Conjugation of an antibody Fv fragment to a virus coat protein: cell-specific targeting of recombinant polyoma-virus-like particles

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The development of cell-type-specific delivery systems is highly desirable for gene-therapeutic applications. Current virus-based vector systems show broad cell specificity, which results in the need to restrict the natural tropism of these viral systems. Here we demonstrate that tumour-cell-specific virus-like particles can be functionally assembled *in vitro* from recombinant viral coat protein expressed in *Escherichia coli*. The insertion of a negatively charged peptide in the HI loop of polyoma VP1 interferes with the binding of VP1 to the natural recognition site on mammalian cells and also serves as an adapter for the coupling of antibody fragments that contain complementary charged fusion peptides.

A recombinant antibody fragment of the tumour-specific anti-(Lewis Y) antibody B3 could be coupled to the mutant VP1 by engineered polyionic peptides and an additional disulphide bond. With this system an entirely recombinant cell-specific delivery system assembled *in vitro* could be generated that transfers genes preferentially to cells presenting the tumour-specific antigen on the cell surface.

Key words: disulphide bond, gene transfer, polyionic peptides, VP1 coat protein.

INTRODUCTION

Most vector systems that have been used in gene therapy so far are derivatives of retroviruses, adenoviruses and adeno-associated viruses [1]. These viruses do not possess an inherent cell-type-specific targeting function. To create a cell-type-specific tropism of vector systems, several attempts have been made to display tumour-specific antibody fragments on the surface of the vectors, which were in most cases retroviruses [2–5]. In a more general approach, an antibody-binding domain of Protein A was inserted into the viral coat of a Sindbis virus vector, allowing the coupling of different antibodies to the vector [6,7]. Furthermore, cell-type-specific targeting based on proteins and peptides other than antibodies has been developed [8–11].

As an alternative to viral vector systems, virus-like particles (VLPs) can be used in gene therapy. These artificial vectors consist only of the recombinantly produced virus shell without any viral genetic information packaged inside. The recombinant expression, especially in *Escherichia coli*, guarantees a high production level of the respective protein and permits the simple generation of functional mutants. One of these VLPs, based on the virus coat protein polyoma VP1, has been used successfully for the cell-type-non-specific targeting and transfection of eukaryotic cells [12–14].

The natural virus shell of polyoma virus consists of three coat proteins. However, Salunke et al. [15] demonstrated that the major coat protein VP1 alone is able to assemble to VLPs. Polyoma VP1 is a pentameric protein that can be produced in *E. coli* in large amounts [16]. The crystal structure of VP1 in complex with sialyl lactose, an analogue of the natural cellular receptor sialic acid [17], has been solved [18,19], providing a basis for the construction of functional variants. The binding activity

of VP1 to sialic acid residues on cell surfaces causes the cell-type-non-specific targeting of the VLPs observed in cell culture. The assembly of VP1 to VLPs can be induced *in vitro* by adding millimolar concentrations of Ca²⁺ ions [15,20]. This assembly has been demonstrated not only for wild-type VP1 (wtVP1) but also for genetically engineered variants [21–24].

In the present study we used VLPs of a variant of polyoma VP1 as vector system. This variant contained a polyionic peptide (Glu₈Cys) inserted into the HI loop of VP1 [24]. To create cell-type-specific targeting of these particles, they were decorated with an Fv fragment of the tumour-specific antibody B3, which specifically recognizes tumour cells presenting the antigen Lewis Y, a sugar residue found on breast tumours and epidermoid tumour cells [25]. This Fv fragment of B3 has already been used to construct immunotoxins containing the enzymically active domain of *Pseudomonas* exotoxin [25–27], which selectively kill the antigen-presenting cells MCF7, A431 and CRL1739 [25]. The Fv fragment was coupled to the VLPs via a specific docking system based on polyionic fusion peptides with a complementary charge and an engineered disulphide bond. The presentation of the tumour-specific antibody fragment on the VLPs led to a retargeting of these modified VLPs towards antigen-presenting cells.

MATERIALS AND METHODS

Production of polyoma VLPs *in vitro*

We recently engineered a mutant of polyoma VP1 with a peptide containing eight glutamic residues and a cysteine residue inserted in the HI loop (VP1-E8C) [24]. This mutant was produced in *E. coli* in the soluble fraction of the cell lysate. The purification scheme for VP1-E8C consists of fractionation by precipitation

Abbreviations used: VLP, virus-like particle; VP1-E8C, virus coat protein VP1 of polyoma virus with a negatively charged peptide consisting of eight glutamic residues and one cysteine residue inserted in the HI loop; wt, wild-type.

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with $(\text{NH}_4)_2\text{SO}_4$ followed by anion-exchange and size-exclusion chromatographies [24]. For the assembly of VLPs, VP1-E8C was dialysed for 2 days at 20 °C against 0.02 M Tris/HCl (pH 7.4)/200 mM NaCl/1 mM CaCl_2 /0.75 M $(\text{NH}_4)_2\text{SO}_4$, on which VLPs form spontaneously [15,20]. For small-scale experiments, VLPs were purified by size-exclusion chromatography on a TSK PW 6000 column (TosoHaas; volume 14 ml, flow rate 0.7 ml/min) equilibrated with 10 mM Tris/HCl (pH 7.4)/10 μM CaCl_2 /150 mM NaCl. The large-scale preparative procedure consisted of a Superdex 200 preparative-grade column (Pharmacia; volume 120 ml, flow rate 1 ml/min) equilibrated in the same buffer.

Preparation of recombinant dsFv-B3-R8C antibody fragment

For cell-type-specific targeting of the VLPs, an Fv fragment of the tumour-specific antibody B3 was chosen (dsFv-B3). This Fv fragment was stabilized by an engineered interchain disulphide bond [28]. The plasmid pUli 39-1 [28], encoding the variable domain VL of the light chain of the antibody B3 fused to *Pseudomonas* exotoxin, was modified by the introduction of a termination codon at the C-terminus of VL, thus creating an open reading frame of the VL domain only. The variable domain VH of the heavy chain of the antibody B3, encoded by pYR 38-2 [28], was modified at the C-terminus by the introduction of a sequence encoding the peptide Arg₅CysPro, thus generating clones for the production of the polyionic-tagged fragment dsFv-B3-R8C.

The VH and VL domains were expressed separately in *E. coli* and isolated as inclusion bodies [29]. Inclusion bodies were refolded to dsFv-B3-R8C for 5 days by the simultaneous dilution of solubilized VH and VL inclusion bodies in 100 mM Tris/HCl (pH 8.5)/1 mM EDTA/0.5 M arginine/1 mM GSH/1 mM GSSG. The protein concentration was 30 $\mu\text{g}/\text{ml}$, with a 5:1 molar ratio of VH to VL. Aggregates and other insoluble components in the renaturation solution were pelleted by centrifugation (42000 g, 30 min) and the soluble protein was concentrated and dialysed against 50 mM Tris/HCl (pH 7.4)/1 mM EDTA/200 mM NaCl (Vario-3-System filtration; Minisette FSQ, cut off 8 kDa). The refolded protein was then purified by cation-exchange (Poros HS; PerSeptive Biosystems) and size-exclusion chromatographies [Superdex 75; Pharmacia; volume 120 ml, flow rate 0.75 ml/min; 0.02 M Tris/HCl (pH 7.4)/200 mM NaCl/5% (v/v) glycerol/1 mM CaCl_2].

Decoration of VLPs of VP1-E8C with the antibody fragment dsFv-B3-R8C

The association of dsFv-B3-R8C and VLPs of VP1-E8C was performed in 0.02 M Tris/HCl (pH 7.4)/200 mM NaCl/5% (v/v) glycerol/1 mM CaCl_2 /1.6 mM GSSG/0.4 mM GSH. As a control, wtVP1 without polyionic sequence was incubated with dsFv-B3-R8C under identical conditions. After 5 h of coupling at 10 °C, the reaction mixtures were applied to a size-exclusion column as described for unconjugated VLPs. VLPs of VP1-E8C, wtVP1 and the conjugated particles were analysed by electron microscopy as described previously [24]. To evaluate the binding of recombinant polyoma VLPs to mammalian cells, haemagglutination assays were performed as described by Gleiter et al. [23].

Association/packaging of DNA in VLPs

The packaging of DNA into VLPs was performed by the procedure of Barr et al. [30]: 1 μg of purified plasmid pEli 92 DNA [31], encoding the bacterial β -galactosidase under the

control of a cytomegalovirus promotor, was incubated with 16 μg of VLPs at 37 °C for 15 min; the mixture was then exposed to an osmotic shock by dilution with 4 vol. of water. Under these conditions the VLPs swell and thereby allow the DNA to enter the particle. Tight binding or incorporation of the DNA to the capsid has been observed under these conditions [12,13]. DNase protection assays were used to analyse DNA binding/packaging. The packaging mixture (1 μg of DNA and 16 μg of VLP) was incubated with 10 m-units of benzonase (Merck, Darmstadt, Germany) in the presence of 10 mM MgCl_2 and 0.5 mM CaCl_2 for 30 min at 37 °C (a unit of benzonase is defined by the manufacturer as the amount of enzyme which results in a change in A_{260} equal to 1 in 30 min at 37 °C in 50 mM Tris (pH 8)/1 mM MgCl_2 /0.1 mg/ml BSA/1 mg/ml sonicated salmon sperm DNA; the absorbance is determined following perchloric acid precipitation). As controls, DNA without protein, or a mixture of 1 μg of DNA and 16 μg of dsFv-B3-R8C, were treated equally. The reaction was stopped by incubation at 65 °C for 15 min. Simultaneously, the VLPs were disassembled in 250 mM EDTA and 10 mM dithiothreitol. The released DNA was then diluted 10000-fold and different fragments on the plasmid were amplified by PCR. The amount of amplified PCR fragment, representative of the presence or absence of protected template DNA, was detected by agarose-gel electrophoresis.

Transfection experiments

The cell lines MCF7 (breast carcinoma) and A431 (epidermoid vulva carcinoma), which express the oligosaccharide Lewis Y, the cell-surface antigen recognized by dsFv-B3, were grown as monolayers in RPMI 1640 medium (ICN) supplemented with 10% (v/v) fetal bovine serum. The Lewis-Y-negative cell lines PA-1 (germ cells) and KB31 (epidermoid cervix carcinoma) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum. All cultures were maintained in a humidified air/ CO_2 (19:1) atmosphere at 37 °C.

VLPs were loaded with plasmid pEli 92, expressing the bacterial β -galactosidase [31]. All transfection experiments were performed in 24-well plates. At 24 h before transfection, 2.5×10^5 cells were seeded in 500 μl of medium in each well. When cells had reached subconfluence (40–60%) the medium was changed (500 μl) and 50 μl of transfection mixture containing 2 μg of DNA per well were added directly. As a control, transfections were performed only with the transfection agent in the absence of DNA. Cells were assayed for the expression of β -galactosidase 48 h after transfection, with the substrate *o*-nitrophenyl D-galactopyranoside ('ONPG') and the β -galactosidase enzyme assay system (Promega) in accordance with the manufacturer's specifications. Cell extracts were prepared by addition of 100 μl of lysis buffer to each well of the 24-well plate, after the removal of medium and washing once with 10 mM potassium phosphate/150 mM KCl (pH 7.4). The absorbance of the reaction products was monitored at 420 nm and corrected for light scattering measured at 620 nm. Results are averages of at least three separate measurements.

RESULTS

Docking of the antibody fragment dsFv-B3-R8C on modified VLPs

VLPs composed of polyoma VP1 were used previously for the transfection of eukaryotic cells [12,13]. The ability of VP1 to bind to sialic acid moieties on mammalian cell surfaces caused cell-type-non-specific targeting of these VLPs to various cell types. To ensure cell-type specificity of polyoma VP1, a new specific

Figure 1 Schematic structure of polyoma VP1 and the dsFv-B-R8C antibody fragment

A monomer of the pentameric VP1 and the VL and VH dimer of the antibody are displayed. The two molecules are coupled via polyionic peptides located at the C-terminus of the VH domain and in the HI loop of the mutant VP1-E8C [24]. This represents a scheme only; sizes are not proportional.

binding function must be established. As the specific binding moiety we chose the disulphide-stabilized dsFv fragment of the monoclonal antibody B3 (dsFv-B3) directed against the antigen Lewis Y [26]. To couple the recombinant antibody to VLPs a polycationic peptide Arg₈CysPro was fused to the C-terminus of the VH domain of the antibody fragment. This module should allow docking of the antibody fragment to an artificial polyanionic site Glu₈Cys inserted in the HI loop of the mutant VP1-E8C [24]. Subsequent covalent cross-linking between the two cysteine residues of the polyionic peptides should render this association irreversible under oxidizing conditions (Figure 1).

As shown in Figure 2, VLPs of VP1-E8C could be separated quantitatively from proteins of lower molecular mass by gel filtration. However, if the antibody fragment dsFv-B3-R8C was incubated with VP1-E8C in the presence of a suitable redox system, a significant portion of the antibody fragments was co-eluted with the VLPs (Figure 2C). This co-elution was caused by the covalent association of the antibody fragment and VP1-E8C via the engineered disulphide bond as demonstrated by non-reducing SDS/PAGE (results not shown). The association was mediated by the polyionic fusion peptides. In the presence of 0.75 M (NH₄)₂SO₄ the high ionic strength of the buffer suppressed the association (results not shown).

VLPs of polyoma VP1 consist of a total of 360 monomers. To analyse how many antibody fragments could be coupled to the

Figure 2 VLPs of VP1-E8C

VP1-E8C at a protein concentration of approx. 0.5 mg/ml was assembled into VLPs by dialysis for 2 days against 0.02 M Tris/HCl (pH 7.4)/0.75 M (NH₄)₂SO₄/1 mM CaCl₂/5% (v/v) glycerol at 20 °C. (A) Electron micrograph of negatively stained VLPs of VP1-E8C (100 µg/ml). The nominal magnification was 100 000-fold. (B) Purification of VLPs by gel filtration. The elution profile is shown of gel filtration of VLPs reconstructed *in vitro* coupled with dsFv-B3-R8C. The elution time of the VLPs corresponds to the size of 50 nm particles. Unbound dsFv-B3-R8C and redox substances were removed. (C) SDS/PAGE analysis of dsFv-B3-R8C-coupled VLPs. The VLP peak obtained by gel filtration was analysed by SDS/PAGE [18% (w/v) gel] under reducing conditions. lane 1, Molecular mass standard [molecular masses indicated (in kDa) at the left]; lane 2, wtVP1; lane 3, VP1-E8C; lane 4, VP1-E8C conjugated with dsFv-B3-R8C; lane 5, dsFv-B3-R8C. (D) Titration of dsFv-B3-R8C with VP1-E8C. VP1-E8C (5 µM), assembled to VLPs, was incubated with different concentrations of dsFv-B3-R8C in 20 mM Tris/HCl (pH 7.4)/200 mM NaCl/5% (v/v) glycerol/1.6 mM GSSG/0.4 mM GSH. This incubation led to aggregation of the VLPs at high concentrations of the antibody fragment, resulting in a loss of detectable particles. After 2 h of incubation the samples were subjected to HPLC gel filtration and the amount of soluble VLPs was analysed. Abbreviation: rel, relative.

Figure 3 Haemagglutination assay for VP1 VLPs

The induction of haemagglutination by VLPs of wtVP1, VP1-E8C and VP1-E8C/dsFv-B3-R8C was determined with sheep erythrocytes. Protein concentrations ranged from 0.25 mg/ml to 2.5 µg/ml (10-fold dilution steps from left to right). VP1-E8C and VP1-E8C/dsFv-B3-R8C showed no haemagglutination, whereas wtVP1 haemagglutinated erythrocytes at a protein concentration of at least 25 ng/ml.

surface of the VLPs, a titration experiment was performed. Figure 2(D) shows the dependence of the amount of VLPs on the concentration of dsFv-B3-R8C used for coupling. The highest molar ratio of dsFv-B3-R8C to VP1-E8C for efficient association proved to be 1:5. Under these conditions approx. 30 antibody fragments could be coupled to one VLP statistically, as judged by densitometric analyses of Coomassie-Blue-stained gels of VLP samples. The use of higher concentrations of dsFv-B3-R8C in the coupling reaction led to a significant aggregation of the VLPs (Figure 2D).

These results demonstrate that the two complementary engineered polyionic sequences in VP1-E8C and dsFv-B3-R8C allowed a highly specific and (via an engineered disulphide bond) covalent coupling of dsFv-B3-R8C to VLPs of polyoma VP1-E8C.

Suppression of cell-type-non-specific binding of VLPs

To guarantee the specific binding of antibody fragments on the surface of the VLPs, a polyionic fusion peptide was inserted in the HI loop of VP1. However, the HI loop was selected for this modification not only because of its solvent-exposed and protruding nature, which permits easy access for other proteins, but also because the sialic-acid-binding site of VP1 is located directly beneath the HI loop. This binding site is responsible for the cell-type-non-specific attachment of wtVP1 to cells. Modification of the HI loop should lead to a suppression of this binding activity. To analyse whether the modification of the VP1 loop inactivated the native cell-binding site of VP1, the remaining cell-binding activity of VP1-E8C via its sialic-acid-binding site was analysed in haemagglutination assays. Figure 3 shows that wtVP1 agglutinated erythrocytes at a minimum concentration of approx. 25 ng/ml. In contrast, no haemagglutination was observed with VP1-E8C, even at a protein concentration of 250 µg/ml. The latter concentration was much higher than that used in transfection experiments (see below). These results showed clearly that the inserted peptide Glu₈Cys abolished cell-type-non-specific binding of VP1-E8C to sialic-acid-presenting cells. However, it should be mentioned that the lack of haemagglutination does not prove the inability of VP1-E8C to bind to any other cell type. It cannot be estimated from the present results whether the insertion of the polyionic peptide directly above the sialic-acid-binding site of VP1 not only decreases the affinity for the respective sialyloligosaccharides but also changes the specificity towards sugar residues that are not present on erythrocytes.

Packaging/association of DNA

wtVP1 possesses a DNA-binding activity at the N-terminus of the protein [32]; capsids of VP1 are able to incorporate DNA *in*

vitro [33]. DNA and plasmids that are packaged in, or at least associated with, VLPs *in vitro* should be protected against DNase digestion [12]. Because VP1-E8C carries a large quantity of negative charges on the surface we checked whether or not VLPs of this mutant were still able to interact with DNA and thus to protect plasmid DNA against DNases. Packaging was performed as described for wtVP1 by an osmotic shock procedure and the VLP-DNA complexes were subjected to the DNase protection assay as described in the Materials and methods section. PCR fragments of the correct size were amplified if the DNA was preincubated via osmotic shock with VLPs consisting of either wtVP1 or VP1-E8C before digestion. In contrast, no significant protection of DNA was observed if the plasmid DNA was preincubated with pentameric VP1 or the isolated antibody fragment (results not shown). This result proved that plasmid DNA became incorporated in, or at least associated with, wild-type and mutant VLPs.

Inhibition of non-specific gene delivery by mutant VLPs

Next we examined whether the recombinant VLPs were suited for gene transfer in cell culture experiments. Using a β -galactosidase-encoding plasmid we compared the transfection efficiencies of the VLPs of wtVP1 and VP1-E8C in the human breast cancer cell line MCF7 (Figure 4A). With wtVP1 as transfection agent, we observed a transfection efficiency of approx. 5%, corresponding to a relative β -galactosidase activity of 70 absorbance units in a colorimetric assay. With VLPs of VP1-E8C (without coupling of dsFv-B3-R8C) the transfection efficiency was less than 1% and not significantly higher than the background signal of DNA alone, which was used as negative control (Figure 4A). This lack of transfection efficiency reflects the suppression of binding to the cell surface by the ionic sequence in the HI loop. This result confirms the results obtained with the haemagglutination assay, which demonstrated the abolition of sialic acid binding of VP1-E8C VLPs on mammalian cells. Identical results were also obtained for the cell line PA-1 (results not shown), which indicated that wtVP1 could be used as a cell-type-non-specific transfection agent, whereas VLPs of the mutant VP1-E8C did not show any significant non-specific gene transfer activity by itself. This suppression of non-specific activity is a prerequisite for the development of a cell-type-specific vector system based solely on the affinity of the target-specific antibody fragments.

Antigen-specific targeting of VLPs of VP1-E8C via dsFv-B3-R8C

With the cell-type-non-specific cell adhesion suppressed for VP1-E8C, coupling of the tumour-specific antibody fragment dsFv-B3-R8C should lead to a specific delivery of these particles to cells presenting the antigen Lewis Y. Using VLPs of VP1-E8C coupled to dsFv-B3-R8C and loaded with plasmid DNA, the expression of β -galactosidase was determined in MCF7 and A431 cells (expressing the B3 antigen Lewis Y on the cell surface) and in PA-1 and KB3-1 cells (Lewis-Y-negative). As controls, the same transfections were performed with separate VP1-E8C/DNA complexes and dsFv-B3-R8C/DNA mixtures respectively. As shown in Figure 4(B), transfection with VLPs of VP1-E8C without dsFv-B3-R8C and with the non-coupled dsFv-B3-R8C resulted in only marginal β -galactosidase expression. This effect was independent of the cell type. However, in MCF7 cells expressing Lewis Y, the VLPs coupled with dsFv-B3-R8C showed a 5-fold higher β -galactosidase activity than transfection assays with VLPs not decorated with the antibody. This β -galactosidase activity corresponded to a transfection efficiency of approx. 3%, demonstrating this cell-type-specific system to be almost as

Figure 4 Cell-type-specific targeting of particles of VP1-E8C/dsFv-B3-R8C

(A) Transfection of MCF7 cells with plasmid pELI 92 by using VLPs of wtVP1, VP1-E8C, and DNA without transfection agent. For quantification of the expression of the plasmid encoding β -galactosidase the enzymic activity was measured with a colorimetric assay. The measured enzyme activity for wtVP1 transfection corresponds to a transfection efficiency of 5%. (B) Gene transfer of β -galactosidase-encoding plasmid in Lewis-Y-positive cells and Lewis-Y-negative cells was performed with VP1-E8C VLPs (first bar, black), dsFv-B3-R8C (second bar, white) and VP1-E8C/dsFv-B3-R8C (third bar, grey) conjugates. The observed β -galactosidase activity was corrected for the β -galactosidase activity of control cells transfected with naked DNA. The error bars represent the S.D. for three independent experiments. (C) Dependence of the transfection efficiency on the amount of VP1-E8C/dsFv-B3-R8C particles. MCF7 cells were transfected with different concentrations of VP1-E8C/dsFv-B3-R8C particles. The ratio of protein to DNA was constant at 16:2 (w/w). (D) Competition for cell attachment of VP1-E8C/dsFv-B3-R8C particles. Transfection of MCF7 cells with VP1-E8C/dsFv-B3-R8C particles carrying the plasmid pELI 92 was competed for by a 20000-fold molar excess of 3'-sialyl-lactose and a 40000-fold molar excess of soluble antigen Lewis Y. The transfection efficiency, measured by β -galactosidase activity, in the absence of a competitor was set at 100%.

efficient as the cell-type-non-specific wtVP1 system. At a constant ratio of VP1 to DNA the efficiency was dependent on the amount of DNA used for the transfection experiments. In the range 625–5000 ng of DNA we observed a dose-dependent linear increase in efficiency with increasing DNA concentration (Figure 4C). For the other Lewis-Y-positive cell line A431, the transfection efficiency was not as good as for MCF7. Nevertheless, in comparison with the cell-type-non-specific background transfection the antibody-mediated transfection of A431 was still significantly higher by a factor of 2.5. In Lewis-Y-negative cell lines the efficiency of dsFv-B3-R8C-coupled VLPs was as low as for the uncoupled system. This suggests that the gene transfer in Lewis-Y-positive cells was based on the antigen–antibody interaction.

To analyse further whether the monitored gene transfer was specifically generated by the coupled antibody fragment, competition experiments were performed with an excess of free antigen, preincubated with the dsFv-B3-R8C-coupled VLPs. The results in Figure 4(D) show that the transfection of MCF7 cells with VP1-E8C/dsFv-B3-R8C conjugates was due to the specific binding of B3 antibody, because its attachment was blocked by an excess of free Lewis Y but not by 3'-sialyl-lactose, which would interact with the sialic-acid-binding site of VP1 [18]. This clearly indicated that the transfection of MCF7 and A431 cells was caused specifically by the antibody–antigen interaction of dsFv-B3-R8C with Lewis Y, proving that this antibody fragment, together with the suppression of the sialic-acid-binding activity of wtVP1, could change VLPs of polyoma VP1 from a non-specific to a cell-type-specific vector system.

DISCUSSION

The development of targeted delivery systems is of utmost interest for gene-therapeutic applications. Various attempts have been made to create such systems, some of which involved the use of recombinant targeting moieties such as antibody fragments. In most cases retroviruses have been developed as delivery systems, with single-chain Fv fragments inserted in a viral coat

protein [2,4,34,35]. The resulting recombinant retroviruses are produced in mammalian cell culture, with the inherent problems of difficult and elaborate production as well as potential viral contaminations of the recombinant therapeutic viruses. Our method for the generation of specific gene-targeting vehicles uses the recombinant polyoma virus coat protein VP1 produced in *E. coli*. This obviates any production and contamination problems associated with mammalian production systems. By inserting polyionic peptides as anchor motifs in VP1 combined with complementary polyionic fusion peptides on recombinant antibodies we could (1) diminish the natural non-specific cell targeting of wild-type polyoma VP1 via sialic acid binding and (2) introduce an additional cell-type-specific tropism with the tumour-specific antibody fragment dsFv-B3-R8C.

Polyoma VP1 binds to sialic acid residues, which are present on almost all eukaryotic cells. Cellular infection by polyoma virus originates from this cell attachment [36]. Similarly, this cell-binding activity can be used for cellular transfection by polyoma VLPs *in vitro* [12,13,27] and *in vivo* [14]. Consequently, blocking of the sialic-acid-binding site by the insertion of a Glu₈Cys peptide in the HI loop of VP1-E8C leads to a substantial loss in cell targeting and gene transfer. The introduction of the targeting domain for the generation of a cell-type-specific gene delivery system was achieved by coupling approx. 30 antibody fragments to VLPs of the variant VP1-E8C. The directed association via polyionic fusion peptides proved to be highly specific and efficient. Some other peptides for inducing a directed association reaction have been invented previously, for example amphiphilic helices [36,37] and leucine zippers [38,39]. However, for two reasons these systems seemed inappropriate for the requirements of our system. First, the association of both the amphiphilic helices and leucine zippers is based on the formation of the secondary structure of each of these elements. For VP1 we showed previously that the insertion of a protein in the HI loop can lead to a marked destabilization of the fused protein [23]. A sub-optimal design of the inserted peptide sequence might result in a complete loss of structure in elements with a single secondary structure, thus preventing a specific association via these peptides. Secondly, both amphiphilic helices and leucine zippers are partly

hydrophobic. Because the VLPs consist of 72 VP1 pentamers, the modification of VP1 with amphiphilic helices or leucine zipper peptides would generate 360 hydrophobic patches on the surface of these particles, which might induce aggregation. In contrast, the polyionic fusion peptides are highly soluble and their interaction does not depend on specific secondary structures.

For possible applications of the modified VLPs as a vector system, the coupling of proteins with the use of polyionic fusion peptides should allow modification of the VLP surface with not merely one functional domain. It should be possible to bind several different proteins with targeting activity or other functions on the VLPs. Coupling via polyionic fusion peptides implies that, independently of the target, the VLP is always the same; it is not necessary to design and optimize a new vector for every new application. A similar approach was chosen by Ohno et al. [6,7], who fused the antibody-binding-domain protein ZZ to the virus coat protein of Sindbis virus and retrovirus. In this case different antibodies can be used to introduce different specificities. However, this approach is limited to antibodies that bind efficiently to protein ZZ. Unfortunately, Fv fragments and other small recombinant antibody forms, which are usually used in phage libraries to generate and select high-affinity antibody fragments against certain targets, cannot be coupled to protein ZZ. These limitations do not hold for the fusion peptides that we have used as anchor groups.

The application of our virus-like system containing a tumour-specific targeting domain for the gene delivery of reporter constructs clearly demonstrated cell specificity: expression of the reporter genes was detected unambiguously in antigen-expressing cells. This expression was 5-fold (MCF7) and 2.5-fold (A431) that of the cell-type-non-specific transfection background. The difference in expression between antigen-positive versus antigen-negative cells is clearly due to antigen-specific gene delivery, because (1) antigen-negative and antigen-positive cells show comparable expression levels with recombinant wild-type particles, and (2) the specific gene transfer can be competed for with excess cognate antigen.

The dsFv-B3 antibody fragment that we have selected as the targeting moiety had already been used for cell-type-specific targeting of immunotoxins. These immunotoxins are highly efficient in killing target cells such as breast (MCF7) or epidermoid (A431) tumour cells, leading even to a significant tumour regression in mice [25,26,40,41]. Using this antibody fragment, coupled to VLPs of VP1-E8C, the tropism of these particles could be changed towards the respective target cells.

However, at present the transfection efficiency is rather low. One reason might be that polyoma VLPs, as demonstrated for wtVP1, are mainly degraded in lysosomes after cellular uptake (U. Schmidt, personal communication). Therefore a possibility of improving the VLP-based gene delivery system would be based on the development of an endosome escape mechanism for these particles. Viruses and other intracellular parasites such as *Listeria* possess a lipase activity that permits their release from the endosomes. Coupling of such a lipase to the surface of the VLPs via polyionic fusion peptides might transfer a natural existing endosomolytic activity to the VLPs.

In summary, we have demonstrated a specific coupling of antibody fragments to the surface of polyoma VLPs via polyionic fusion peptides with a complementary charge. The coupling principle used here might be of general interest to any engineered directed association reaction. In the present study the docking of a tumour-specific anti-(Lewis Y) antibody fragment led to a retargeting of the modified polyoma particles towards antigen-presenting cells, thus demonstrating the basic concept for the development of a cell-type-specific, non-viral vector system.

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