Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 348 (2006) 278–287
BBRC
www.elsevier.com/locate/ybbrc

Disulfide bond formation in NGR fiber-modified adenovirus is essential for retargeting to aminopeptidase N

Dragomira Majhen [a,b], Jelka Gabrilovac [c], Marc Eloit [b], Jennifer Richardson [b], Andreja Ambriović-Ristov [a,*]

[a] *Laboratory for Genotoxic Agents, Division of Molecular Biology, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia*
[b] *UMR 1161 Virologie INRA-AFSSA-ENVA, Ecole Nationale Vétérinaire D'Alfort, 7 avenue du Général de Gaulle, 94704 Maisons Alfort Cedex, France*
[c] *Laboratory for Experimental Haematology Immunology and Oncology, Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia*

Received 12 July 2006
Available online 20 July 2006

Abstract

The peptide motif NGR (asparagine–glycine–arginine) is known to bind to aminopepidase N (APN). We have constructed five adenoviruses (Ads) bearing NGR in the HI loop of the adenoviral fiber protein. We compared the targeting properties of the NGR peptide within different amino acid environments and showed that their cellular receptor(s) were not identical. Ads containing NGR within potentially cyclic sequences flanked by cysteines retargeted viruses mainly to APN, while Ads containing NGR within linear sequences not containing cysteines retargeted Ads mainly to $\alpha_v\beta_3$ integrin, albeit with a lower affinity. Finally, we show evidence that disulfide bond formation within an Ad bearing the CDCNGRCFC sequence is essential for retargeting to APN, suggesting that this sequence does indeed assume a cyclic structure which facilitates NGR binding to APN. Therefore, our study underscores the importance of cysteine residues flanking targeting peptides for not only affinity but also specificity of the retargeted Ad.

Keywords: Adenovirus type 5; Retargeting; NGR; Aminopeptidase N; Integrin $\alpha_v\beta_3$; Disulfide bond

Tumor gene therapy aimed at inhibition of angiogenesis has potential to overcome two fundamental problems associated with standard chemotherapy: (i) poor accessibility and (ii) drug resistance of malignant cells [1]. Vectors based on adenovirus type five (Ad) have been intensively investigated for tumor gene therapy. However, these vectors suffer a major limitation, their nonspecific distribution in tissue after *in vivo* gene transfer [2], arising as a consequence of their mechanism of cell entry [3–6]. One strategy for genetic retargeting of Ad [for a review, see 7,8] is based on the modification of Ad tropism by genetic incorporation of a targeting ligand into the adenovirus fiber protein. The Ad fiber is a homotrimer with each subunit consisting of three domains: the amino-terminal tail, which associates with the penton base protein; the shaft, which consists of a motif of approximately 15 residues that is repeated 22 times; and the knob, which interacts with the cellular receptor [9,10]. The resolution of the three-dimensional structure of the fiber knob enabled Krasnykh and colleagues to identify the so-called HI-loop as an appropriate location for incorporation of targeting moieties [11,12]. Arap and colleagues [13] screened phage displaying random peptide libraries for the ability to home selectively to tumor vasculature in mice. Such selection permitted the identification of peptide motifs capable of targeting to tumor vasculature: an RGD-4C (arginine–glycine–aspartic acid flanked by 4 cysteines and referred to as double-cyclic due to a potential for forming two disulfide bonds) and an NGR (asparagine–glycine–arginine) motif in a linear NGRAHA and potentially cyclic CVLNGRMEC form. The RGD-4C peptide bound to α_v integrins, while the NGR peptide bound to

* Corresponding author. Fax: +385 1 4561 177.
E-mail address: andrea@irb.hr (A. Ambriović-Ristov).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.07.051

aminopeptidase N (APN, CD13). It has been shown that the only vascular structures with detectable APN are blood vessels undergoing angiogenesis, including tumor vasculature [14]. APN could therefore be an appropriate target for tumor gene therapy aimed at inhibition of angiogenesis [13,15–17]. Both linear NGRAHA and potentially cyclic CVLNGRMEC peptides have enabled targeting of viral particles to endothelial cells [18,19], suggesting that peptide cyclization through formation of a disulfide bridge is not necessary for the targeting properties of NGR peptides. Here we show that Ads containing NGR within a potentially cyclic sequence bound more efficiently to APN, while Ads containing NGR within a linear sequence bound more efficiently to $\alpha_v\beta_3$ integrin. In addition, we provide evidence for disulfide bond formation within Ad bearing a CDCNGRCFC sequence that is required for targeting properties of NGR.

Materials and methods

Cell lines. The HEK (human embryonic kidney cell line)-293 [20], RD-human embryonal rhabdomyosarcoma (ATCC CCL-136), and human laryngeal carcinoma (HEp2) cell lines, as well as HEp2-derived clones with graded expression of $\alpha_v\beta_3$ integrin, HEp2-$\alpha_v\beta_3$-K4, HEp2-$\alpha_v\beta_3$-K16, and HEp2-$\alpha_v\beta_3$-K1 [21], were used. Cells were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FCS, Invitrogen, USA) (HEK-293 and RD cells) or 10% bovine serum (BS, Invitrogen, USA) (HEp2 and HEp2-derived clones) at 37 °C with 5% CO_2.

Genetic engineering and production of Ad vectors. All molecular cloning steps were performed by using standard techniques [22]. Enzymes were purchased from Roche Molecular Biochemicals, Germany. The plasmid pQE16.knobΔHI was a gift from Victor Krasnykh (University of Alabama at Birmingham, Birmingham, Alabama) and has been described previously [11]. This plasmid contains a unique *Eco*RV restriction site in place of the deleted portion of the sequence encoding the HI loop. This plasmid was digested with *Eco*RV and duplexes of oligonucleotides were inserted, thereby generating pAd5FibTVTM5, pAd5FibTVTM6, pAd5FibARAP, pAd5FibNGR4G, and pAd5FibNGR4C. Five pairs of oligonucleotides were used: TVTM-5U (5′-CACACTAAACGGTACACAGGAAACAGGAGACACAACTGCACTAAACGGACGAATGGAATCACCAC C-3′) and TVTM-5D (5′-GGTGGTGATTCCATTCGTCGTTTTAGTGCAGTTGTGTCTCCTGTTTCCTGTGTACCGTTTAGTGTG-3′); TVTM-6U (5′-CACACTAAACGGTACACAGGAAACAGGAGACACAACTGCACTAAACGGACGAGAAGAATCACCACC-3′) and TVTM-6D (5′-GGTGGTGATTCTTCTCGTCCGTTTAGTGCAGTTGTGTCTCCTGTTTCCTGTGTACCGTTTAGTGTG-3′); ARAP-U (5′-CACACTAAACGGTACACAGGAAACAGGAGACACAACTTGCAACGGCCGCTGCGTGAGCGGCTGCGCCGGCCGCTGCCC-3′) and ARAP-D (5′-GGGCAGCGGCCGGCGCAGCCGCTCACGCAGCGGCCGTTGCAAGTTGTGTCTCCTGTTTCCTGTGTACCGTTTAGTGTG-3′); NGR4C-U (5′-CACACTAAACGGTACACAGGAAACAGGAGACACAACTTGTGACTGCAACGGACGATGTTTCTGCCC-3′) and NGR4C-D (5′-GGGCAGAAACATCGTCCGTTGCAGTCACAAGTTGTGTCTCCTGTTTCCTGTGTACCGTTTAGTGTG-3′); NGR 4G-U (5′-CACACTAAACGGTACACAGGAAACAGGAGACACAACTGGAGACGGAAACGGACGAGGATTCGGACC-3′) and NGR4G-D (5′-GGTCCGAATCCTCGTCCGTTTCCGTCTCCAGTTGTGTCTCCTGTTTCCTGTGTACCGTTTAGTGTG-3′). The incorporation of oligonucleotides into dephosphorylated plasmids was ascertained by using PCR primers as follows: pHIloopU, 5′-GTAACATTGTCAGTCAAGTTTAC-3′ and pHIloopD, 5′-GATGTGGCAAA TATTTCATTAATG-3′. Plasmids that gave rise to PCR products larger than those of controls were purified and sequenced. The NGR-containing segments of the fiber gene constructions were introduced into Ad backbone by homologous recombination in *Escherichia coli* BJ 5183 (recBC sbcBC) as described elsewhere [23]. All constructed Ads (Ad5TVTM5, Ad5TVTM6, Ad5ARAP, Ad5NGR4C, and Ad5NGR4G) as well as wild type Ad5RSVwt contain a β-galactosidase reporter gene driven by the Rous sarcoma virus promoter in the E1 region [24]. They were amplified in HEK-293 cells, banded in CsCl gradients, and stored at −80 °C in aliquots. The virus particle concentration was measured by optical density according to the protocol described by Mittereder et al. [25]. Adenovirus blue forming units (b.f.u.) were determined on HEK-293 cells as described by Ambrović-Ristov et al. [26].

Determination of $\alpha_v\beta_3$, $\alpha_v\beta_5$, APN, and CAR levels by flow cytometry. Flow cytometry was used to analyze expression of $\alpha_v\beta_3$, $\alpha_v\beta_5$, APN, and CAR on RD cells. Briefly, adherent cells were grown in tissue culture dishes, detached with Versene (Invitrogen, USA), and washed twice with PBS. Membrane fluorescence staining for $\alpha_v\beta_3$ was performed with monoclonal antibodies against $\alpha_v\beta_3$ (23C6, Pharmingen, USA) labeled with fluorescein isothiocyanate (FITC). For $\alpha_v\beta_5$ PE-labeled monoclonal antibodies against $\alpha_v\beta_5$ (P1F6) (Chemicon, USA) were used. Isotype control samples were incubated with FITC-conjugated mouse IgG_1 (Sigma, Germany). Membrane fluorescence staining for APN was performed with monoclonal antibodies against APN (WM15, Pharmingen, USA), and PE-conjugated anti-mouse antibody (DAKO, USA). For determination of CAR expression, cells were incubated with anti-CAR mouse monoclonal antibody, clone RmcB (Upstate cell signaling solutions, USA) and PE-conjugated anti-mouse antibody (DAKO, USA). Isotype control samples for APN and CAR measurements were incubated with mouse IgG_1 (Sigma, Germany) followed by PE-conjugated anti-mouse antibody (DAKO, USA).

Transduction efficacy. For transduction, 2×10^4 (RD) or 10^4 (HEp2 or HEp2-derived $\alpha_v\beta_3$-expressing cell lines) [21] cells were plated in 96-well plates. Twenty-four hours later cells were infected for 1 h in a total volume of 50 μl (in triplicate or quadruplicate) with serial twofold dilutions of Ad5RSVwt, Ad5TVTM5, Ad5TVTM6, Ad5ARAP, Ad5NGR4C, and Ad5NGR4G normalized for physical particle number. For experiments in which the effect of the reducing agent DTT on the biological functions of vector particles was examined, all Ads were incubated before infection with different concentrations of DTT (10, 4, and 2 mM) for 1 h at 37 °C. Viruses were then removed and fresh medium was added. Twenty-four hours after infection, cells were fixed with 0.5% glutaraldehyde and stained for β-galactosidase expression. The number of stained cells was determined by light microscopy. Blue cells in RD cell line were counted for approximate m.o.i. 10^4 or 5×10^3 pp/cell, while in HEp2 cell line blue cells were counted for m.o.i. 2.5 or 1.25×10^3 pp/cell.

Inhibition of the transduction of RD cells by NGR-bearing adenoviruses in the presence of synthetic peptides. The synthetic peptides used in the inhibition of transduction assay were produced by Epytop (France). Sequences were as follows: CNGRC, CRGDC, and control peptide CARAC. Cells (2×10^4 RD cells or 10^4 HEp2 or HEp2-$\alpha_v\beta_3$ expressing cells) were plated in 96-well plates and twenty-four hours later the effect of CNGRC and CRGDC peptides on the transduction efficacy was determined. Peptides (40 μl of a 15 μg/ml solution) were added to cells and incubated for 1 h at 37 °C. Then, serial twofold dilutions of Ad5RSVwt, Ad5TVTM5, Ad5TVTM6, Ad5ARAP, and Ad5NGR4C normalized for physical particle number were added. After 1 h the mixture of viruses and peptides was removed from cells and fresh medium was added. Twenty-four hours after infection, cells were fixed with 0.5% glutaraldehyde and stained for β-galactosidase expression. The number of stained cells was determined by light microscopy.

Treatment of RD cells with TGF-β1. For treatment with TGF-β1, 3×10^5 RD cells were plated in 35 mm dishes and 24 h later TGF-β1 was added at final concentrations of 2.4, 1.2, 0.6, and 0.15 ng/ml, and in a total volume of 2.5 ml. Seventy-two hours after beginning TGF-β1 treatment, cells were detached with trypsin and the expression of surface molecules was analyzed by flow cytometry, while for transduction and inhibition of transduction assays RD cells (2×10^4 per well) were plated in 96-well plates. Protocols were essentially the same as described previously, except that we infected TGF-β1 treated RD cells 6 h after plating.

Statistical analysis. All data were analyzed by unpaired Student's *t*-test and expressed as means ± standard error of the mean. Data were considered significant when $P < 0.05$. All experiments were performed in triplicate and repeated on at least three independent occasions.

Results

Characterization of Ads bearing NGR sequences

We initially constructed four Ad vectors designated Ad5TVTM5, Ad5TVTM6, Ad5ARAP, and Ad5NGR4C by insertion of four different NGR-bearing peptides (Table 1). Two of these (TVTM5 and TVTM6 - from tumor vasculature targeting motifs) constituted congeners of linear NGR motifs present in the ninth fibronectin type III repeat. The third (ARAP) and the fourth (NGR4C) peptide sequences contained cysteine residues and are referred to as cyclic. The ARAP sequence has been identified by Arap and colleagues [13] as a tumor-binding peptide, while the other, NGR4C, was chosen by analogy with an RGD4C peptide that binds integrins. To verify that peptide insertions did not affect incorporation of fiber protein into Ads particles, Western analysis was performed using equal particle numbers of all Ads including Ad5RSVwt. All Ads were shown to contain a similar amount of the fiber protein (data not shown). The infectivity indices obtained on HEK-293 cells (ratio of number of physical particles and number of blue forming units per milliliter) of all constructed Ads (Table 1) were comparable, ranging from 40 to 52, suggesting that insertions in the HI-loop of the fiber knob domain did not substantially affect the structure of these fibers. It should be noted that at least two different batches of each Ad were purified and verified in the same manner.

Insertion of NGR motif into Ad fiber protein increases transduction of an APN-positive embryonal rhabdomyosarcoma cell line

To investigate tropism of NGR-bearing Ads, an embryonal rhabdomyosarcoma cell line (RD) was used. RD cells express high levels of APN and $\alpha_v\beta_5$ integrin, and low levels of CAR and $\alpha_v\beta_3$ integrin (Table 2). For transduction of RD cells all viruses were adjusted to the same number of physical particles per cell (pp/cell). Both by counting β-galactosidase positive cells under the light microscope (Fig. 1, grey bars marked as C) and by measurement of β-galactosidase activity (data not shown), we observed that Ads bearing NGR sequences transduced RD cells more efficiently than wild type Ad5RSVwt. In addition, Ads containing NGR within potentially cyclic sequences showed a higher transduction efficacy (2.4- and 2.2-fold for Ad5NGR4C and Ad5ARAP, respectively) than those containing NGR within linear sequences (1.4 for both Ad5TVTM5 and Ad5TVTM6) (Fig. 1). While Ad5NGR4C showed statistically higher retargeting than Ad5ARAP, a statistically significant difference in the transduction efficacy of Ad5TVTM5 and Ad5TVTM6 was not observed.

To verify that NGR-bearing Ads transduce RD cells more efficiently due to the inserted NGR sequences, we studied transduction in the presence of the synthetic CNGRC peptide. NGR-containing peptides have been shown to bind not only to APN, but also to integrins, albeit with a lower affinity than that of RGD-containing peptides for integrins [27,28]. Moreover, it has been shown that tumor homing of the NGR phage was partially inhibited by the RGD4C peptide, although this peptide was only 10–20% as potent as CNGRC [13]. We therefore included the synthetic peptide CRGDC among peptides tested for their capacity to inhibit transduction. As shown in Fig. 1, neither CNGRC nor CRGDC peptides influenced trans-

Table 2
Expression of APN, CAR, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on cells

Cells	APN	CAR	$\alpha_v\beta_3$	$\alpha_v\beta_5$
RD (rhabdomyosarcoma)	+	±	±	+
HEp2 (laryngeal carcinoma)	–	++	–	++
HEp2-$\alpha_v\beta_3$K4	–	++	±[a]	++
HEp2-$\alpha_v\beta_3$K16	–	++	±[a]	++
HEp2-$\alpha_v\beta_3$K1	–	++	±[a]	++

Assignment of expression levels: –: negative (compared to isotype control); ±, weak: below 50% of positive cells, +: positive, more than 50% of positive cells; and ++: highly positive, 100% of positive cells.
[a] HEp2-$\alpha_v\beta_3$K4 < HEp2-$\alpha_v\beta_3$K16 < HEp2-$\alpha_v\beta_3$K1.

Table 1
Characteristics of adenovirus preparations[a]

VIRUS	Insert amino acid sequence	b.f.u./ml[c]	pp/ml[b]	Infectivity[d] index
Ad5RSVwt		$2.64 \pm 0.31 \times 10^{10}$	1.065×10^{12}	40.3
Ad5TVTM5	ALNGRMESP	$2.70 \pm 0.45 \times 10^{10}$	1.176×10^{12}	43.5
Ad5TVTM6	ALNGREESP	$2.45 \pm 0.31 \times 10^{10}$	1.047×10^{12}	42.7
Ad5ARAP	CNGRCVSGCAGRC	$2.40 \pm 0.32 \times 10^{10}$	1.164×10^{12}	48.5
Ad5NGR4C	CDCNGRCFC	$4.42 \pm 0.52 \times 10^{10}$	2.270×10^{12}	51.4
Ad5NGR4G	GDGNGRGFG	$2{,}05 \pm 0.52 \times 10^{11}$	$1{,}056 \times 10^{13}$	51.5

[a] Each line represents a separate preparation of virus.
[b] The virus particle concentration (physical particles per milliliter) was measured by optical density (1U of optical density at 260 nm corresponds to 1.1×10^{12} particles per milliliter).
[c] Blue forming units (b.f.u.) per milliliter were determined at 20 h post-infection of HEK-293 cells by staining for β-galactosidase expression.
[d] Infectivity index is the ratio of number of pp/ml and b.f.u./ml.

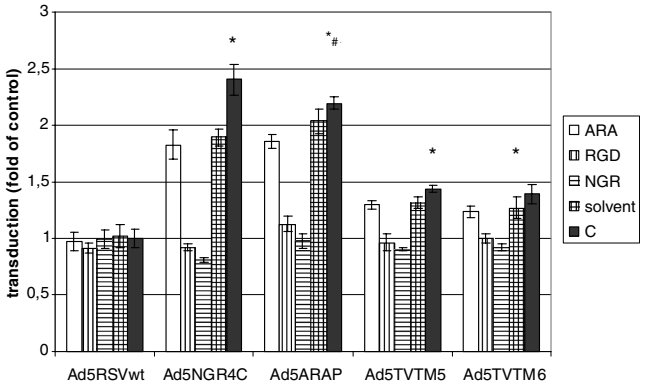

Fig. 1. Transduction of RD cells by wild type and NGR-bearing Ads and inhibition of the transduction of RD cells by NGR-bearing Ads in the presence of synthetic peptides. 2×10^4 RD cells were plated in 96-well plates. Twenty-four hours later, 15 μg/ml of peptides CNGRC, CRGDC or a control peptide CARAC (designated as NGR, RGD, and ARA, respectively) was added to cells and incubated for 1 h at 37 °C. Then, cells were infected for 1 h with 2-fold serial dilutions of Ads, and 24 h after infection cells were stained using X-gal and blue cells were counted. Transduction efficacy for each Ad (bars marked as C) is expressed as the number of blue cells relative to that for Ad5RSVwt. $^*P < 0.0002$ versus Ad5RSVwt; $^{\#}P < 0.0015$ versus Ad5NGR4C. For inhibition of the transduction of RD cells by NGR-bearing Ads in the presence of synthetic peptides results for each Ad are given as the number of blue cells relative to that for Ad5RSVwt. Data of one of two experiments that yielded similar results are presented.

duction by wild type Ad5RSVwt. An unrelated peptide, CARAC, as well as the solvent used for reconstitution of all peptides, had little effect on the transduction efficacy of NGR-bearing Ads. By contrast, both CNGRC and CRGDC reduced the transduction efficacy of Ads containing NGR within linear or cyclic motifs to that of wild type Ad. This finding suggests that in addition to APN, NGR-bearing Ads may use one of the RGD-binding integrins for transduction.

Insertion of NGR motif into Ad fiber protein increases transduction of cells expressing $\alpha_v\beta_3$ integrin

Potential retargeting of Ads bearing NGR to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins was next examined by using HEp2 cells, which express a large amount of $\alpha_v\beta_5$ but do not express $\alpha_v\beta_3$ on the cell surface (Table 2). We recently isolated HEp2 derived clones with graded expression of $\alpha_v\beta_3$ and found that the expression of $\alpha_v\beta_3$ correlated with Ad transduction efficacy [21]. We reasoned that if NGR insertion could retarget Ads to $\alpha_v\beta_3$ integrin, they should transduce these cell lines more efficiently than wild type Ad.

Regarding the parental HEp2 cell line, Ad5RSVwt, Ad5NGR4C, and Ad5TVTM5 (Fig. 2A) transduced HEp2 cells to the same extent, indicating that insertion of NGR-containing sequences did not confer advantage as regards transduction *via* $\alpha_v\beta_5$ integrin or any other molecule expressed on the surface of the parental HEp2 cell line. The results of a transduction assay performed using HEp2 and three HEp2-derived cell lines with graded increased expression of $\alpha_v\beta_3$ are presented in Fig. 2B, where results

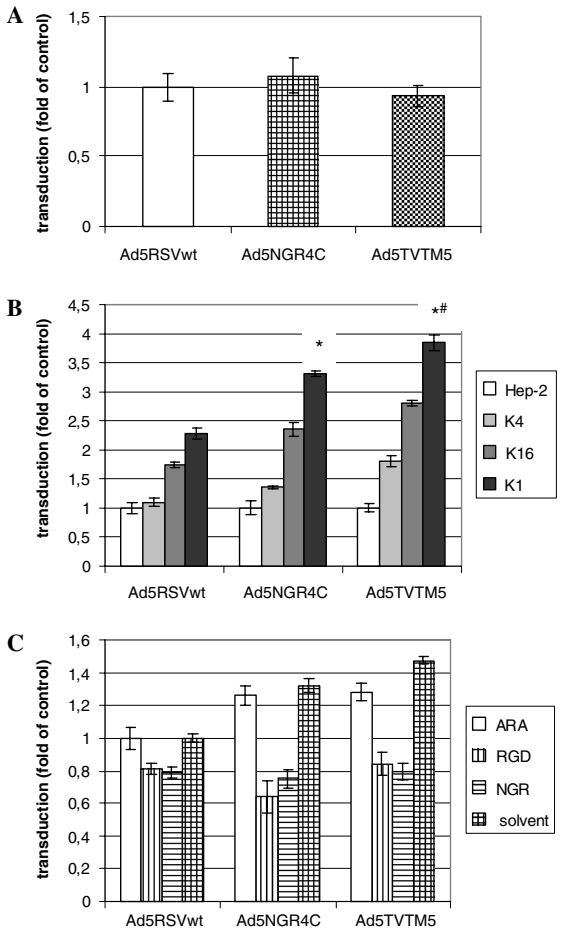

Fig. 2. Increased transduction of HEp2 clones with graded expression of $\alpha_v\beta_3$ by NGR-bearing Ads. (A) Transduction of HEp2 cells by Ad5RSVwt and NGR-bearing Ads; (B) transduction of HEp2-derived cell lines with graded expression of $\alpha_v\beta_3$ ($\alpha_v\beta_3$ expression: K4 < K16 < K1) by Ad5RSVwt and NGR-bearing Ads; (C) inhibition of transduction of HEp2-$\alpha_v\beta_3$K1 cells, which express the highest amount of $\alpha_v\beta_3$, by Ad5RSVβgal and NGR-bearing Ads in the presence of CNGRC, CRGDC, and control CARAC (designated as NGR, RGD, and ARA, respectively) peptides. 10^4 cells were plated in 96-well plates. Twenty-four hours later peptides (CNGRC, CRGDC, and a control peptide CARAC, all at a concentration of 15 μg/ml) were added to cells and incubated for 1 h at 37 °C. Then, cells were infected for 1 h at 37 °C with 2-fold serial dilutions of Ads, and 24 h after infection cells were stained using X-gal and blue cells were counted. Results for transduction (A,B), for each Ad, are given as number of blue cells relative to that for Ad5RSVwt. Results for inhibition of transduction, for each Ad, are given as number of blue cells relative to that for Ad5RSVwt in the presence of solvent used to dissolve peptides. Each point represents the mean ± standard deviation of at least three independent determinations. $^*P < 0.0001$ versus Ad5RSVwt; $^{\#}P < 0.002$ versus Ad5NGR4C.

for each virus are presented as relative to the transduction efficacy for HEp2 cells. The transduction efficacy of wild type Ad5RSVwt increased with increasing $\alpha_v\beta_3$ expression, due to increased internalization mediated by interaction between the RGD motif in penton base and $\alpha_v\beta_3$ integrin. However, Ads containing NGR (within linear and cyclic motifs) transduced each $\alpha_v\beta_3$-expressing clone even more efficiently than the wild type Ad5RSVwt. In cells (HEp2-$\alpha_v\beta_3$-K1) expressing the highest amount of $\alpha_v\beta_3$,

transduction by the NGR-bearing Ads Ad5NGR4C and Ad5TVTM5 was higher (1.45- and 1.68-fold, respectively), than that of wild type Ad. The relative increase in transduction by NGR-bearing Ads was substantially more modest in HEp2 cell lines than in RD cells. It should be borne in mind that HEp2 cells express CAR on the cell surface (Table 2), and that the efficacy of transduction depends on expression of both CAR and α_v integrins. Indeed, we used approximately four times less virus (m.o.i.) for transduction of HEp2 cells than for RD cells (see Materials and methods). The more modest increase in retargeting in HEp2 cells expressing $\alpha_v\beta_3$ integrins was thus probably related to the higher basal level of transduction, owing to CAR expression. Nevertheless, unlike observations made in RD cells whereby Ads containing NGR within cyclic sequences transduced cells (presumably *via* APN) more efficiently than those containing NGR within linear sequences, in HEp2-$\alpha_v\beta_3$-K1 cells Ads containing a linear NGR motif were more potent (presumably *via* $\alpha_v\beta_3$-integrin) than Ads containing a cyclic motif. Thus it appeared that the relative transduction efficiency of Ads bearing NGR within linear versus cyclic sequences differed as regards transduction *via* $\alpha_v\beta_3$-integrin or APN.

To investigate further the interaction of NGR-bearing Ads with $\alpha_v\beta_3$-integrin, we performed an inhibition of transduction assay using CNGRC and CRGDC synthetic peptides on HEp2-$\alpha_v\beta_3$-K1 cells, which expressed the highest amount of $\alpha_v\beta_3$ (Fig. 2C). As regards Ad5RSVwt, both CNGRC and CRGDC peptides decreased transduction by Ad5RSVwt below the level observed in the presence of the unrelated peptide CARAC. We suppose that this small (but statistically significant) effect may be due to an inhibition of internalization mediated by interaction of the viral RGD motif in the penton base and α_v integrins on the cell surface. As observed for Ad5RSVwt, both peptides CNGRC and CRGDC abolished increased transduction of NGR-bearing adenoviruses to the level observed with wild type, while the unrelated peptide CARAC exerted a slight effect or did not influence transduction at all.

Up-regulation of APN and $\alpha_v\beta_3$ integrin mediated by TGF-β1 increases retargeting index for Ad containing NGR within a cyclic but not linear motif

Treatment with TGF-β1 strongly up-regulates APN in human monocytic cells [29] and the myelo-monocytic cell line HL-60 [30]. In order to address the role of APN in transduction of NGR bearing Ads, we treated RD cells with different concentrations of this cytokine so as to increase APN expression. The influence of such treatment on all principal molecules implicated in Ad entry was examined. RD cells expressed a high amount of APN and $\alpha_v\beta_5$ integrin (Table 2), and therefore their expression may be presented as mean fluorescence intensity (MFI) (Fig. 3A). The expression of CAR and $\alpha_v\beta_3$ integrin, however, was weak (Table 2), and therefore the modulation of these molecules by TGF-β1 treatment is presented as percentage of positive cells (Fig. 3A). As shown in Fig. 3A, treatment with 2.4 ng/ml TGF-β1 up-regulated APN 2.2-fold. In addition to APN, TGF-β1 also increased $\alpha_v\beta_3$ in a dose-dependent manner (Fig. 3A). Treatment of RD cells with TGF-β1 also induced a slight increase in $\alpha_v\beta_5$ integrin expression (Fig. 3A; MFI 21 vs 25 in treated cells). In contrast to APN and $\alpha_v\beta_3$, TGF-β1 down-regulated expression of CAR on RD cells from 19.7% to 7–8% CAR positive cells, at all tested TGF-β1 concentrations without considerable difference between doses of TGF-β1 applied (Fig. 3A).

We next tested the effect of TGF-β1 treatment on adenoviral transduction. We observed a decrease in transduction efficacy for wild type Ad5RSVwt in a dose dependent manner, which may be attributable to diminution of CAR or to reduced RD cell proliferation (data not shown). The second explanation seems more probable, since the down-regulation of CAR, induced by TGF-β1, did not show the same dose-response pattern as the level of transduction. Moreover, at the smallest dose of TGF-β1 (0.15 ng/ml), which still strongly down-regulated CAR but no longer diminished cell proliferation (data not shown), the level of transduction of treated RD cells was comparable to that of control RD cells (data not shown).

Results of transduction experiments are expressed for each Ad as the number of transduced cells relative to that obtained for wild type Ad5RSVwt (Fig. 3B), so as to correct for any effect on transduction efficacy that was not the consequence of NGR insertion. We hypothesized that if NGR-bearing Ads retarget to APN, the retargeting index (ratio of transduction efficacy for NGR-bearing Ads as compared with wild type Ad) should increase in proportion to APN up-regulation. As shown in Fig. 3B, for Ad5NGR4C containing NGR within a cyclic sequence and at the highest concentration of TGF-β1, we observed a substantial increase in retargeting index (from 2.6- to 5.6-fold). In contrast, for Ad5TVTM5 containing NGR within a linear sequence, only a moderate increase in retargeting index (from 1.4- to 1.8-fold) was observed.

Retargeting of Ad5NGR4C is dependent on formation of disulfide bonds

Next, we wished to address the existence of disulfide bonds within the cysteine-containing sequences. We treated Ad5RSVwt, Ad5NGR4C (representative of Ads containing NGR within potentially cyclic sequences) and Ad5TVTM5 (representative of Ads containing NGR within linear sequences) with the reducing agent DTT and measured the transduction efficacy. As shown in Fig. 4A, DTT treatment had no effect on the transduction efficacy of wild type Ad5RSVwt and Ad5TVTM5. By contrast, DTT treatment abolished the increased transduction efficacy of Ad5NGR4C, containing NGR in a sequence bounded by cysteines, in a dose dependent manner.

The NGR-bearing peptides inserted into Ad5TVTM5 and Ad5NGR4C differ not only in their capacity to form disulfide bonds but also in their amino acid composition.

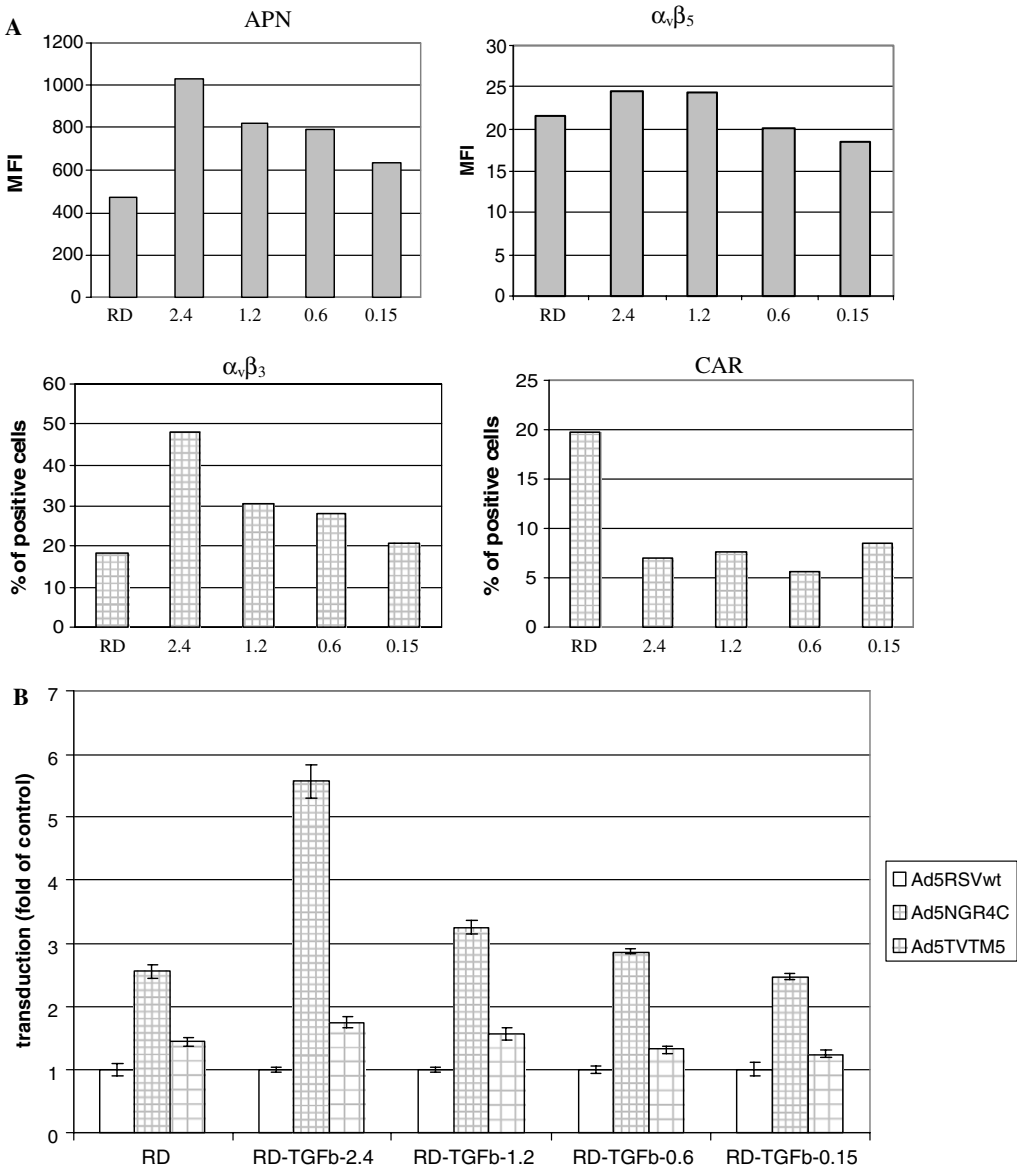

Fig. 3. Treatment of RD cells with TGF-β1 up-regulates APN and increases retargeting. (A) Influence of TGF-β1 on surface expression of molecules implicated in adenoviral entry ($\alpha_v\beta_3$, $\alpha_v\beta_5$, and CAR) and APN. RD cells were incubated with TGF-β1 (2.4, 1.2, 0.6, and 0.15 ng/ml) for 72 h. Control cells were incubated in medium. Cells were then detached with trypsin, adjusted to the same density, incubated with specific antibodies, and analyzed by flow cytometry. Primary antibodies used were as follows: FITC-labeled anti-$\alpha_v\beta_3$ integrin (MAb 23C6); PE- labeled anti-$\alpha_v\beta_5$ integrin (MAb P1F6), and MAbs WM15 against APN and RmcB against CAR. The secondary reagent was PE-labeled anti-mouse antibody. Isotype control samples were incubated with mouse IgG1 of an unrelated specificity. For molecules whose expression was detected on the entire cell population (APN and $\alpha_v\beta_5$), the results are presented as MFI of each sample minus MFI of the respective isotype control. For molecules whose expression was detected only on a subpopulation of cells ($\alpha_v\beta_3$ and CAR), the results are presented as the percentage of positive cells. Data of one of two experiments that yielded similar results are presented. (B) Influence of TGF-β1 on transduction efficacy of RD cells by Ad5RSVwt and NGR-bearing Ads, Ad5NGR4C (representative of Ads containing NGR within cyclic sequences) and Ad5TVTM5 (representative of Ads containing NGR within linear sequences). RD cells were incubated with TGF-β1 at the indicated concentrations (2.4, 1.2, 0.6, and 0.15 ng/ml) for 72 h. Control cells were incubated in medium. Cells were then detached with trypsin and 2×10^4 RD cells were plated in 96-well plates. Six hours later cells were infected for 1 h with 2-fold serial dilutions of Ad, 24 h after infection cells were stained using X-gal and blue cells were counted. Results for each Ad are given as the number of blue cells relative to that for Ad5RSVwt.

In order to determine whether differences in transduction efficacy were attributable to disulfide bond formation, we constructed a vector (Ad5NGR4G) identical to Ad5NGR4C in all but the four cysteines, which were replaced by glycines (characteristics of preparation of this Ad are presented in Table 1). We observed that Ad5NGR4G exhibited transduction efficacies similar to those of other Ads (Ad5TVTM5 and Ad5TVTM6) containing NGR within linear sequences. It transduced RD cells slightly more efficiently (1.3-fold) than wild type Ad5RSVwt, while Ad5NGR4C in the same experiment showed a 2.4-fold higher transduction efficacy (Fig. 4B). Finally, we tested the effect of TGF-β1 treatment on transduction efficacy, using the highest TGF-β1 concentration,

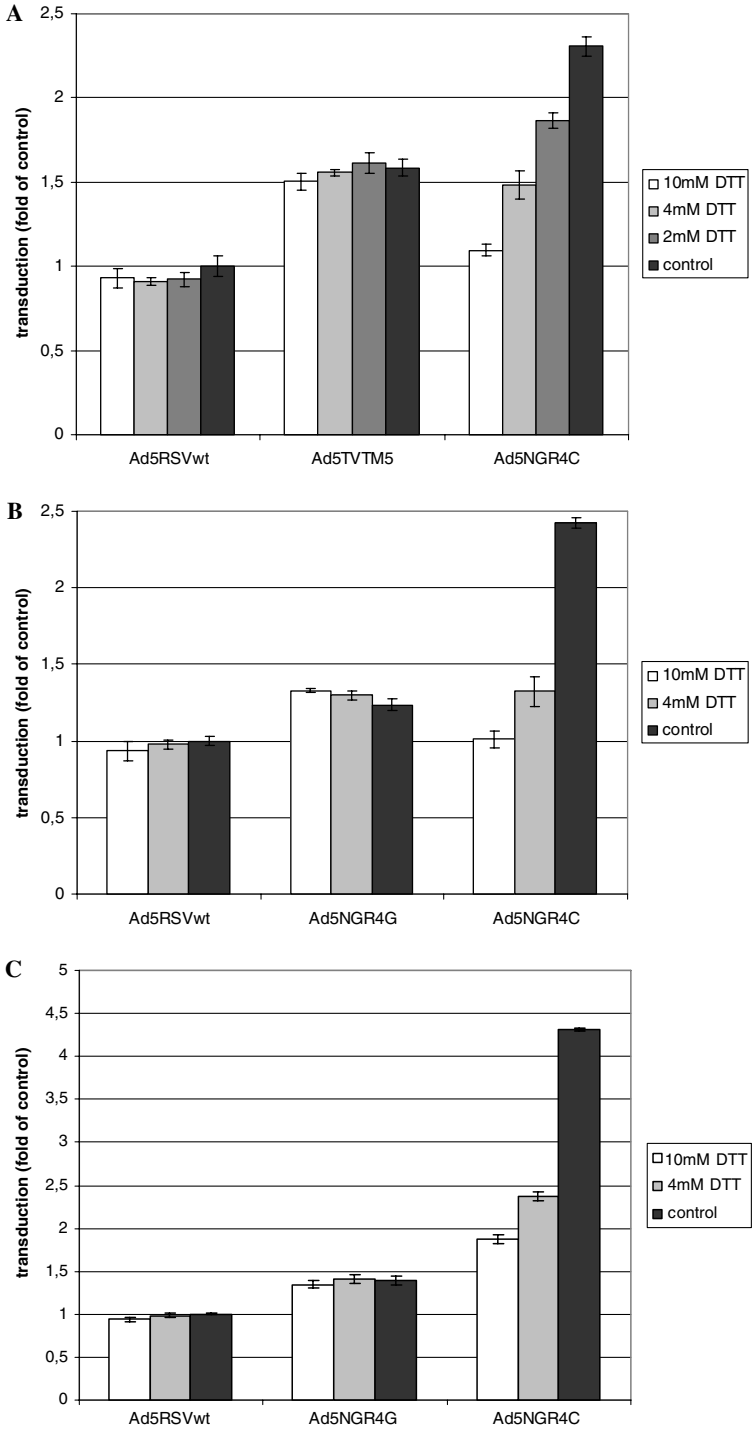

Fig. 4. Retargeting of Ad5NGR4C is dependent on formation of disulfide bonds. (A) Abolishment of increased transduction of RD cells by Ad5NGR4C after treatment with reducing reagent DTT. (B) Transduction efficacy of Ad5NGR4G and Ad5NGR4C differing only in cysteine residues and influence of treatment with DTT. (C) Influence of TGF-β1 and DTT treatment on transduction efficacy of RD cells by Ad5RSVwt, Ad5NGR4C, and Ad5NGR4G. 2×10^4 RD cells were plated in 96-well plates. Twenty-four hours later, Ads were treated for 1 h at 37 °C with different concentrations of DTT (2, 4, and 10 mM). Immediately after the DTT treatment, cells were infected for 1 h with 2-fold serial dilutions of Ads, and 24 h after infection cells were stained using X-gal and blue cells were counted. For measurement of influence of TGF-β1 on transduction efficacy, RD cells were incubated with TGF-β1 at the concentration 2.4 ng/ml for 72 h, then plated in 96-well plates and infected 6 h later. Results for each Ad are given as the number of blue cells relative to that for Ad5RSVwt without the addition of reducing agents. Data of one of two experiments that yielded similar results are presented.

2.4 ng/ml, tested previously (Fig. 3). As shown in Fig. 4C, for Ad5NGR4C we observed a substantial increase in retargeting index (from 2.4- to 4.3-fold) due to up-regulation of APN. In contrast, only a moderate increase in retargeting index (from 1.2- to 1.4-fold) was observed for Ad5NGR4G. Within the same experiment we treated all

three Ads (Ad5RSVwt, Ad5NGR4G, and Ad5NGR4C) with the reducing agent DTT and measured the transduction efficacy. DTT treatment had no effect on the transduction efficacy of wild type Ad5RSVwt and Ad5NGR4G, but abolished the increased transduction efficacy of AdNGR4C, in both TGF-β1 treated and untreated RD cells, in a dose dependent manner (Fig. 4B and C). Therefore, Ads containing NGR within cyclic sequence, due to the presence of cysteine residues, bound more efficiently to APN, while Ads containing NGR within a linear sequence bound more efficiently to $\alpha_v\beta_3$ integrin. Together, these data indicate that the formation of disulfide bond(s) between cysteines flanking NGR is crucial for the retargeting potential of the CDCNGRCFC sequence.

Discussion

To modify the host range of natural Ad, fiber modifications of potentially therapeutic vectors have been proposed. While short peptides derived from protein ligands that bind molecules of interest are attractive candidates for this purpose, the binding specificity may depend on the sequence surrounding the peptide and the resulting conformation. This is known to be the case for RGD motifs and their specificity for particular integrins [31,32]. Arap and colleagues [13] identified, by *in vivo* selection of phage display libraries, the NGR-containing peptide CNGRCVSGCAGRC as binding to tumor vasculature. They tested other peptides that contain the NGR motif, including a linear peptide, NGRAHA, and a synthetic peptide, CNGRC, that was cyclized *in vitro*. All peptides showed homing to tumor vasculature. Colombo and coworkers [17] described that both GNGRG and CNGRC targeted TNF to tumors; however, the antitumor activity of cyclic CNGRC-TNF was >10-fold higher than that of linear GNGRG-TNF. Linear and cyclic NGR peptides have been used for viral targeting [18,19]. Liu and coworkers [19] showed that incorporation of linear NGR-containing peptides into envelope escort proteins of MoMuLV enhanced retrovirus binding to human endothelial cells to a greater extent than potentially cyclic peptides. However, it is unclear from these studies whether lower efficiency of cyclic peptides was related to a poor incorporation of the targeting motif into virions or to a lower targeting efficiency. Mizuguchi and coworkers [33] constructed an Ad, AdNGR-L2, bearing an NGR peptide identical to our Ad5ARAP and showed, in CAR deficient LN444 cells which express moderate amounts of APN, a level of transduction approximately 100 times higher than that of wild type Ad.

Here, we generated and tested a series of four NGR-bearing, fiber HI-loop-modified adenoviruses in an effort to target APN. We used the HI loop of the fiber knob, since it is exposed at the surface and not directly involved in homotypic fiber contacts and CAR binding [34–36]. We show that incorporation of the NGR motif, within both linear and cyclic sequences, into the HI-loop of fiber protein resulted in retargeting of Ads in RD cells (Fig. 1). Such retargeting was the result of the NGR insertion, since the peptide CNGRC completely abolished increased transduction efficacy of NGR-bearing adenoviruses (Fig. 1). However, we showed that the CRGDC peptide also abolished increased transduction efficacy for all NGR-bearing Ads to the level of wild type Ad. The question that arises from these data is whether these peptides, CNGRC and CRGDC, inhibited transduction as a result of binding to the same or different molecule(s). In this regard, it is well known that RGD-containing peptides can cause cell detachment upon binding to integrins. We reasoned that if CNGRC bound preferentially to APN, it should not cause detachment to the same extent as the CRGDC peptide. As expected, we observed substantial detachment of RD cells only after addition of CRGDC, while the addition of CNGRC had no effect (data not shown). It is thus likely that these peptides bound preferentially to different molecules, although cross-reactivity cannot be excluded.

The increased transduction efficacy of NGR-containing Ads that we observed is less pronounced than that reported for MoMuLV Env escort proteins [19] or NGR-containing Adeno-associated virus [18] or even for Ad bearing the same NGR peptide, selected by phage display [33], as the Ad5ARAP Ad used in our study. Nevertheless, for meaningful comparison of recombinant Ads bearing NGR peptides, all viruses should be compared using the same APN-expressing cell type. From our results it is clear that transduction efficacy of Ad5NGR4C depends on APN expression (Fig. 3B). Therefore, if the expression of APN in endothelial cells in angiogenesis is high, this should promote retargeting.

Our results showed that on RD cells Ads containing NGR within cyclic sequences had a higher retargeting index than those containing NGR within linear sequences. We detected, a significant, albeit small, difference in transduction efficacy between two cyclic peptides that differ in flanking sequences (Fig. 1). Peptides NGR4C and ARAP, inserted into the HI-loop of Ad fiber protein, differ in length, amino acid sequence, and position of cysteine residues. This small difference in transduction efficacy may be the consequence of the proximity of NGR to a disulfide bond and subsequent accessibility for APN. The NGR peptides TVTM5 and TVTM6, which approximate the ninth type fibronectin III repeat, facilitated targeting upon presentation in the context of MoMuLV env escort proteins [19]. Our results are in line with those obtained by Colombo and coworkers [17], who showed that a cyclic NGR peptide binds to APN more effectively than a linear one.

We hypothesized that for NGR sequences, flanking cysteines are necessary for optimal interactions with the APN on RD cells. Increased transduction of Ad5NGR4C was completely abolished by DTT treatment, while the same treatment had no influence on Ad5RSVwt or Ad5TVTM5 transduction efficacy (Fig. 4A). To test our hypothesis more fully, we constructed a vector (Ad5NGR4G) identical

to Ad5NGR4C except that all four cysteines were replaced by glycines. Ad5NGR4G exhibited transduction efficacies similar to those previously described for Ad5TVTM5 and Ad5TVTM6 containing NGR within linear sequences. Moreover, Ad5NGR4G-mediated transduction was insensitive to DTT treatment, confirming that the formation of disulfide bonds in Ad5NGR4C is crucial for APN binding (Fig. 4B and C). Their position and exact number, however, are as yet undetermined. Somewhat unexpectedly, the efficiency of transduction after reduction of disulfide bonds fell below that mediated by the linear peptide (Fig. 4A and B), except after TGF-β1 where retargeting is largely increased (Fig. 4C). Thus reduction of cysteines, by disrupting secondary structure, may diminish binding to both possible targets, APN and $\alpha_v\beta_3$ integrin. Indeed, Colombo and coworkers [17] showed that peptide cyclization through disulfide bridge formation has an indirect role in NGR-mediated binding by stabilizing the conformation of the NGR motif.

Our study underscores the importance of cysteine residues flanking targeting peptides for not only affinity but also specificity of the retargeted Ad. Similarly Pierschbacher and Ruoslahti [31] first showed with cyclic RGD peptides that modifying peptide conformation can change its selectivity for the two receptors. The cyclic peptide was found to inhibit attachment to vitronectin at a 10-fold lower molar concentration than the same peptide before cyclization. At the same time, the cyclic peptide was ineffective at inhibiting attachment to fibronectin, and prior to cyclization showed no difference in activity from the prototype peptide. However, Belousova et al. [37] incorporated the aforementioned RGD motif into the HI-loop of Ad fiber protein and showed equal efficiencies of gene transfer for all RGD-containing vectors, regardless of the presence of cysteine residues.

NGR-containing Ads transduced HEp2-$\alpha_v\beta_3$ integrin expressing cells more efficiently than wild type Ad. An Ad containing NGR within a linear sequence was more efficient in $\alpha_v\beta_3$ integrin mediated transduction than an Ad containing NGR within a cyclic one (Fig. 2B). Importantly, both CNGRC and CRGDC peptides completely abolished increased transduction efficacy in comparison with wild type Ad5RSVwt (Fig. 2C).The question that arises from the aforementioned data is whether the observed retargeting on RD cells is the consequence of NGR motif affinity for APN, $\alpha_v\beta_3$ integrin or both. RD cells have a very small amount of $\alpha_v\beta_3$ on the cell surface (Table 2; Fig. 3A, only 18% of cells were $\alpha_v\beta_3$ positive) and it is not likely that this amount would suffice for retargeting of more than 2.4-fold. Moreover, the relationship between motif structure and extent of binding to RD cells (Fig. 1) differed from that observed in the HEp2 model of $\alpha_v\beta_3$ binding (Fig. 2B), suggesting that the cellular receptor(s) for NGR motifs within cyclic and linear sequences were not identical. We showed that up-regulation of APN on the surface of RD cells after TGF-β1 treatment (Fig. 3A) substantially increased the retargeting index for an Ad5NGR4C containing NGR within a cyclic sequence (from 2.6-fold to 5.6-fold; Fig. 3B), confirming that NGR within cyclic sequences retargeted Ad mainly to APN. A weak retargeting of Ad containing NGR within cyclic sequence to $\alpha_v\beta_3$ integrin was also evidenced (Fig. 2B). The retargeting index for Ad5TVTM5, as well as for Ad5NGR4G containing NGR within a linear sequence, increased to a small extent (from 1.4- to 1.8-fold; Fig. 3B and 1.2 to 1.4-gold; Fig. 4C) after TGF-β1 treatment. While we suppose that this was the consequence of preferential retargeting to $\alpha_v\beta_3$ integrin, we cannot exclude that Ad containing NGR within a linear sequence retargeted to APN with some degree of affinity.

The construction of a vector that specifically homes to tumor-associated vessels may be useful in targeting therapies specifically to tumors. Since in activated endothelial cells both APN and $\alpha_v\beta_3$ integrin are up-regulated [38], NGR-containing Ads could be suitable vectors for tumor gene therapy aimed at inhibition of angiogenesis. In summary, this study provides further support for the use of peptides identified by phage display in the design of retargeted vectors. Moreover, increased retargeting of Ads containing NGR within cyclic sequences underscores the need for individual analysis of all inserted motifs, and in particular as regards specificity and binding efficacy. Further study will be required to determine whether NGR-containing Ads are capable of mediating *in vivo* targeting.

Acknowledgments

The Ministry of Science, Education and Sport of the Republic of Croatia (Grant Nos. 0098076 and 0098094) and Institut National de la Recherche Agronomique of French Republic funded this work. We thank Martine Monteil (National Veterinary School of Alfort, Maisons Alfort, France) for providing us with antipenton monoclonal antibodies, Ljiljana Krajcar for technical assistance, and Maja Osmak for helpful discussion. Dragomira Majhen was a recipient of an EMBO short-term fellowship (April–July, 2003) and a French government fellowship (November–December, 2004). Andreja Ambriović-Ristov and Marc Eloit were supported by NATO Science Programme, Collaborative Linkage Grant (2002–2004).

References

[1] A.J. Zurita, W. Arap, R. Pasqualini, Mapping tumor vascular diversity by screening phage display libraries, J. Control. Release 91 (2003) 183–186.

[2] G.R. Nemerow, Cells receptors involved in adenovirus entry, Virology 274 (2000) 1–4.

[3] J.M. Bergelson, J.A. Cunningham, G. Droguett, E.A. Kurt-Jones, A. Krithivas, J.S. Hong, M.S. Horwitz, R.L. Crowell, R.W. Finberg, Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5, Science 275 (1997) 1320–1323.

[4] E. Li, S.L. Brown, D.G. Stupack, X.S. Puente, D.A. Cheresh, G.R. Nemerow, Integrin αvβ1 is an adenovirus coreceptor, J. Virol. 75 (2001) 5405–5409.

[5] G.R. Nemerow, D.A. Cheresh, T.J. Wickham, Adenovirus entry into host cells: a role for αv integrins, Trends Cell. Biol. 4 (1994) 52–55.
[6] T.J. Wickham, P. Mathias, D.A. Cheresh, G.R. Nemerow, Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment, Cell 73 (1993) 309–319.
[7] V. Krasnykh, I. Dmitriev, J.G. Navarro, N. Belousova, E. Kashentseva, J. Xiang, J.T. Douglas, D.T. Curiel, Advanced generation adenoviral vectors possess augmented gene transfer efficiency based upon coxsackie adenovirus receptor-independent cellular entry capacity, Cancer Res. 60 (2000) 6784–6787.
[8] D. Majhen, A. Ambriović-Ristov, Adenoviral vectors-how to use them in cancer gene therapy? Virus Res. 119 (2006) 121–133.
[9] L.J. Henry, D. Xia, M.E. Wilke, J. Deisenhofer, R.D. Gerard, Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*, J. Virol. 68 (1994) 5239–5246.
[10] N. Louis, P. Fender, A. Barge, P. Kitts, J. Chroboczek, Cell binding domain of adenovirus serotype 2 fiber, J. Virol. 68 (1994) 4104–4106.
[11] I. Dmitriev, V. Krasnykh, C.R. Miller, M. Wang, E. Kashentseva, G. Mikheeva, N. Belousova, D.T. Curiel, An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism, J. Virol. 72 (1998) 9706–9713.
[12] V. Krasnykh, I. Dmitriev, G. Mikheeva, C.R. Miller, N. Belousova, D.T. Curiel, Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob, J. Virol. 72 (1998) 1844–1852.
[13] W. Arap, R. Pasqualini, E. Ruoslahti, Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model, Science 279 (1998) 377–380.
[14] R. Pasqualini, E. Koivunen, R. Kain, J. Lahdenranta, M. Sakamoto, A. Stryhn, R.A. Ashmun, L.H. Shapiro, W. Arap, E. Ruoslahti, Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis, Cancer Res. 60 (2000) 722–727.
[15] H.M. Ellerby, W. Arap, L.M. Ellerby, R. Kain, R. Andrusiak, G.D. Rio, S. Krajewski, C:R: Lombardo, R. Rao, E. Ruoslahti, D.E. Bredesen, R. Pasqualini, Anti-cancer activity of targeted pro-apoptotic peptides, Nature Med. 5 (1999) 1032–1038.
[16] F. Curnis, A. Sacchi, L. Borgna, F. Magni, A. Gasparri, A. Corti, Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13), Nat. Biotechnol. 18 (2000) 1185–1190.
[17] G. Colombo, F. Curnis, G.M. De Mori, A. Gasparri, C. Longoni, A. Sacchi, R. Longhi, A. Corti, Structure–activity relationships of linear and cyclic peptides containing the NGR tumor-homing motif, J. Biol. Chem. 277 (2002) 47891–47897.
[18] M. Grifman, M. Trepel, P. Speece, L.B. Gilbert, W. Arap, R. Pasqualini, M.D. Weitzman, Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids, Mol. Ther. 3 (2001) 964–975.
[19] L. Liu, W.F. Anderson, R.W. Beart, E.M. Gordon, F.L. Hall, Incorporation of tumor vasculature targeting motifs into moloney murine leukemia virus env escort proteins enhances retrovirus binding and transduction of human endothelial cells, J. Virol. 74 (2000) 5320–5328.
[20] F.L. Graham, J. Smiley, W.C. Russell, R. Nairn, Characteristics of a human cell line transformed by DNA from human Ad5, J. Gen. Virol. 75 (1977) 133–139.
[21] A. Ambriović-Ristov, J. Gabrilovac, T. Čimbora-Zovko, M. Osmak, Increased adenoviral transduction efficacy in human laryngeal carcinoma cells resistant to cisplatin is associated with increased expression of integrin $\alpha_v\beta_3$ and coxsackie adenovirus receptor, Int. J. Cancer. 110 (2004) 660–667.
[22] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
[23] C. Chartier, E. Degryse, M. Gantzer, A. Dieterle, A. Pavirani, M. Mehtali, Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*, J. Virol. 70 (1996) 4805–4810.
[24] S. Arrabal, M. Touchard, F. Mouthon, B. Klonjkowski, J.P. Deslys, D. Dormont, M. Eloit, Nervous and nonnervous cell transduction by recombinant adenoviruses that inducibly express the human PrP, Biochem. Biophys. Res. Commun. 285 (2001) 623–632.
[25] N. Mittereder, K.L. March, B.C. Trapnell, Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy, J. Virol. 70 (1996) 7498–7509.
[26] A. Ambriović-Ristov, S. Mercier, M. Eloit, Shortening adenovirus type 5 fiber shaft decreases the efficiency of postbinding steps in CAR-expressing and nonexpressing cells, Virology 312 (2003) 425–433.
[27] E. Koivunen, B. Wang, E. Ruoslahti, Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins, Bio/Technol. 13 (1995) 265–270.
[28] J.M. Healy, O. Murayama, T. Maeda, K. Yoshino, K. Sekiguchi, M. Kikuchi, Peptide ligands for integrin $\alpha_v\beta_3$ selected from random phage display libraries, Biochemistry 34 (1995) 3948–3955.
[29] A. Kehlen, J. Langner, D. Riemann, Transforming growth factor-β increases the expression of aminopeptidase N/CD13 mRNA and protein in monocytes and monocytic cell lines, Adv. Exp. Med. Biol. 477 (2000) 49–56.
[30] J. Gabrilovac, D. Breljak, B. Čupić, A. Ambriović-Ristov, Regulation of aminopeptidase N (EC3.4.11.2; APN; CD13) by interferon-γ on the HL-60 cell line, Life Sci. 76 (2005) 2681–2697.
[31] M.D. Pierschbacher, E. Ruoslahti, Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion, J. Biol. Chem. 262 (1987) 17294–17298.
[32] E. Ruoslahti, RGD and other recognition sequences for integrins, Annu. Rev. Cell Dev. Biol. 12 (1996) 697–715.
[33] H. Mizuguchi, N. Koizumi, T. Hosono, N. Utoguchi, Y. Watanabe, M.A. Kay, T. Hayakawa, A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob, Gene Ther. 8 (2001) 730–735.
[34] D. Xia, L.J. Henry, R.D. Gerard, J. Deisenhofer, Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution, Structure 2 (1994) 1259–1270.
[35] P.W. Roelvink, G. Mi Lee, D.A. Einfeld, I. Kovesdi, T.J. Wickham, Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae, Science 286 (1999) 1568–1571.
[36] M.C. Bewley, K. Springer, Y.B. Zhang, P. Freimuth, J.M. Flanagan, Structural analysis of the mechanism of adenovirus binding to its human cellular receptor CAR, Science 286 (1999) 1579–1583.
[37] N. Belousova, V. Krendelchtchikova, D.T. Curiel, V. Krasnykh, Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein, J. Virol. 76 (2002) 8621–8631.
[38] R. Pasqualini, E. Koivunen, E. Ruoslahti, αv integrins as receptors for tumor targeting by circulating ligands, Nat. Biotechnol. 15 (1997) 542–546.