Targeted gene delivery into $\alpha_9\beta_1$-integrin-displaying cells by a synthetic peptide

Holm Schneider[a,*], Richard P. Harbottle[a], Yasuyuki Yokosaki[b], Philipp Jost[a], Charles Coutelle[a]

[a] *Cystic Fibrosis Gene Therapy Research Group, Section of Molecular Genetics, Division of Biomedical Sciences, Imperial College School of Medicine, Sir Alexander Fleming Building, London SW7 2AZ, UK*
[b] *Department of Laboratory Medicine, National Hiroshima Hospital, 513 Jike, Saijoh, Higashi-Hiroshima 739-0041, Japan*

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Abstract We have investigated the usefulness of two small synthetic peptides comprising either a linear or a cyclic PLAEIDGIEL domain and a DNA-binding moiety of 16 lysine residues to mediate gene transfer selectively into $\alpha_9\beta_1$-integrin-displaying cells. Such specific gene delivery could only be achieved with the peptide containing the cyclic PLAEIDGIEL domain. However, inclusion of the cationic liposome Lipofectamine into the peptide/DNA complexes resulted for both peptides in efficient gene transfer with significant targeting specificity. Naturally, the integrin $\alpha_9\beta_1$ is present only in a few highly specialised tissues and abundant throughout the human airway epithelia in vivo. Targeting gene vectors to this integrin therefore appears a useful approach to gene therapy of lung diseases such as cystic fibrosis. As the integrin $\alpha_9\beta_1$ is associated with tissue differentiation during foetal development and may cause resurgence of the foetal phenotype in colon cancers, such vectors may also be applicable for prenatal and cancer gene therapy.

Key words: $\alpha_9\beta_1$-Integrin; DNA transfer; Peptide; Receptor-mediated; Gene therapy

1. Introduction

Integrin $\alpha_9\beta_1$, the cell surface receptor for the extracellular matrix proteins tenascin C [1] and osteopontin [2] and the vascular cell adhesion molecule-1 [3], is found in highly specialised tissues, such as squamous epithelia, smooth and skeletal muscles and hepatocytes [4]. It has also been reported to be abundant throughout the human airway epithelia in vivo, irrespective of any particular clinical status [5], which makes it interesting for targeted drug or gene delivery to treat lung diseases such as cystic fibrosis. During foetal development $\alpha_9\beta_1$-integrin is associated with the onset of tissue differentiation suggesting a role in the maturation and/or maintenance of these tissues [6]. Its presence in certain carcinoma cell lines has been interpreted as a resurgence of the foetal phenotype in a subset of colon cancers [7]. Hence, $\alpha_9\beta_1$-integrin-targeting gene therapy vectors may also be applicable for prenatal [8] and cancer gene therapy.

Recently we localised the $\alpha_9\beta_1$-integrin-binding site of tenascin C to the B-C loop of its third fibronectin type III repeat (TNfn3) and showed that a synthetic Pro-Leu-Ala-Glu-Ile-Asp-Gly-Ile-Glu-Leu-Thr-Tyr peptide (PLAEIDGIELTY) displaced $\alpha_9\beta_1$-integrin-displaying cells completely from binding to recombinant TNfn3 [9,10]. This peptide, therefore, appeared a promising candidate domain for the development of gene therapy vectors targeting the integrin $\alpha_9\beta_1$. Here we show that targeted gene delivery into $\alpha_9\beta_1$-integrin-displaying cells can be achieved with a synthetic peptide comprising a cyclic CPLAEIDGIELC domain and a DNA-binding moiety of 16 lysine residues.

2. Materials and methods

2.1. Cell lines, plasmids and synthetic peptides

The human colon carcinoma cell line SW480 either displaying $\alpha_9\beta_1$-integrin after being stably transformed with pcDNAIneoα_9 or lacking this integrin (mock-transfectants) [1] was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 U/ml) and G418 (1 mg/ml). All tissue culture reagents were purchased from Life Technologies, Paisley, UK.

The pGEX expression plasmid encoding a TNfn3/glutathione *S*-transferase fusion protein [11] was obtained from Dr. Kathryn L. Crossin (Scripps Research Institute, La Jolla, CA, USA). Recombinant TNfn3 was produced in *Escherichia coli* and purified as described previously [9].

The peptide PLAEIDGIELTY was purchased from Genosys Biotechnologies Inc., Cambridge, UK. The peptides K[16]-GGPLAEIDGIELGA, K[16]-GCPLAEIDGIELCA (disulphide-cyclised) and K[16] were synthesised by Affiniti Research Products, Exeter, UK. Their ability to bind DNA was shown by adding increasing amounts of peptide to a constant amount of plasmid DNA followed by analysis on a 1% agarose gel indicating the amount of peptide required for the complete retardation of the electrophoretic mobility of 1 μg plasmid DNA (= 1 retardation unit).

2.2. Competitive cell binding inhibition assays

Individual wells of non-tissue culture-treated polystyrene 96-well microtitre plates were coated by incubation with recombinant TNfn3 for 1 h at 37°C, washed three times with phosphate-buffered saline (PBS) and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. $\alpha_9\beta_1$-Integrin-displaying SW480 cells, harvested when sub-confluent, were washed with DMEM and re-suspended in DMEM containing 0.5% BSA. Aliquots of the cell suspension were pre-treated by mixing with the indicated peptides for 15 min at 4°C. Following addition of 5×10^4 cells to each well, the plates were spun in a centrifuge at $10\times g$ for 5 min and incubated for 1 h at 37°C, 5% CO_2. Non-adherent cells were removed by centrifugation of the plate top-side down at $48\times g$ for 5 min. The attached cells were fixed with 1% formaldehyde and stained with 0.5% (w/v) crystal violet [12]. Excess stain in the wells was thoroughly washed away with PBS. After air-drying the cells were solubilised in 2% Triton X-100 and quantified by measuring the absorbance at 595 nm.

*Corresponding author. Fax: (44) (171) 5943015.
E-mail: h.schneider@ic.ac.uk

Abbreviations: TNfn3, third fibronectin type III repeat of tenascin C; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FCS, foetal calf serum

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2.3. Gene transfer experiments

The American firefly luciferase gene under the control of a simian virus 40 promoter and enhancer in the plasmid vector pGL3 (Promega, Madison, WI, USA) was used as reporter gene. Plasmid DNA was purified from overnight cultures of transformed *E. coli* DH5α using an endotoxin extraction kit (Qiagen, Hilden, Germany). Cells were seeded in tissue culture-treated 24-well plates at a density of 5×10^4/well and incubated for approximately 12 h until 50% confluent. Peptide-DNA and peptide-DNA-LipofectAMINE complexes were prepared in 50 μl of serum-free OptiMem medium (Life Technologies, Paisley, UK). The cells were washed and incubated in OptiMem for 30 min at 37°C before addition of the complexes and further incubation for 4 h. The medium was then replaced by DMEM containing 10% FCS supplemented with penicillin (100 U/ml), streptomycin (100 U/ml) and G418 (1 mg/ml) and the incubation was continued for an additional 44 h. Luciferase activity was determined after harvesting the cells in 200 μl of reporter lysis buffer (Promega, Madison, WI, USA) using the luciferase assay kit from Promega and a Berthold luminometer.

2.4. Statistical analysis

Each result of gene transfer experiments represents the mean (±S.E.M.) of at least four independent assays each performed in sextuplicate. Statistical analysis was done using the non-parametric rank test of Wilcoxon. Probability values <0.05 were considered to be statistically significant.

3. Results and discussion

Aiming to improve the targeting of our established non-viral integrin-mediated gene transfer systems [13,14] to airway epithelia, we synthesised two peptides each comprising a receptor-targeting domain (either disulphide-cyclised CPLAEIDGIELC or linear GPLAEIDGIELG) and a DNA-binding moiety consisting of 16 lysine residues.

Previous binding competition studies had revealed that a linear version of the PLAEIDGIEL domain on its own blocked the adhesion of $\alpha_9\beta_1$-integrin-displaying cells to recombinant TNfn3 better than a cyclic one. This indicated that the linear structure was advantageous due to its ability to adapt to conformational requirements for that binding (data not shown). Surprisingly, in first gene transfer assays specific gene delivery into $\alpha_9\beta_1$-integrin-displaying SW480 cells could only be achieved with the cyclic CPLAEIDGIELC-K[16] peptide and the transfection efficiency was relatively low when compared with other systems for receptor-mediated gene transfer developed in our lab [14,15]. Therefore we investigated whether the positively charged polylysine tail influences the binding of the PLAEIDGIEL domain to $\alpha_9\beta_1$-integrin. These experiments showed that the coupling of K[16] to the linear GPLAEIDGIELG domain leads to a drastic weakening of its binding to $\alpha_9\beta_1$-integrin. While the linear peptide without K[16] tail displaced $\alpha_9\beta_1$-integrin-displaying SW480 cells completely from their binding to recombinant TNfn3, its equivalent with K[16] tail at a concentration of 1 mM reduced that binding only by 33%. In contrast, the peptide with the cyclic head group and K[16]tail at the same concentration diminished the adhesion of $\alpha_9\beta_1$-integrin-displaying SW480 cells by 57% (Fig. 1).

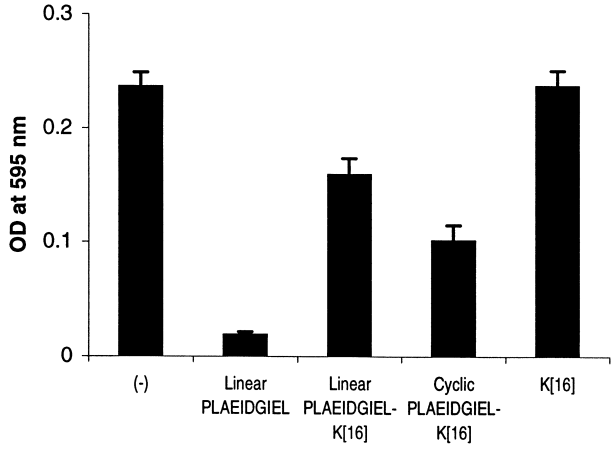

Fig. 1. Effects of different peptides containing a PLAEIDGIEL domain on the attachment of $\alpha_9\beta_1$-integrin-displaying SW480 cells to TNfn3. Individual wells of 96-well plates were coated with recombinant TNfn3 and blocked with BSA. 5×10^4 $\alpha_9\beta_1$-integrin-displaying SW480 cells pre-treated with the indicated peptides were added to each well (triplicate wells for each peptide) and the plates were incubated for 1 h at 37°C, 5% CO_2. Non-adherent cells were removed by centrifugation of the plate top-side down. The attached cells were fixed with 1% formaldehyde, stained with crystal violet, solubilised in 2% Triton X-100 and quantified by measuring the absorbance at 595 nm. Error bars indicate the standard deviation.

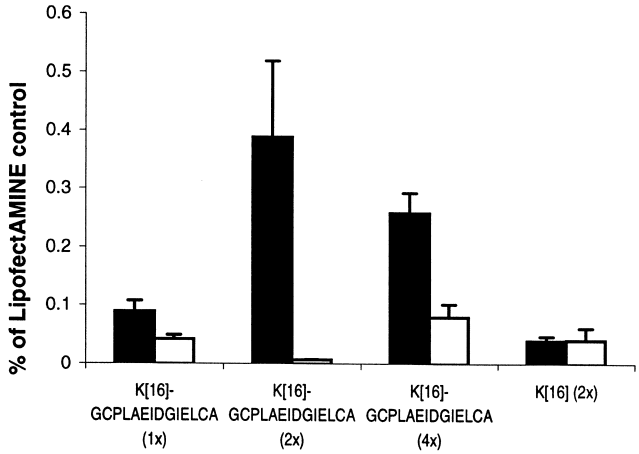

Fig. 2. $\alpha_9\beta_1$-Integrin-mediated delivery of a luciferase reporter gene plasmid using a cyclic K[16]-GCPLAEIDGIELCA peptide. Peptide-pGL3 complexes containing one, two or four retardation units of the peptide (retardation unit = the amount of peptide required for complete retardation of the electrophoretic mobility of 1 μg of plasmid DNA) formed in HEPES-buffered saline were incubated with semi-confluent cells on 24-well dishes for 4 h at 37°C. Following further incubation for an additional 44 h, luciferase activity in the cell lysates was determined. Each column represents the average result of four experiments each performed in sextuplicate (black columns: SW480 cells displaying $\alpha_9\beta_1$-integrin; white columns: SW480 cells lacking this integrin). Error bars indicate the S.E.M.

The difference between the linear and the cyclic peptide may be due to a conformation change of the negatively charged linear head group after coupling with the positively charged K[16] tail, e.g. the shape and function of the head group may be neutralised like a closed pocket knife, which appears to occur to a lesser extent with the disulphide-constrained cyclic head group.

Repeated gene transfer assays confirmed that the peptide with the cyclic CPLAEIDGIELC domain mediates specific gene delivery into $\alpha_9\beta_1$-integrin-displaying SW480 cells (Fig. 2), whereas there was no consistently significant difference between cells displaying $\alpha_9\beta_1$ and cells lacking this integrin when the linear peptide was used (data not shown). Complexes formed with two retardation units of the cyclic peptide

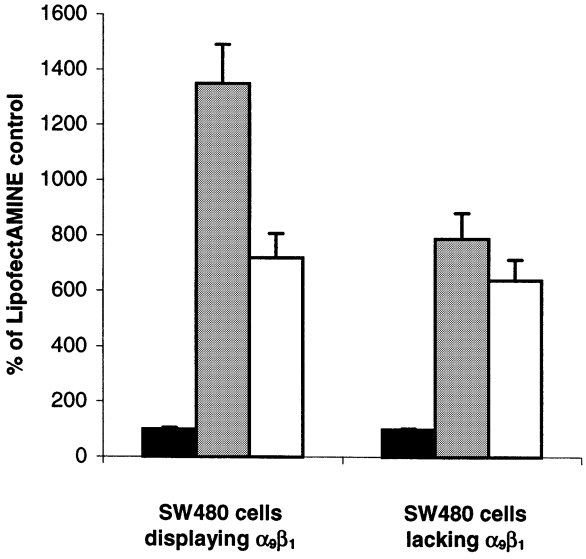

Fig. 3. Liposome-mediated enhancement of the transfection by a cyclic K[16]-GCPLAEIDGIELCA peptide. Peptide-pGL3 complexes (containing two retardation units of the peptide) were allowed to form complexes with 24 μg LipofectAMINE/μg DNA in HEPES-buffered saline for 15 min at room temperature. The complexes were added to semi-confluent cells followed by incubation for 4 h at 37°C. 48 h after gene delivery the luciferase activity in the cell lysates was determined. Each column represents the average result of six experiments each performed in sextuplicate (black columns = LipofectAMINE/DNA complexes; grey columns = K[16]-GCPLAEIDGIELCA/LipofectAMINE/DNA complexes; white columns = K[16]/LipofectAMINE/DNA complexes). Error bars indicate the S.E.M.

were found to deliver a luciferase reporter gene one order of magnitude more efficiently into $\alpha_9\beta_1$-integrin-displaying cells than complexes formed with two retardation units of K[16] (Fig. 2). However, the cationic liposome LipofectAMINE that served as assay standard gave on average 200-fold higher reporter gene expression values than the optimal amount of the cyclic peptide on $\alpha_9\beta_1$-integrin-displaying cells.

As time course studies revealed highly $\alpha_9\beta_1$-integrin-specific transfection after vector-cell contact for 120–240 min, but increasing unspecific transfection with longer contact times (data not shown), we standardised our gene transfer assays at cell contact times of 2–4 h. We tried to improve the efficiency of the gene transfer by including the cationic lipid LipofectAMINE into the peptide/DNA complexes, which has been demonstrated to enhance the transfection mediated by our RGD-prototype system for integrin-mediated gene transfer [16]. Optimisation experiments showed that the gene transfer efficiency achieved with LipofectAMINE can be increased more than one order of magnitude by complexing the cyclic peptide with LipofectAMINE and DNA using lipid/DNA ratios between 12:1 and 24:1 (w/w). However, as with our model system [16] this increase in efficiency was associated with reduced, but still significant ($P < 0.05$) cell specificity (Fig. 3). Thus, in the presence of LipofectAMINE internalisation of the complex occurs to a large extent by an integrin-independent mechanism. Nevertheless, with respect to in vivo application of $\alpha_9\beta_1$-integrin-targeting peptides such combination appears to be a promising approach.

Interestingly, the lipid enhancement also allowed targeting specificity to be achieved with the linear peptide. Pooled data from four experiments each performed in sextuplicate showed 2.1-fold better transfection with K[16]-GGPLAEIDGIELGA/LipofectAMINE than with K[16]/LipofectAMINE on $\alpha_9\beta_1$-integrin-displaying cells ($P < 0.05$) and 1.9-fold higher efficiency of K[16]-GGPLAEIDGIELGA/LipofectAMINE on $\alpha_9\beta_1$-integrin-displaying cells than on control cells lacking this integrin ($P < 0.05$).

In contrast to previously developed peptides for integrin-mediated gene transfer [13,14,17,18] which display the arginine-glycine-aspartic acid (RGD) motif recognised by a broad variety of receptors, PLAEIDGIEL-containing vectors target only one receptor, the integrin $\alpha_9\beta_1$. Thus, the results of this study prove the possibility of generating vectors for the targeting of individual integrins.

The peptide vectors developed so far represent the first generation of synthetic virus-like particles: they mimic viruses by using integrins to enter cells and also by literally 'packaging' DNA within a complex of a size equivalent to a virus, and thus protecting the DNA from degradation by nucleases [14]. Our peptides, however, avoid some crucial problems inherent in viral gene therapy vectors, such as limited capacity for foreign DNA, contamination of virus preparations with wild type/helper virus, and induction of immune responses (Harbottle et al., unpublished data). They can be considered to be modular multifunctional systems with greater flexibility because they allow the combination of any given plasmid DNA with several peptides harbouring different functions, which can be adapted to a particular gene transfer request, in a given disease [19]. As internalisation into cells may occur by a phagocytosis-like 'zippering-up' mechanism without specific limitation to the size of the particle [14], systems for integrin-mediated gene transfer appear to be suitable for efficient introduction of mammalian artificial chromosomes into cells.

The development of targeted non-viral vectors is one of the main current strategies in gene therapy research. Although systems for receptor-mediated gene transfer have not yet reached the gene transfer efficiency of the best viral vectors, combination with liposomes may facilitate approaching it. The possibility to synthesise and modify well-defined gene therapy vectors may therefore be a major advantage of our synthetic peptides and the data reported here stand as proof of principle for that.

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