

Helper-Dependent Adenoviral Vectors Containing Modified Fiber for Improved Transduction of Developing and Mature Muscle Cells

JONATHAN L. BRAMSON,^{1,2} NATALIE GRINSHTEIN,^{1,2} ROBERT A. MEULENBROEK,³
JOHN LUNDE,^{4,5} DAYANTHA KOTTACHCHI,^{6,7} IAN A. LORIMER,^{6–8} BERNARD J. JASMIN,^{3–5}
and ROBIN J. PARKS^{3,5,6,8}

ABSTRACT

Adenoviruses (Ads) have shown great utility as vectors for the delivery of genes to mammalian cells, partly because of their ability to infect a wide range of different cell types independent of the replicative state of the cell. However, Ads do not transduce mature muscle efficiently because of low levels of the natural viral primary receptor, the coxsackie virus and adenovirus receptor, on the surface of adult muscle cells. In this study, we have addressed whether incorporation of polylysine [p(K)] or arginine-glycine-aspartic acid (RGD) placed in the H-I loop of the adenoviral fiber protein can improve helper-dependent Ad vector (hdAd) transduction of mature muscle cells. We show that incorporation of the p(K) motif into the fiber of early region 1 (E1)-deleted Ad results in enhanced transduction of undifferentiated and differentiated C2C12 cells relative to a virus, containing a wild-type fiber (12- and 21-fold enhancement, respectively). Incorporation of the RGD motif resulted in only a 60–70% increase in transduction efficiency in these cells. The two fiber modifications were then incorporated into helper viruses for use in the Cre-*lox* system for generating hdAd, and the resulting retargeted Ad vectors, which encoded the β -galactosidase reporter gene (β -Gal), demonstrated enhanced transduction of C2C12 cells in culture. Although hdAdpK also showed enhanced infection of mature mouse muscle *in vivo*, hdAdRGD did not. All hdAd vectors elicited only minor anti-Ad immune responses, compared with an E1-deleted control vector, but each vector elicited strong anti- β -Gal immunoreactivity. Our results demonstrate that hdAd with modified cell tropism can be generated efficiently and, in the case of polylysine-modified hdAd, can lead to improved transduction of adult muscle cells *in vivo*.

OVERVIEW SUMMARY

In this study, we have addressed whether incorporation of polylysine [p(K)] or arginine-glycine-aspartic acid (RGD) in the H-I loop of fiber can improve helper-dependent Ad vector (hdAd) transduction of mature muscle cells. Although incorporation of either the p(K) or RGD motif resulted in up to a 21-fold improvement in muscle cell transduction *in vitro*, only the p(K) modification resulted in improved mature muscle cell transduction *in vivo*, although to a lesser

degree (about 1.8-fold increase over unmodified vector). Taken together, these data indicate that hdAd transduction of mature muscle can be achieved through fiber modification.

INTRODUCTION

ADENOVIRAL (Ad) VECTORS have become popular gene transfer vehicles because of their many desirable characteris-

¹Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, L8N 3Z5 Canada.

²Centre for Cancer Therapeutics, McMaster University, Hamilton, ON, L8N 3Z5 Canada.

³Molecular Medicine Program, Ottawa Health Research Institute, Ottawa, ON, K1H 8L6 Canada.

⁴Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, K1H 8M5 Canada.

⁵Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON, K1H 8M5 Canada.

⁶Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, ON, K1H 8M5 Canada.

⁷Cancer Research Group, Ottawa Regional Cancer Centre, Ottawa, ON, K1H 1C4 Canada.

⁸Department of Medicine, University of Ottawa, Ottawa, ON, K1H 8M5 Canada.

tics, including high-level transgene expression, high-level transduction of many different cell types and tissues regardless of their replicative state, and favorable safety profile (see Amalfitano and Parks, 2002). Ad attachment to a cell is mediated through a primary interaction between the knob domain of the fiber virion protein with a specific cellular receptor, termed the coxsackie virus–adenovirus receptor (CAR) (Bergelson *et al.*, 1997, 1998). Ad internalization is mediated through a secondary interaction between the virion penton protein and $\alpha_v\beta_3$ -integrin or $\alpha_v\beta_5$ -integrin located in the cell membrane (Wickham *et al.*, 1993, 1994). Cells that contain no or low levels of either the primary or secondary Ad receptor are transduced at a greatly reduced frequency (Goldman *et al.*, 1996; Nalbantoglu *et al.*, 1999). Indeed, if the levels of CAR are artificially increased, through transient expression from a plasmid or through generation of a transgenic mouse with elevated CAR expression, Ad transduction is proportionally increased (Nalbantoglu *et al.*, 1999, 2001). Thus, the ability of Ad to act as an efficient gene delivery vehicle is intimately tied to its ability to bind to and enter the target cell.

There have been numerous attempts to increase Ad transduction of previously refractory tissues (i.e., those expressing low levels of the Ad receptor), or to retarget the virus to an alternative receptor in order to enhance cell type-specific transduction. There are two main targeting strategies: noncovalent attachment of targeting ligands and genetic modification of capsid proteins. Noncovalent methods have involved the use of bispecific antibodies (one binding the Ad virion, the other binding the desired cellular ligand), antibody–receptor ligand complexes, or the mixing of chemically modified Ad virions with biotinylated ligand (Wickham *et al.*, 1996; Rogers *et al.*, 1997; Smith *et al.*, 1999). Although these methods do work, there are concerns about batch-to-batch variation in the degree of modification between different vector preparations. Genetic modification involves cloning of the targeting ligand directly into one of the virion coat proteins (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Krasnykh *et al.*, 1998). Because the structure of the Ad fiber knob has been solved, and extensively analyzed by amino acid mutagenesis (Roelvink *et al.*, 1999; Kirby *et al.*, 2000), several groups are concentrating on incorporating targeting ligands into the H-I loop of knob protein (Krasnykh *et al.*, 1998). This region is not essential for binding to CAR and, thus, modification of this region is not expected to alter the natural ability of the virus to propagate. Furthermore, H-I modifications can be combined with other knob mutations that do disrupt CAR binding (Roelvink *et al.*, 1999; Kirby *et al.*, 2000; Jakubczak *et al.*, 2001), thereby reducing Ad infection promiscuity as well as redirecting binding.

Perhaps the two most often utilized Ad modifications involve incorporation into fiber of a polylysine tract [p(K)] or an arginine-glycine-aspartic acid (RGD) motif, neither necessarily designed to increase Ad cell type specificity; rather, they are intended to increase the level of Ad transduction, especially of cells that have low levels of, or no, CAR (Wickham *et al.*, 1997). Because an RGD motif present in Ad penton protein is responsible for binding to $\alpha_v\beta_3$ - or $\alpha_v\beta_5$ -integrins, stimulating Ad internalization (Bai *et al.*, 1993; Wickham *et al.*, 1993), it was believed that incorporating this motif into Ad fiber would make infection a one-step process. The RGD modification of fiber results in increased virus transduction of endothelial and

smooth muscle cells (Wickham *et al.*, 1997). This modification has also been incorporated into the fiber of helper-dependent Ad vectors, and results in improved transduction of certain cancer cell lines (Biermann *et al.*, 2001), but not fetal muscle (Bilbao *et al.*, 2003). The p(K) modification allows the virus to bind heparan sulfate proteoglycans located on the cell surface, and has been shown to increase Ad transduction by as much as 500-fold in macrophages, endothelial cells, smooth muscle cells, fibroblasts, and T cells (Wickham *et al.*, 1997). This modification is also reported to increase Ad transduction of mature muscle (Bouri *et al.*, 1999), although not in all studies (van Deutekom *et al.*, 1999; Cao *et al.*, 2001).

In this paper, we examine the ability of p(K) or RGD modification of the Ad fiber protein to enhance transduction of muscle. We have constructed helper viruses, which contain genetically modified fiber encoding either the p(K) or RGD modification, for use in the Cre–*loxP* system for generating helper-dependent Ad vector (hdAd) (Parks *et al.*, 1996). We have used these helper viruses to generate hdAd vectors deleted of all viral protein-coding sequences and encoding an *Escherichia coli* β -galactosidase expression cassette. We demonstrate that inclusion of these modifications in the hdAd fiber leads to an increase in muscle cell transduction *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell and virus culture

All cell culture media and reagents were obtained from GIBCO Laboratories (Grand Island, NY). 293 cells (Graham *et al.*, 1977) were grown in monolayer in minimum essential medium supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), amphotericin B (Fungizone; 2.5 mg/ml), and 10% fetal bovine serum (complete medium). Recombinant Ad helper viruses were grown and titered on 293 cells, as previously described (Ng and Graham, 2002). The 293-derived cell line that stably expresses the Cre recombinase, 293Cre4 (Chen *et al.*, 1996), was propagated in complete medium supplemented with G418 (0.4 mg/ml). C2C12 cells were grown in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal bovine serum, and confluent plates were switched to medium supplemented with 2% horse serum in order to induce differentiation. C2C12 cells were allowed to differentiate for 4 days before infection.

The E1-deleted, first-generation Ad vectors used in these studies were constructed by a combination of conventional cloning and RecA-mediated recombination (Chartier *et al.*, 1996; He *et al.*, 1998), and are all of the general structure shown in Fig. 1. fgAdwt is an E1/E3-deleted Ad vector that contains the *E. coli* β -galactosidase gene under the regulation of the murine cytomegalovirus immediate-early promoter/enhancer (MCMV) and the simian virus 40 polyadenylation sequence [p(A); pA in Fig. 1] replacing the E1 deletion. fgAdwt is based on human Ad serotype 5, with the exception of the fiber-coding sequence, which is from human Ad serotype 2. The fiber gene of fgAdwt contains an oligonucleotide linker, 5'-CT-AGCTTCGGTAAAAAGAAGAAAAA GAAAAAGGATCC-CGAAG (and its complement), cloned into the *SpeI* site of the gene, which introduces a unique *BstBI* cloning site in the plas-

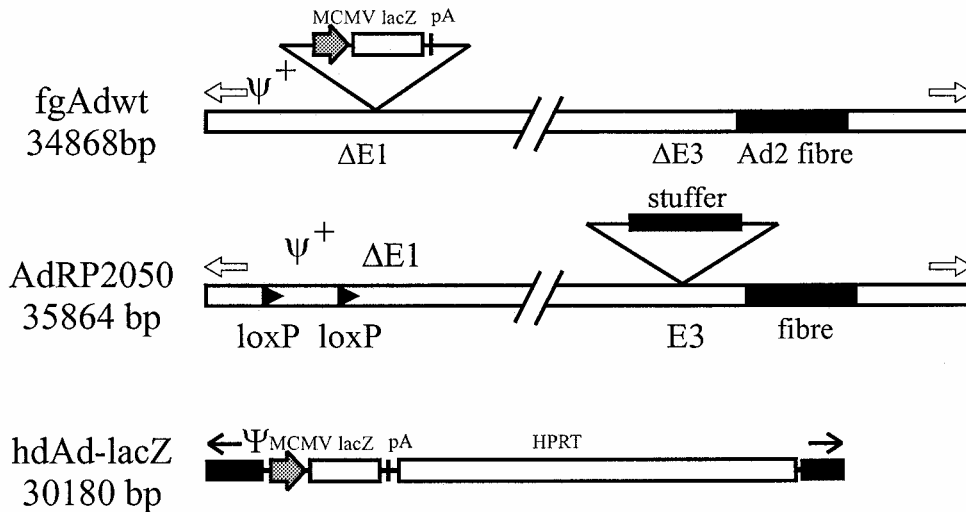


FIG. 1. Structure of adenovirus vectors used in this study. The E1/E3-deleted, first-generation Ads (fgAds) used in this study encode the *E. coli* β -galactosidase gene (*lacZ*) under the regulation of the murine cytomegalovirus immediate-early promoter/enhancer (MCMV) and the simian virus 40 polyadenylation sequence (pA) replacing the E1 deletion. fgAdwt is based on human Ad serotype 5, with the exception of the fiber-coding sequence, which is from human Ad serotype 2 and contains a unique *Bst*BI restriction site with the region encoding the H-I loop of fiber knob. fgAdpK and fgAdRGD contain a heptalysine or RGD motif, respectively, within the H-I loop of fiber knob, as described in Table 1. The helper viruses used in this study are similar in structure to AdRP2050, although two derivatives were generated that contained a heptalysine (AdRP2149) or RGD (AdRP2150) motif within the H-I loop of fiber knob. AdRP2050 contains the viral packaging signal flanked by *loxP* sites and the wild-type E3 sequence with an insertional interruption by a segment of intron 2 from the human low-density lipoprotein receptor-related protein (LRP5). The helper-dependent Ad vector encoding an MCMV-*lacZ* expression cassette, hdAd-*lacZ*, has been described elsewhere (Parks *et al.*, 1999a; Hubberstey *et al.*, 2002).

mid used to generate fgAdwt, and alters the fiber protein as indicated in Table 1. fgAdpK and fgAdRGD contain a heptalysine or RGD motif, respectively, within the H-I loop of knob, as described in Table 1. The particle-to-PFU (plaque-forming unit) ratios for the various viruses were 19:1, 79:1, and 84:1 for fgAdwt, fgAdpK, and fgAdRGD, respectively.

The helper viruses used in these studies were as follows. AdRP2050 contains the viral packaging signal flanked by *loxP* sites, and is similar in structure to AdLC8cluc (Parks *et al.*, 1996); however, AdRP2050 does not contain the luciferase expression cassette in E3 but, rather, contains the wild-type E3 sequence with an insertional interruption of 2890 bp from intron 2 of the human low-density lipoprotein receptor-related protein (LRP5) inserted in the *Xba*I site located at bp 28593 on the conventional Ad5 map (Fig. 1). Helper virus AdRP2149 is similar in structure to AdRP2050, but has a fiber-coding sequence identical to that of fgAdpK (Table 1). AdRP2150 is similar in structure to AdRP2050, but contains a fiber-coding sequence identical to that of fgAdRGD (Table 1).

Helper-dependent Ad vectors were propagated and titered as previously described (Parks *et al.*, 1996). To avoid, during vector amplification, any complications due to differences among the various helper viruses in terms of 293Cre infection efficiency, we used our unmodified helper virus (AdRP2050) to serially amplify the vectors, and used the fiber-modified helpers only during large-scale preparation. hdAd-*lacZ* is deleted of all Ad protein-coding sequences, and contains an MCMV-*lacZ*-pA expression cassette identical to that of the fgAd used in this study (Fig. 1). To maintain the size of the vectors above the limit for efficient DNA packaging (~28 kb; Parks and Graham, 1997), hdAd-*lacZ* contains a ~22-kb fragment of eukaryotic DNA derived from the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene, as described elsewhere (Parks *et al.*, 1999a; Hubberstey *et al.*, 2002). The titer of the hdAd was determined as *lacZ*-transducing units or blue-forming units (BFU) on 293 cells. For the hdAd vectors hdAdwt and hdAdRGD, the particle-to-BFU ratio was 7:1, whereas the ratio for hdAdpK was 10:1.

TABLE 1. FIBER-MODIFIED VIRUS

Modification	E1-deleted Ad	Helper virus	Fiber sequence ^a
Wild type	fgAdwt	AdRP2050	TLNGT S FEA S ESTE
p(K)	fgAdpK	AdRP2149	TLNGT S F G KKKKKK K D P EA S ESTE
RGD	fgAdRGD	AdRP2150	TLNGT S F A CD C R G DC F CG I EA S ESTE

^aThe amino acid sequence of the altered area of the fiber knob region is shown. Amino acids that correspond to the normal Ad2 sequence are shown in small caps; inserted amino acids are shown in boldface type.

Cell infection and blocking with soluble knob

Purified, amino-terminal hexahistidine (6×His)-tagged Ad2 knob (amino acids 406 to 601) was prepared as described elsewhere (Kottachchi, 2001). 293 cells or undifferentiated or differentiated C2C12 cells were overlaid for 1 hr at 37°C with phosphate-buffered saline (PBS) with or without Ad2 knob at 1 mg/ml. After incubation, the overlay was removed and cells were infected with a multiplicity of infection (MOI) of 5 (as determined by BFU count on 293 cells) with the various viruses, and the infection was allowed to proceed for 1 hr, at which time fresh medium was added. Eighteen hours later, the cells were stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (Parks *et al.*, 1996), or the cells were harvested and assayed for β -galactosidase (β -Gal) expression (Parks *et al.*, 1999b). For *in vitro* experiments, β -Gal activity is shown as micromoles of *o*-nitrophenyl- β -galactoside (ONPG) hydrolyzed per milliliter per hour.

In vivo expression studies

C57BL/6J mice (10 to 12 weeks old) or BALB/c mice (both obtained from Charles River Laboratories, Wilmington, MA) were anesthetized with halothane, and injected with 10^8 BFU of the various viruses in a volume of 25 μ l in both the right and left tibialis anterior (TA) muscle. Two or 3 days later, the mice were killed and the TA muscle was removed and immediately frozen in liquid nitrogen and stored at -80°C . For β -Gal determinations, the muscle samples were prepared and assayed as previously described (Parks *et al.*, 1999a).

ELISpot assay and anti- β -galactosidase ELISA

Spleens and lymph nodes (pooled population from the popliteal, inguinal, and ileac lymph nodes) were harvested 10 days after Ad-mediated gene delivery and tested for immune reactivity by enzyme-linked immunospot (ELISpot) assay, as previously described (Bramson *et al.*, 2003). Cells were stimulated for 18 hr with either TPHPARIGL, an immune-dominant β -Gal peptide that binds to H-2 L^d, or IPQSLDSWWTSL, an irrelevant H-2 L^d-binding peptide derived from hepatitis B surface antigen (HbsAg). To calculate the number of antigen-specific spot-forming cells (SFCs), numbers of spots in the negative control wells were subtracted from numbers of spots in the specific antigen-stimulated wells and normalized to 10^6 splenocytes. Serum was obtained by cardiac puncture 21 days postimmunization. Ad- and β -Gal-specific IgG1 and IgG2a were measured as described previously (Bramson *et al.*, 2003; Yang *et al.*, 2003).

RESULTS

Modification of Ad fiber leads to increased transduction of undifferentiated or differentiated C2C12 cells

To determine whether the p(K) or RGD modifications led to enhanced CAR-independent transduction of muscle cells *in vitro*, we infected 293 and undifferentiated or differentiated C2C12 cells in culture, either in the presence or absence of competing soluble knob. If binding of the cells was dependent on

CAR, infection should be inhibited when CAR is blocked by preincubation with knob. However, if cell binding is independent of CAR, the presence of soluble knob should have little or no effect on virus infection. As shown in Fig. 2, all three viruses were able to infect 293 cells and led to similar levels of β -Gal expression. Preincubation of 293 cells with soluble knob led to a dramatic reduction in transduction, represented by a >4000-fold decrease in β -Gal expression for vector containing wild-type fiber (2660 ± 270 versus 0.64 ± 0.25 units of β -Gal activity for cells incubated without and with soluble knob, respectively), and a 250-fold (4350 ± 20 versus 16.9 ± 1.5) and 650-fold (4190 ± 16 versus 6.43 ± 1.17) decrease in expression levels for p(K)- and RGD-modified vector, respectively. These data suggest that the majority of binding for the three viruses on 293 cells is through a CAR-dependent mechanism. When similar experiments were performed on undifferentiated C2C12 cells, we observed a significant difference in the number of *lacZ*-transduced cells for the modified vectors (data not shown). fgAdpK showed an approximately 12-fold higher level of β -Gal expression compared with fgAdwt (201 ± 23 versus 17.3 ± 0.7 for fgAdpK and fgAdwt, respectively), whereas fgAdRGD mediated only a 60% increase in gene expression relative to virus containing wild-type fiber. Blocking CAR before viral infection led to a >100-fold decrease in expression from fgAdwt, but to only a 12- and 27-fold decrease in expression from p(K)- and RGD-modified vectors, respectively, as determined by β -Gal activity assay (Fig. 2). Similar results were observed in differentiated C2C12 cells, where modification with p(K) and RGD led to a 21- and 1.7-fold increase in gene expression (Fig. 2). Taken together, these data indicate that, although both the p(K) and RGD fiber modifications can lead to increased transduction of muscle cells in culture, the p(K) modification leads to a greater level of infection. These data are in agreement with previously published studies (Bouri *et al.*, 1999; van Deutekom *et al.*, 1999; Cao *et al.*, 2001).

hdAdpK leads to enhanced transduction of muscle cells in vitro

Our data with fgAd suggested that vectors modified with the p(K) or RGD motif could increase transduction of muscle cells *in vitro*. To determine whether this was also true for retargeted hdAd, we designed helper viruses, for use in the Cre-*loxP* system for generating hdAd (Parks *et al.*, 1996), with fiber modifications identical to those used with our fgAd. We then generated genetically identical hdAd vectors with capsids that contained fiber that was either wild type, p(K) modified, or RGD modified. Differentiated or undifferentiated C2C12 cells in 35-mm dishes were infected with 10^6 BFU of hdAd (based on blue-forming units on 293 cells), and the quantity of β -Gal produced in the cultures was determined 24 hr later (Fig. 3). We observed an \sim 5-fold increase in β -Gal expression from hdAd vector containing the p(K) and RGD modifications on undifferentiated C2C12 cells (0.302 ± 0.025 , 1.53 ± 0.09 , and 1.39 ± 0.06 units of β -Gal activity for hdAdwt, hdAdpK, and hdAdRGD, respectively). This increased level of expression correlated with an increase in the number of blue cells after X-Gal staining (data not shown). Although hdAdpK mediated a similar level of enhanced transduction in differentiated C2C12 cells (\sim 4-fold), we observed only a 1.6-fold increase in β -Gal

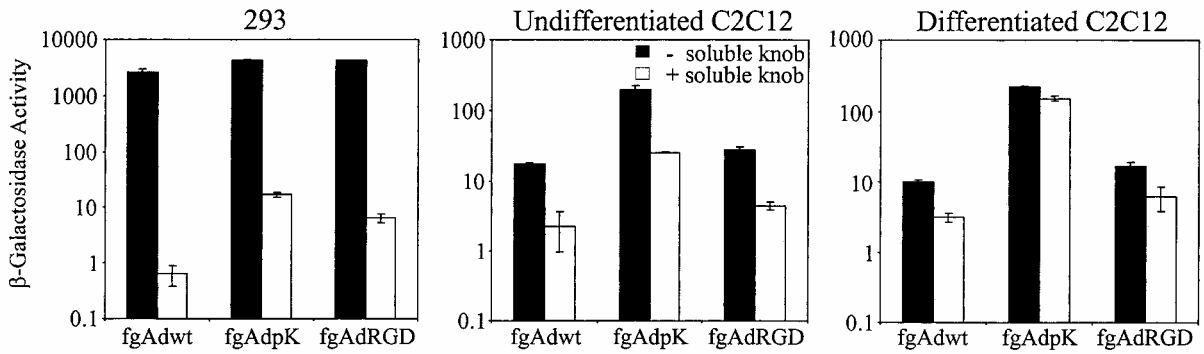


FIG. 2. β -Gal expression mediated by fgAdwt, fgAdpK, and fgAdRGD in cell lines. 293 cells (*left*), and either undifferentiated C2C12 cells (*middle*) or differentiated C2C12 cells (*right*) were preincubated with PBS or soluble knob protein, and infected (MOI of 5) with fgAdwt, fgAdpK, or fgAdRGD. Eighteen hours later, the cells were lysed and the resulting crude protein extracts were assayed for β -Gal activity (shown as micromoles of ONPG hydrolyzed per hour per milliliter of lysate). These data are representative of two experiments.

expression from hdAdRGD compared with hdAdwt. Thus, modification of hdAd to contain either a p(K) or RGD motif in fiber leads to enhanced levels of transduction of both undifferentiated and undifferentiated C2C12 *in vitro*, similar to that observed with fgAd (Fig. 2).

Muscle transduction *in vivo*

We wished to determine whether incorporation of p(K) or RGD motif into fiber of hdAd could also lead to enhanced transduction of muscle cells *in vivo*. We injected 10^8 BFU of hdAdwt, hdAdpK, or hdAdRGD into the tibialis anterior muscle of adult C57BL/6J mice and examined the level of β -Gal expression 2 days later. Once again, we observed increased expression mediated from hdAdpK compared with hdAdwt (Fig. 4, left); however, expression was only approximately 1.8-fold higher [(29.5 ± 4.3) versus $(16.5 \pm 5.5) \times 10^5$ RLU for

hdAdpK and hdAdwt, respectively]. hdAdRGD did not show enhanced transduction of muscle cells *in vivo*; indeed, expression for hdAdRGD was reduced to almost half of that observed for hdAdwt [$(9.72 \pm 3.34) \times 10^5$ RLU]. Similar results were observed in BALB/c mice 3 days after infection (Fig. 4, right). Therefore, we conclude that modification of hdAd to incorporate a heptalysine motif in fiber leads to a slight increase in transduction efficiency of mature muscle in mice.

Immune responses to gene delivery by fiber-modified hdAd

Modification of Ad fiber with p(K) has been shown to increase Ad transduction of a variety of cell types, including macrophages (Wickham *et al.*, 1997). Because macrophages can act as professional antigen-presenting cells (APCs), we were concerned that the improved transduction mediated by hdAdpK in

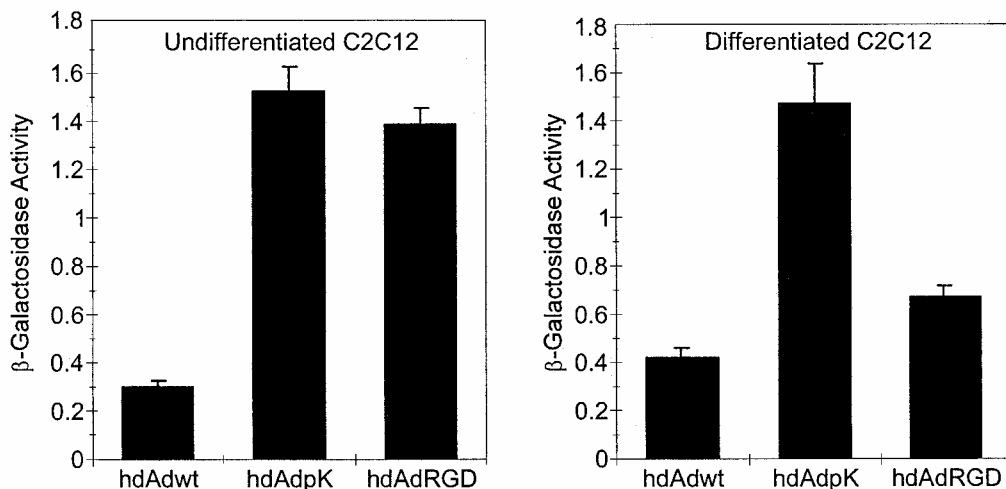


FIG. 3. Improved hdAd transduction of C2C12 cells mediated by fiber modification with p(K) and RGD. Undifferentiated or differentiated C2C12 cells were infected with 10^6 lacZ-transducing particles (as determined on 293 cells) of hdAdwt, hdAdpK, or hdAdRGD and, 24 hr later, the infected cells were harvested and assayed for β -Gal activity (shown as micromoles of ONPG hydrolyzed per hour per milliliter of lysate). These data are representative of three experiments.

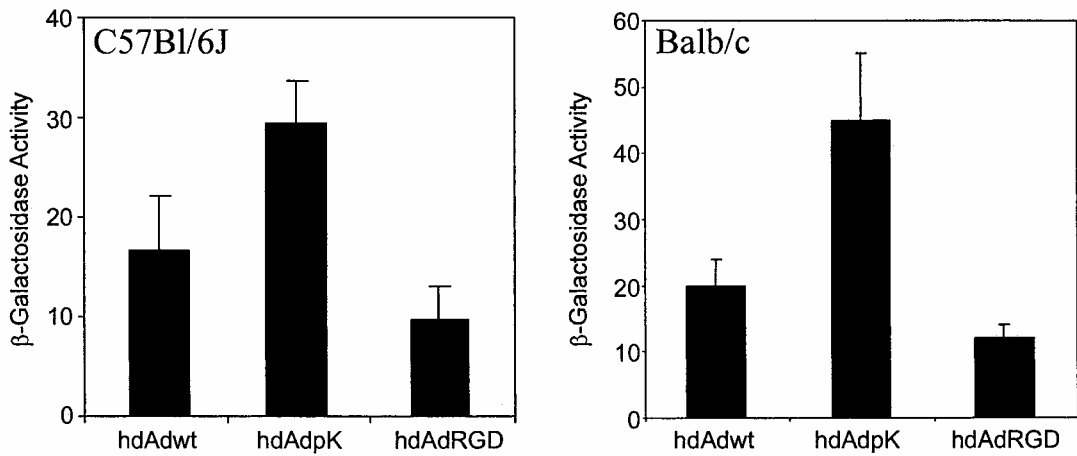


FIG. 4. Improved hdAd transduction of mature mouse muscle through modification of the Ad fiber protein. Adult C57BL/6J mice (*left*, 6 mice per group, 12 muscles) or BALB/c mice (*right*, 3 mice per group, 6 muscles) were injected with 10^8 *lacZ*-transducing units of hdAdwt, hdAdpK, or hdAdRGD in each tibialis anterior (TA) muscle. Forty-eight or 72 hr later the mice were killed, the TA muscle was removed, and assays for β -Gal activity (shown as relative light units [RLU] $\times 10^5$) were performed.

muscle might also lead to increased transduction of macrophages, or other APCs, leading to enhanced immune responses to our transgene product, β -Gal. To examine this possibility, we injected the TA muscle of BALB/c mice with 10^8 BFU of hdAdwt, hdAdpK, or hdAdRGD and examined colony formation 10 days postinfection in an ELISpot assay using cells derived from splenocytes and draining lymph nodes. We observed that hdAdpK did not lead to higher immune activation (Fig. 5); indeed, the number of spot-forming cells (SFCs) in the ELISpot assay was 2- to 4-fold lower for hdAdpK compared with hdAdwt. hdAdRGD led to immune activation intermediate between that of hdAdwt and hdAdpK. Thus, the improved transduction of muscle mediated by hdAdpK was not accompanied by enhanced cellular immunity.

We also examined whether infection with either of the modified viruses led to enhanced antibody production, or to a shift

in the antibody profile. As shown in Fig. 6, expression of β -Gal from all the viruses resulted in antibody production, although hdAdpK resulted in higher levels of IgG2a and IgG1 compared with hdAdwt (IgG2a, $13,400 \pm 3200$ U/ml and IgG1, 6100 ± 1300 U/ml versus IgG2a, $23,700 \pm 4600$ U/ml and IgG1, 1700 ± 600 U/ml for hdAdpK and hdAdwt, respectively). In contrast, β -Gal expression from hdAdRGD resulted in similar levels of antibody ($16,900 \pm 3500$ and 2800 ± 1400 U/ml for IgG2a and IgG1, respectively) compared with hdAdwt. Although all the mice exhibited anti- β -Gal antibodies, either no, or weak, anti-Ad immunoreactivity was detected in most of the mice treated with the hdAd vectors (Fig. 7). All mice treated with fgAdwt demonstrated a robust anti-Ad antibody response. Thus, for the hdAd used in this study, it appears that the anti-transgene antibody response correlates with gene expression (Fig. 4), but the cellular response does not (Fig. 5). The impli-

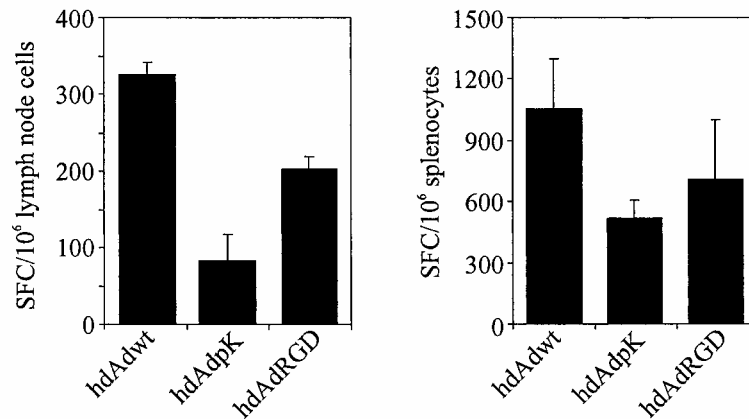


FIG. 5. ELISpot assay for immune reactivity to β -Gal. BALB/c mice were injected with 10^8 *lacZ*-transducing units of hdAdwt, hdAdpK, or hdAdRGD and, 10 days later, the mice were killed and spleens and lymph nodes were harvested and tested for immune reactivity by ELISpot assay, as described in Materials and Methods.

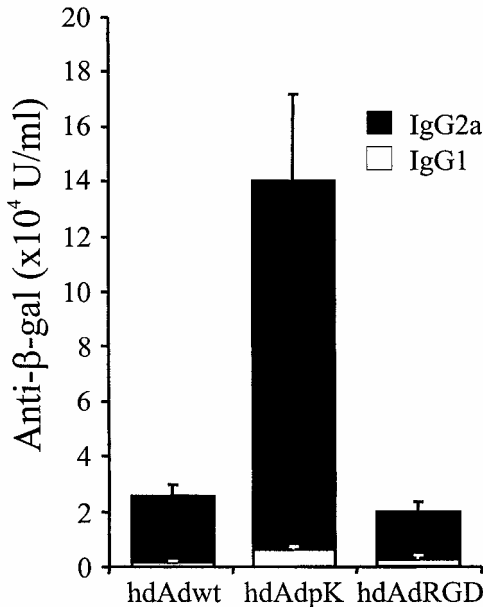


FIG. 6. β -Galactosidase antibody levels. BALB/c mice were injected with 10^8 *lacZ*-transducing units of hdAdwt, hdAdpK, or hdAdRGD and, 21 days later, serum samples were collected and assayed for IgG2a and IgG1 antibodies.

cations of this discrepancy for long-term gene expression remain to be determined.

DISCUSSION

Ads have been used in numerous preclinical studies to deliver reporter or therapeutic transgenes to muscle. Ad was viewed initially as one of the few vector systems that had the cloning capacity to accommodate the large dystrophin cDNA, although developments of minidystrophin cDNAs (Wang *et al.*, 2000; Harper *et al.*, 2002) mean that other vector systems, such as adeno-associated virus with a 5-kb cloning capacity, may now be used to develop therapeutics directed toward Duchenne muscular dystrophy (DMD). Nevertheless, the large cloning capacity of Ads, in particular helper-dependent Ad vectors that are deleted of all viral coding sequences, makes this vector system attractive for inclusion of large amounts of foreign DNA, such as large upstream regulatory regions for tissue-specific expression (Schiedner *et al.*, 1998; Aurisicchio *et al.*, 2000; Maione *et al.*, 2000), or for inclusion of on/off regulatory systems (Burcin *et al.*, 1999). Unfortunately, Ads do not transduce mature muscle efficiently, which is reportedly due to developmental downregulation of CAR (Nalbantoglu *et al.*, 1999) or low levels of appropriate integrins (Acsadi *et al.*, 1994; Huard *et al.*, 1995), although the actual reason for poor transduction of muscle may be more complex (Cao *et al.*, 2001). The ability to manipulate Ad such that it can bind to other cellular receptors suggests that the barrier to poor transduction may be easily overcome.

We have shown that incorporation of a polylysine or RGD motif into the H-I loop of the fiber knob domain of fgAd or hdAd resulted in up to a 12-fold increase in virus transduction

of muscle cells in culture. These data illustrate that, by adding small motifs to the fiber protein that allows the virus to bind to the cell through alternative receptors, improved muscle cell transduction can be achieved. Using an hdAd vector modified to contain an RGD motif in the H-I loop of fiber, Biermann *et al.* (2001) demonstrated that genetic modification of fiber could be used to enhance Ad transduction of ovarian cancer cells. Using the same vector, Bilbao *et al.* (2003) investigated the potential of an RGD-modified hdAd to enhance transduction of fetal muscle after *in utero* gene transfer. These researchers observed that the fiber modification actually reduced transduction of muscle; however, CAR levels are relatively high on immature muscle and thus no benefit was achieved through genetic modification of fiber. The RGD motif did enhance transduction of CHO cells, which do not express CAR, and we have observed similar results with our vectors (data not shown). However, the enhanced transduction that we observed for muscle cells *in vitro* was not completely recapitulated in our animal experiments, where we observed only an 80% increase in transduction and gene expression from hdAdpK. Similar results were observed by others (Bouri *et al.*, 1999; van Deutekom *et al.*, 1999; Cao *et al.*, 2001). It is likely that poor attachment of Ad to muscle cells is only one impediment to efficient muscle transduction (discussed below). Nevertheless, our data clearly illustrate that improved transduction of mature muscle can be achieved by virus retargeting.

In addition to showing improved infection of muscle cells *in vivo*, hdAdpK also demonstrated an interesting immune profile: the anti- β -Gal CTL response was reduced (Fig. 5), but the antibody response was elevated (Fig. 6) compared with animals treated with hdAdwt. The reasons for the altered immune profile remain unclear. Although loss of Ad-transduced cells is likely due to many factors, the CTL response, either directed toward viral or transgene antigen, is thought to play a predominant role (Tripathy *et al.*, 1996; Yang *et al.*, 1996). In general, persistent, long-term transgene expression correlates with reduced, or absent, immune responses to the therapeutic protein (Tripathy *et al.*, 1996; Morral *et al.*, 1997). Whether the change in immune response for hdAdpK has any effect on persistence of transgene expression *in vivo* remains to be determined. Interestingly, we observed that the anti-Ad antibody response elicited by the hdAd vectors was reduced compared with the antibody response generated by intramuscular injection with fgAd (Fig. 7). In a previous study, we noted that intravenous administration of hdAd elicited significant, serotype-specific antibodies that prevented efficient vector readministration (Parks *et al.*, 1999b); this block could be overcome by switching the serotype of the hdAd used for the second administration. The differences in these two studies may reflect differences in the efficiency of delivery of Ad antigen to B cell-rich areas by the two different routes of administration, intramuscular versus intravenous. On the basis of the results herein, we predict that, although the duration of gene expression may not be extended with hdAdpK vector because of comparable anti-transgene responses, the reduced anti-Ad response may permit multiple administrations of the vector to muscle tissue. This is an important consideration for treatment of muscle disease, such as DMD, where repeat vector administration would likely be required to provide life-long expression of the dystrophin. We are currently addressing this possibility in murine models.

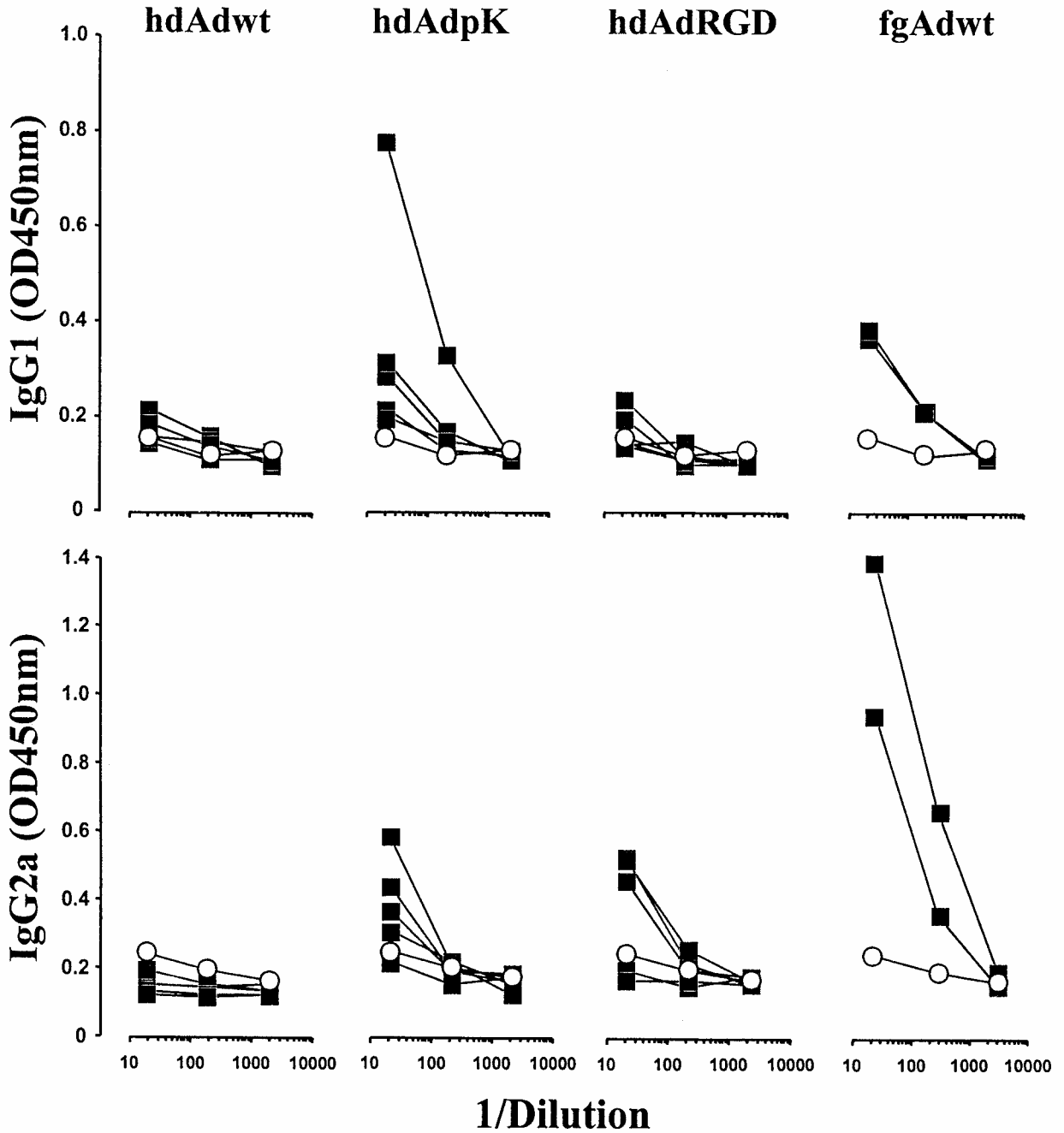


FIG. 7. Adenovirus antibody levels. C57BL/6 were injected with 10^8 *lacZ*-transducing units of hdAdwt, hdAdpK, or hdAdRGD, or with fgAdwt, and 21 days later serum samples were collected and assayed for IgG2a and IgG1 anti-Ad antibodies by ELISA. Solid squares, Ad-immune animals; open circles, naive serum. *Top*: IgG1. *Bottom*: IgG2a. Data are presented as optical density at 405 nm.

Developmental downregulation of CAR on mature muscle cells, resulting in loss of the primary cellular receptor for Ad binding, is likely only one barrier to successful Ad transduction of muscle. Huard and co-workers have suggested that one of the main barriers to Ad transduction of muscle is the presence of the basal lamina (BL) surrounding the muscle (Feero *et al.*, 1997; van Deutekom *et al.*, 1999; Cao *et al.*, 2001). The BL presents

a physical barrier to Ad transduction, preventing the virus from reaching the cell surface. In contrast, adeno-associated virus (AAV) is not hindered by the BL, because the diameter of an AAV virion (20–30 nm) is slightly smaller than the average pore size of the BL (~40 nm; Yurchenco, 1990); both are significantly less than the diameter of Ad (~100 nm). Thus, although we have identified a motif that, when incorporated into hdAd

fiber knob, can significantly increase Ad attachment to, and infection of, muscle cells, we have addressed only one of several impediments to efficient Ad transduction of muscle. Improvements to the method of vector delivery, which allow the virus greater access to the cell surface, are required before Ad can reach its full potential for muscle-directed therapies.

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Address reprint requests to:

Dr. Robin J. Parks
Room 4A115, 501 Smyth Road
Molecular Medicine Program
Ottawa Health Research Institute
Ottawa, Ontario, Canada K1H 8L6

E-mail: rparks@ohri.ca

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