CAR, integrins and HSG with and without Arg–Gly–Asp (RGD) inserted at the HI loop of the fiber were studied. As was previously observed with CAR-ablated vectors, CAR and integrin double binding-ablated vectors transduced hepatocytes less efficiently in vitro but not in vivo. On the contrary, the role of HSG on Ad5 infectivity was evident in vitro only when CAR binding was abrogated, but the shaft mutation that ablated HSG binding on the background of a normal capsid was sufficient to abrogate liver transduction in vivo. The insertion of amino acids RGD at the HI loop in a shaft-mutated fiber only partially rescued integrin-mediated infectivity. These results indicate that the shaft mutation precluded HSG binding and affected the structure of the fiber. The insertion of ligands at the hexon or protein IX may be required to benefit from the fiber shaft mutation-detargeting properties.

INTRODUCTION

Except for some types of tumours where intratumoral injection can result in a therapeutic outcome such as glioblastoma, the efficacy of virotherapy and gene therapy of cancer depends on the ability of viruses or vectors to reach disseminated tumour cells upon systemic administration. Adenovirus type 5 (Ad5) is a suitable candidate to use in the clinic as a vector for gene therapy or a virus for virotherapy because it can be produced at high titres and efficiently transduces and replicates in a broad range of cell types. However intravenous administration of Ad5 vectors results in rapid blood clearance and liver transduction (Alemany & Curiel, 2001; Alemany et al., 2000b). This liver tropism precludes the use of Ad5 to target other cell types by intravenous injection.

To develop Ad5 retargeted from liver to disseminated cancer cells, hepatocyte tropism of adenoviral vectors needs to be ablated (liver detargeting) and new specific ligands for cancer cells must be presented on the virus capsid (tumour targeting). Ad5 infects cells by a mechanism that involves at least two sequential virus–cell interactions, each one mediated by distinct capsid proteins. First fiber knob domain binds the primary coxsackievirus and adenovirus receptor (CAR) (Bergelson et al., 1997) and then the Arg–Gly–Asp (RGD) motif of the penton base interacts with cellular integrins αVβ3 and αVβ5 promoting a rapid adenovirus cell entry into clatrin-coated vesicles (Nemerow & Stewart, 1999; Wickham et al., 1993).

Several point mutations of the AB, DE or FG loop of the fiber knob domain have been reported that successfully ablate fiber–CAR binding (Roelvink et al., 1999). Surprisingly most studies on CAR-binding ablated vectors show that these vectors transduced liver as efficiently as non-modified vectors despite that all of them drastically reduced hepatocyte transduction in vitro (Alemany & Curiel, 2001; Einfeld et al., 2001; Leissner et al., 2001; Martin et al., 2003; Mizuguchi et al., 2002; Smith et al., 2002). It was expected that residual entry by CAR-binding ablated vectors was mediated by integrins. However, integrin-binding ablated vectors that have been developed also fail to reduce liver transduction (Einfeld et al., 2001; Mizuguchi et al., 2002; Smith et al., 2003b). With regard to the liver detargeting
properties of vectors with the double ablation of CAR and integrin binding, controversial results have been presented (Einfeld et al., 2001; Martin et al., 2003; Smith et al., 2003b).

Recently, the heparan sulfate glycosaminoglycans (HSG) putative-binding site KTKT of the Ad5 fiber shaft domain has been shown to be involved in Ad5 liver transduction in mice, rats and non-human primates (Nicol et al., 2004; Smith et al., 2003a, b).

In contrast to strategies based on conjugating ligands to the capsid, the genetic modification of fiber or other exposed capsid proteins to exhibit tumour-specific ligands is better suited for clinical application of oncolytic vectors, since these modifications will be passed to virus progeny allowing intratumoral spreading (Alemany et al., 2000a).

The insertion of RGD motif at the HI loop of the fiber knob confers on Ad5 a CAR-independent cell entry mechanism that results in a different biodistribution profile upon systemic administration (Dmitriev et al., 1998; Reynolds et al., 1999). Since integrins are a large family of adhesive receptors involved in leukocyte homing at inflammatory sites but also in the spread and adhesion of tumour cells, RGD represents a good candidate ligand to test selective tumour retargeting strategies.

In summary, understanding the role of Ad5–cell interactions on liver detargeting and the combination of detargeting modification with tumour-selective ligands presented on the fiber or other capsid proteins is crucial to drive therapeutic vectors to disseminated cancer after systemic administration. In this study, we injected intravenously Ad5 unable to bind CAR, integrin and heparan sulfate and analysed their biodistribution. We then analysed the ability of RGD insertion into HI loop of fiber to rescue the infectivity of the abladed adenoviral vectors on human tumour cells. We describe that HSG-binding fiber shaft domain is involved in liver transduction, but HSG binding alone cannot explain all the detargeting obtained when this domain is deleted. In addition, deletion of this fiber shaft domain impairs the presentation of RGD in the HI loop of the fiber and it may be more compatible with insertion of this ligand at other capsid locations.

### METHODS

**Adenoviral vectors.** All vectors contain a green fluorescent protein (GFP) and a luciferase (Luc) expression cassette domain under the constitutive cytomegalovirus promoter replacing E1. AdGFPLucY477A-6his and double-ablated Track-Luc (DATL) contain six histidine (his) residues at the C-terminal of the Ad5 fiber protein. Recombinant Ad5 vectors: AdGFPLuc, AdGFPLucY477A, AdGFPLucY477A-6his and DATL have been described previously (Alemany & Curiel, 2001; Nettelbeck et al., 2004).

To obtain AdTLRGD, luciferase from pGL3 (Promega) was cloned into HindIII–XbaI of pAdTrackCMV shuttle vector (He et al., 1998). This shuttle vector pAdTrackCMVLuc was digested with Pshl and ADLRG was introduced into the HI loop of fiber by site directed mutagenesis using oligos ADLRGup 5'-GACACACCTTGGTGGGGAGGGAGCTTGC-3' and ADLRGdown 5'-TGACACTTGGCAGAAGACATCAGTCAGAATGTTGCCTG-3' as described previously (Krasnykh et al., 1998). To obtain pVK50TL, the E1 region of pVK50 (Krasnykh et al., 1998) was replaced by a double expression cassette (TL) from the pAdTrackCMVLuc shuttle vector by homologous recombination.

Genomes of new recombinant adenoviral vectors were generated by homologous recombination between the shuttle vectors with the modified fibers (pShuttleG, pShuttleYG, pShuttleeG and pShuttleYGR) and Swal+Pcal digested pVK50TL. Finally, pAdTLG, pAdTLYG, pAdTLGR and pAdTLYGR were Pac digested and transfected into 211B cells to generate AdTLG, AdTLYG, AdTLGR and AdTLYGR. The packaging cell line 211B constitutively expresses the wild-type Ad5 fiber and produces viral particles (vp) that contain both wild-type and mutated fibers. To increase the spreading of adenoviral vectors, rounds of amplification were done in the presence of polybrene as described previously (4 µg ml⁻¹; Sigma) (Smith et al., 2003b). The last amplification round was done in 293 cells to generate vp containing only mutated fibers.

Ad5 were purified by CsCl gradients and dialysed in PBS/Cl/Mg (PBS + ) with 10 % glycerol (v/v) (Invitrogen). The protein titre was determined by optical density and all mutations were confirmed by sequencing purified viral DNA. Protein content of purified adenoviral vectors was confirmed by silver nitrate staining of denatured proteins loaded on SDS-polyacrylamide gel (Fig. 1). Quantitative analysis with Quantity One software (Bio-Rad) indicated that the GA mutation did not impair fiber assembly. For example, the ratio of fiber to core protein for AdTLYG compared to the same virus without the GA mutation (AdGFPLucY477A) was 1:462 and 1:322, respectively.

**Cell lines.** 293 and A549 human lung cancer cells were obtained from the ATCC. NP9 and NP18 human pancreatic tumour cell lines were established in our laboratory from perpetuated xenografts orthotopically implanted into nude mice (Villanueva et al., 1998). 293-6his and 211B were kindly provided by Joanne T. Douglas (Department of Pathology, Division of Human Gene Therapy, University of Alabama at Birmingham, USA) and Dan J. Von

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![Fig. 1. Protein content of purified virions. About $8 \times 10^6$ vp of purified virions were boiled and separated by SDS-PAGE and then stained with silver nitrate. An adenoviral vector lacking fiber was used as a control (AdTLRGP).](Image)
Seggern (Department of Immunology, IMM19, The Scripps Research Institute, La Jolla, CA, USA), respectively (Douglas et al., 1999; Von Seggern et al., 1998). 293, A549 and NP9 cells were maintained in DMEM containing 5% fetal bovine serum (FBS) and penicillin–streptomycin [PS, (100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹); Gibco-BRL]. NP18 cells were maintained in RPMI 1640, 10% FBS, PS and 1-glutamine (1/100 dilution from Gibco-BRL). 293-6his and 211B cells were maintained in DMEM, 10% FBS and hygromycin (250 µg ml⁻¹) for 293-6his and neomycin (400 µg ml⁻¹) for 211B. Murine hepatocytes were isolated from male BALB/c mice by a two-step collagenase perfusion method (Grompe et al., 1992) and seeded in collagen-coated plates.

**Adenovirus-mediated transduction assays.** One day before infection, 25,000 hepatocytes, 293, 293-6his, A549, NP18 or NP9 cells were seeded per well in 96-well plates. Virus was added at the vp per cell indicated in the figure legends in 60 µl growth medium and incubated with cells for 2 h at room temperature. Next, the virus-containing medium was changed and 293 and 293-6his cells were incubated for 20 h and other cell types for 2 days in fresh medium at 37 °C to allow virus internalization and reporter gene expression. Cells were lysed by adding 50 µl cell lysis reagent per well (Promega) and freeze–thawed once. Lysates were centrifuged at 13,000 g for 15 min at 4°C. Total photon emission (in units of relative light units per µg protein) was quantified using a Bio-Rad protein assay. Results are expressed in RLU (relative light units per mg protein). The Mann–Whitney test was used to analyse if differences among groups were statistically significant.

For heparin inhibition assays, 5 × 10⁴ vp per cell of each vector were preincubated with increasing doses of heparin from 0 to 100 µg ml⁻¹ (Sigma) in DMEM with 0.1% BSA (Sigma) for 1 h at 37°C to allow adenovirus binding to heparin. Then adenoviral vectors were added to A549 cells for 1 h at 4°C to prevent virus internalization. The virus-containing medium was changed to fresh medium and 2 days later cells were processed as above to quantify luciferase activity and protein content.

**Biodistribution of adenoviral vectors.** CAR-binding vector (AdGFPLuc) or CAR and integrin double-ablated vector (DATL) were injected via tail vein into C57/BL6 mice (four animals per group, 5 × 10¹⁰ vp per mouse). Animals were kept and manipulated in accordance with recommendations of the Federation of European Laboratory Animal Science Associations for the proper use of laboratory animals. Two days after injection, organs and tissues were sectioned and snap-frozen. Frozen tissues were ground to a fine powder using a pestle and mortar, and cooled in liquid nitrogen. Organ powders were weighed, and 1 ml luciferase cell culture lysis reagent (Promega) and measured in a luminometer (Berthold Junior; Berthold). Protein concentration of supernatant was quantified using Bio-Rad protein assay. Results are expressed in RLU (relative light units per µg protein). The Mann–Whitney test was used to analyse if differences among groups were statistically significant.

**Live imaging of transgene expression.** Unmodified fiber vector (AdGFPLuc), fiber shaft–mutated vector (AdTLG) or a vector containing both mutations (AdTLYG) were administered intravenously into BALB/c nude mice (four animals per group, 5 × 10¹⁰ vp per mouse). Three days after injection, mice were anaesthetized and 100 µl 10 mM D-Firefly luciferin (Xenogen) solution was administered intraperitoneally. In vivo bioluminescence was measured using an in vivo imaging system (IVIS; Xenogen). Images were analysed using Living Image 2.20.1 software (Xenogen) overlaid on Igor Pro 4.06A software (Wavemetrics). Total photon emission (in units of photon s⁻¹) was quantified using Living Image 2.20.1. After in vivo quantification, biodistribution of adenoviral vectors in tissues was done as described above. Statistical significance was determined using the Mann–Whitney test.

**Subcutaneous tumour xenograft model in nude mice.** Eight million A549 cells were xenografted under the skin of each flank in anaesthetized mice (male atimic nu/nu mice, 8–10 weeks old). When the nodules reached 60–100 mm³, a single dose of 5 × 10¹⁰ vp of unmodified fiber vector (AdGFPLuc) and fiber shaft-mutated vector containing RGD into HI loop (AdTLGR) were intravenously administrated into mice (five animals per group). Biodistribution of adenoviral vectors in tissues was done as described above. Statistical significance was determined using the Mann–Whitney test.

**Quantification of Ad5 receptors by flow cytometry.** A549, NP9 and NP18 cells at 80% of confluence were detached from plates using 0.1 mM EDTA and washed with PBS. Then 10⁰ cells were resuspended in 100 µl PBS+/+ with 1% BSA and incubated for 1 h at 4°C. Next, cells were incubated with anti-CAR (SN hybridoma RmCB, diluted 1/5), anti-α5 integrins (SN hybridoma L230, diluted 1/5) or anti-immunoglobulin (Ig) G (as a negative control, diluted 1/5; DAKO) for 1 h at 4°C. Cells were washed twice with PBS++ and incubated with anti-mouse IgG-fluorescein isothiocyanate secondary antibody (goat anti-mouse Alexa Fluor 488; 1/300 final dilution) for 1 h at 4°C. Cells were washed twice again and resuspended in PBS++, and analysed in duplicate by flow cytometry with an Epics XL cytometer (Coulter).

**RESULTS**

**CAR and integrin-binding double ablation does not reduce hepatic transduction.**

We demonstrated previously that CAR ablation was not sufficient to reduce the high levels of transduction observed in vivo upon endovenous administration of Ad5 (Alemany & Curiel, 2001). As a next step towards eliminating other virus–cell interactions that may account for the efficient liver transduction we have mutated both CAR and integrin binding in a vector expressing Luc and GFP (double-ablated Track-Luc, DATL).

Transduction efficiency of DATL in 293 cells and isolated murine hepatocytes was measured. In both cell types DATL showed over a 1000-fold transduction deficiency compared with a vector with the same expression cassette but with unmodified capsid (AdGFPLuc) and a 10-fold transduction deficiency compared with the CAR-ablated vector AdGFPLucY477A-6his. These transduction defects in vitro served also as a functional validation of the mutations. In contrast, DATL infected as efficiently as AdGFPLuc 293 cells expressing a 6his receptor, a result that validates the use of this artificial receptor system to propagate and measure the bioactivity of ablated vectors (Fig. 2). CAR and integrin-binding ablation does not completely abrogate Ad5 infection and therefore additional virus–receptor interactions may be involved.

Biodistribution of DATL was compared to AdGFPLuc. Each vector was administrated intravenously into C57/BL6 mice (5 × 10¹⁰ vp per mouse). Two days later, animals were sacrificed and the organs and tissues were processed for...
luciferase analysis. As shown in Fig. 3, lung, heart, kidney and ovary were transduced to much lower levels with DATL than AdGFPLuc. However, CAR and integrin-binding double ablation did not change the preferential transduction levels observed in spleen and liver. This pattern of expression indeed shows a different biodistribution, but with regard to liver transduction, double ablation of CAR and integrin binding is not sufficient to detarget Ad5.

**Effect of fiber shaft mutation on Ad5 infectivity in vitro**

Contrary to CAR and integrin binding, a major role in liver detargeting has been associated to the putative KKTK HSG-binding site of the fiber shaft (Smith et al., 2003b). We introduced the GA mutation (KKTK→GATK) in the fiber shaft domain to eliminate this putative-binding site to HSG. This mutation was introduced in a wild-type fiber background and in a CAR-ablated fiber background to evaluate the effect of fiber shaft mutation alone or in combination with CAR-binding ablation (AdTLG and AdTLYG, respectively).

To study the transduction properties of shaft modified Ad5, we performed transduction experiments in A549 lung cancer cells. A549 cells express the three receptors that have been postulated to mediate cell transduction (CAR, integrins and HSG) (Dechecchi et al., 2000; Smith et al., 2002). A549 cells were infected with increasing doses of unmodified AdGFPLuc, CAR-binding ablated AdGFPLucY477A, fiber shaft-mutated AdTLG or the vector with both Y477A and GA mutations AdTLYG. The GA fiber shaft mutation reduced Ad5 infectivity. Compared with unmodified AdGFPLuc, A549 transduction was 10-fold lower with AdTLG and 1000-fold lower with AdTLYG (Fig. 4a). Of note, the decrease in transduction efficiency caused by the shaft mutation was much more important when binding to CAR was ablated. In a wild-type background the shaft mutation reduced A549 transduction 10-fold (AdGFPLuc vs AdTLG) but in a CAR-ablated background the shaft mutation reduced transduction 50-fold (AdGFPLucY477A vs AdTLYG). This indicates that the role of HSG becomes more important when the virus cannot enter cells using CAR.

**Fiber shaft mutation abrogates Ad5 binding to heparan sulfates**

To demonstrate that the GA mutation affected the interaction with heparan sulfates and this interaction accounts for the reduced infectivity of vectors with the GA mutation, we performed inhibition assays with heparin, an analogue competitor of HSG. Unmodified AdGFPLuc, CAR-binding...
Ablated AdGFPLucY477A, fiber shaft-mutated AdTLG and the vector with both Y477A and GA mutations, AdTLYG, were preincubated with increasing concentrations of heparin. Luciferase quantification was measured 2 days after infection. As shown in Fig. 4(b), a low dose of heparin (0.1 μg ml⁻¹) is sufficient to reduce transduction efficiency of CAR ablated Ad5 (AdGFPLucY477A) but heparin does not affect infectivity of unmodified vector (AdGFPLuc) or Ad5 containing GA mutation (AdTLG and AdTLYG). These results confirm that heparan sulfates are Ad5 receptors and that the GA fiber shaft mutation ablates this heparan sulfate recognition. However, this entry pathway only becomes evident when CAR binding is ablated (Fig. 4b).

More importantly, fiber shaft mutation has effects other than HSG binding. Fiber shaft mutation reduces transduction efficiency to a greater extent than heparin inhibition (AdGFPLucY477A, Fig. 4b) both for AdGFPLuc and AdGFPLucY477A. However, heparin does not inhibit AdGFPLuc (Fig. 4b). These findings indicate that not all the reduction of infectivity associated to the GA mutation can be attributed to heparan sulfate binding and that this fiber shaft mutation has other effects on Ad5 infectivity besides HSG binding.

**Fiber shaft mutation abrogates liver transduction**

To evaluate the role of the fiber shaft mutation on liver transduction, we injected $5 \times 10^{10}$ vp of each vector (unmodified vector AdGFPLuc, fiber shaft-mutated vector AdTLG and the vector containing both Y477A and GA mutations, AdTLYG) in BALB/c nude mouse via tail vein. Biodistribution of recombinant Ad5 was quantified both directly in animals by molecular imaging and in tissue cell extracts. As shown by live imaging in Fig. 5(a), mice that received recombinant Ad5 with shaft-mutated fibers (AdTLG and AdTLYG) presented lower levels of transgene expression than mice that received recombinant Ad5 with unmodified fibers (AdGFPLuc). Luciferase quantification in vitro revealed a different biodistribution profile of adenoviral vectors carrying shaft-mutated fibers (Fig. 5b). Lung, heart, spleen and liver were transduced at much lower levels than with unmodified vectors.

These results indicate that fiber shaft mutation is sufficient to abrogate liver transduction in mice. Although the combination of fiber shaft mutation and CAR-binding ablation seemed to detarget Ad5 to a greater extent than the fiber shaft mutation alone, the differences between AdTLG and AdTLYG were not statistically significant. In consequence, both AdTLG and AdTLYG may become ideal liver-detargeted vector backbones in an attempt to target Ad5 incorporating tumour-selective ligands.

**RGD in HI loop only partially rescues the low infectivity of fiber shaft mutant Ad5 in cancer cell lines**

To explore the possibility of using fiber shaft-mutated Ad5 for tumour targeting, we assayed the capability of ligands such as RGD to rescue infectivity in a fiber shaft-mutated vector with and without CAR-binding ablation. We performed comparative transduction efficiency assays of Ad5 containing the GA mutation with or without RGD inserted at the HI loop of the fiber knob. We used cell lines expressing various levels of CAR as well as αv integrins measured by flow cytometry. With regard to CAR expression levels they could be classified NP18 > A549 > NP9 (respective values of peak count 228, 85·5 and 51·1) and with regard to integrin expression NP18 = A549 > NP9 (respective values of peak count 668, 751 and 190·5). In all the cell lines tested, RGD partially rescued infectivity in a shaft-mutated fiber when
CAR binding was ablated (mutant AdTLYG, Fig. 6), indicating that RGD inserted into HI loop is able to recognize cellular integrins despite the presence of fiber shaft mutation and independent of CAR. RGD only efficiently rescued the infectivity of Ad5 unable to bind heparan sulfates in A549, a cell line with high levels of \( \alpha \)V integrins and moderate levels of CAR. However, Ad5 containing the shaft-mutated fiber and the RGD insertion into HI loop did not enter cells as efficiently as their counterparts without the fiber shaft mutation. This indicated that the shaft mutation reduced the efficacy of a ligand inserted at the HI loop of the fiber knob to mediate Ad5 entry. This reduced efficiency could preclude an effective \textit{in vivo} targeting of the vectors detargeted with the GA mutation.

In fact, an \textit{in vivo} biodistribution study, in mice with subcutaneous A549 tumour showed this to be the case. The AdTLGR virus detarget the liver but did not show an improved tumour-targeting compared with non-modified Ad5 vector, AdGFPLuc. (Fig. 7).

**DISCUSSION**

In this study, we focused on developing retargeted Ad5. To abrogate liver transduction we developed Ad5 unable to...
bind CAR, integrin and HSG receptors. To redirect untargeted Ad5 to cancer cells we inserted the tumour-selective ligand RGD into HI loop of the fiber knob domain. The fiber shaft mutation was sufficient to abrogate liver transduction and spare normal tissues, but has an important effect on fiber structure that does not allow optimal targeting strategies based on the fiber knob domain modification.

Despite the fact that Ad5 entry into cells is mediated by binding CAR and integrins, CAR-binding ablation or integrin-binding ablation were not sufficient to abrogate hepatocyte transduction in mice. Our in vitro and in vivo assays to study transduction properties of the double binding-ablated Ad5, DATL, show that double binding-ablated vectors also fail to reduce liver transduction despite decreased transduction levels observed in vitro in hepatocytes. Reports in favour and against the liver-detargeting properties of CAR and integrin double-ablated vectors have been published (Einfeld et al., 2001; Martin et al., 2003; Smith et al., 2003b). Our results support those against the role of CAR and integrins in liver transduction.

The Ad5 cell entry mechanism is well known; however, the role of HSG is still unclear. Further inhibition of CAR, integrins and HSG may help to elucidate the role of HSG on adenovirus entry. Recently, HSG have been described as Ad2 and Ad5 receptors able to mediate initial adenovirus binding to cells (Dechecchi et al., 2000, 2001) and represent an alternative entry pathway for CAR-binding ablated vectors (Smith et al., 2003b). HSG are co-receptors of a broad range of animal viruses (Rostand & Esko, 1997). Our data from heparin inhibition assays confirm that HSG are Ad5 receptors. Heparin at low dose reduces the transduction efficiency of the CAR-ablated vector but not that of non-modified vector. These data correlate with previously described observations (Smith et al., 2002) and support a major role of HSG on Ad5 cell entry when it does not enter cells by CAR.

Residues KTKK of fiber shaft domain have been proposed as a putative HSG-binding site and we demonstrate using heparin inhibition assays that the fiber shaft mutation KTKK to GATK precludes Ad5 HSG binding.

The transduction deficiency caused by the fiber shaft mutation is greater than that caused by heparin, indicating that this fiber shaft mutation has effects other than HSG binding. The shaft mutation reduces transduction efficiency of non-modified vector (AdGFPLuc vs AdTLG) despite that pre-incubation of non-modified vector with heparin does not affect transduction efficiency. This differential effect of the mutation and heparin is also observed in CAR-ablated vectors. A possible explanation is that fiber shaft mutation alters the structure of fiber. The HSG-binding site KTKK is close to the fiber hinge that allows bending of the fiber shaft. Flexibility of the fiber shaft is required for the virus capsid to simultaneously bind CAR and integrins (Wu et al., 2003). Both residues in the mutation KK to GA are involved in the structure of the fiber shaft domain; the first K maintains the basic framework of the structure by intrachain hydrogen bonds and the second K forms stabilizing hydrophobic patches at greater radius of triple β-spiral (van Raaij et al., 1999). The structural defect still allows partial knob recognition because an Ad5 containing both the GA fiber shaft mutation and the Y477A CAR-ablation mutation transduces cells less efficiently than Ad5 containing only the fiber shaft mutation (AdTLYG vs AdTLG in Fig. 4). Taking into account the role of HSG when CAR binding is missing and the structural effects of the shaft mutation, we attribute the lower transduction levels of AdTLG versus AdGFPLuc to defects on fiber structure and the lower transduction levels of AdTLYG versus AdGFPLucY477A to combined defects on fiber structure and HSG-binding ablation.

It is important to note the differences obtained in vitro and in vivo. In vitro, CAR and integrins are primary receptors of Ad5 entry and HSG role as a receptor is only evident when adenoviral vector is unable to bind CAR or integrin. However, in vivo liver transduction in mice is CAR and integrin independent and the fiber shaft plays a major role in liver transduction. These results are consistent with data previously reported (Smith et al., 2003b). It has been proposed that blood complement factors can be involved in liver transduction in vivo (Shayakhmetov et al., 2004). The unexpectedly remarkable effect that the shaft mutation has in vivo may be related to changes in fiber structure coupled to blood factors that mediate capsid entry into hepatocytes by interaction with the fiber knob. Taken together, the important role of the knob on liver detargeting and the minor role of HSG on infectivity, our data are quite incompatible with the idea that GA mutation abrogated HSG binding without any affect on the knob. It remains to be studied whether blood factors interact less efficiently with
GA mutated Ad5 capsid. It is also not clear how these in vivo data will extrapolate to humans, a question that needs to be investigated in clinical trials.

On the other hand, despite that RGD allows an efficient alternative CAR-independent cell entry pathway (Dmitriev et al., 1998), when RGD is inserted in a shaft-mutated fiber only partially rescues infectivity in cell lines that express integrins at high levels. This structural effect of the shaft mutation in knob-mediated transduction has important implications in targeting and may imply that the ligand needs to be inserted at the pIX or hexon. Our results have not detected improved tumour-targeting of AdTLGR and AdTLYGR compared to AdGFPLuc in A549 subcutaneous tumour xenotransplanted in mouse.

We are currently mutating residues around the KKKT putative HSG-binding site to distinguish the contribution of HSG binding and structural effects on liver detargeting. We are also placing RGD at other capsid sites such as the hypervariable region 1 of hexon or protein IX to allow for targeting in a GA shaft-mutated fiber. In this regard, RGD has been inserted into the hexon and pIX for tumour-targeting purposes (Vellinga et al., 2004; Vigne et al., 1999).

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