

Zellspezifischer Gentransfer mittels Varianten des Hüllproteins VP1 des murinen Polyomavirus

Dissertation zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.) der
Mathematisch-Naturwissenschaftlich-Technischen Fakultät
(mathematisch-naturwissenschaftlicher Bereich)
der Martin-Luther-Universität Halle-Wittenberg

vorgelegt von
Stefan Gleiter
aus Laupheim
2002

urn:nbn:de:gbv:3-000003474

[<http://nbn-resolving.de/urn/resolver.pl?urn=nbn%3Ade%3Agbv%3A3-000003474>]

Promotionsgesuch eingereicht am: 19.März 2002

Diese Arbeit wurde angeleitet von Herrn Prof. Dr. Rainer Rudolph.

Gutachter:

1: Prof. Dr. Rainer Rudolph

2: PD. Dr. Helge Taubert

3: Prof. Dr. Frank Emmrich

Tag der öffentlichen Verteidigung: 06. Juni 2002

Die vorliegende Arbeit wurde von Oktober 1998 bis März 2002 am Institut für Biotechnologie in der Arbeitsgruppe für Proteintechnologie unter der Anleitung von Prof. Dr. Rainer Rudolph und Dr. Hauke Lilie angefertigt.

Bei Prof. Dr. Rainer Rudolph möchte ich mich für das ständige Interesse am Fortgang meiner Arbeit und seine nicht endende Diskussionsbereitschaft danken. Außerdem möchte ich mich bei Ihm dafür bedanken, daß er mich nicht „gefeuert“ hat, obwohl ich einige seiner zündenden Ideen nicht in die Tat umgesetzt habe, sondern diese noch immer für nachfolgende Generationen an Doktoranden zur Verfügung stehen.

Ganz herzlich möchte ich mich bei Hauke Lilie bedanken. Er war immer ein geduldiger Ansprechpartner bei allen Probleme und Sorgen, die mit VP1 auftraten und hat mich in nur jeder erdenklichen Weise unterstützt. Seine ständige Diskussionsbereitschaft und seine Fähigkeit, auch aus den absoluten Rückschlägen noch den positiven Gesichtspunkt herauszukitzeln, haben maßgeblich zum Gelingen der Arbeit beigetragen. Ich werde auch nie die Meßreihen bei einer guten Flasche Wein vor dem Fluori vergessen.

Ganz besonderer Dank gilt der VP1-Truppe Kay, Tobias und Thomas. Vielen Dank für Eure Unterstützung, Diskussion und Aufmunterung, wenn VP1 seine häßlichen Seiten zeigte. Allen aktuellen und ehemaligen Mitgliedern des Labor 168 sei für die schöne Arbeitsatmosphäre gedankt. Besonders Martin, der mich mit seiner Leidenschaft für das Basketballspiel ab und zu aus dem Laboralltag retten konnte.

Frank Broda möchte ich ganz herzlich für die beiden „VP1-Bildchen“ danken, die ich alleine niemals hätte herstellen können.

Bettina und Gerd Hause möchte ich für die Unterstützung bei der LSM und der Elektronenmikroskopie danken.

Ganz besonderer Dank gilt Uli Brinkmann, der mit seinen Ideen und Einfällen viel zum Fortgang des Projektes beigetragen hat.

Alex und Dietmar möchte ich für die fruchtbare Zusammenarbeit bei den FACS Analysen und ihre kritische Diskussion der erhobenen Zellkulturdaten herzlich danken.

Gerald Böhm und allen Mitarbeitern seiner Gruppe (inzwischen Firma) sei für die vielen Ideen und Anregungen und die gute Zusammenarbeit gedankt.

Bei A. Engel und K.H. Sellinger von der Firma Roche möchte ich mich für den anti-CEA Antikörper und Herceptin bedanken.

Prof. S. Ståhl und M. Larsson sei für das Protein Z codierende Plasmid gedankt.

Prof. I. Pastan und Prof. B. Garcea möchte ich mich für die Originalplasmide für den B3 bzw. die Originalsequenz von VP1 bedanken.

Ein ganz besonderes Dankeschön gilt Jeannette Winter, die es immer wieder geschafft hat, meinen Blutdruck in geregelte Bahnen zu leiten und die mich immer wieder motiviert hat und an meine Arbeit geglaubt hat.

Ich möchte mich auch noch bei einer der wichtigsten Personen am Institut bedanken: Fr. Weichelt. Mit nie endender Energie hat sie für die Fortsetzung von Arbeitsverträgen gesorgt und mich mit einer Unzahl Eddings und Kugelschreibern versorgt, die sich immer nach zwei Tagen im Labor in Luft aufgelöst haben.

Nicht zuletzt möchte ich bei meinen Eltern bedanken, die mich immer mit allem unterstützt haben und ich hoffe, Ihre Geduld nicht über Gebühr strapaziert zu haben.

1	Zusammenfassung	1
2	Einleitung	4
2.1	Molekulare Therapien	4
2.2	Proteintherapie	4
2.3	Gen- und Krebstherapie	7
2.3.1	Krankheitsbilder	9
2.3.1.1	Adenosin-Desaminase Mangel	9
2.3.1.2	Zystische Fibrose	9
2.3.1.3	Duchenne Muskeldystrophie (DMD)	10
2.3.1.4	Arthritis	10
2.3.1.5	Lysosomale Speicherkrankheiten	10
2.3.1.6	Krebs	11
2.4	Vektoren für die Gen- und Krebstherapie	14
2.4.1	Virale Vektoren	15
2.4.1.1	Retroviren	15
2.4.1.2	Adenovirale Vektoren	17
2.4.1.3	Herpes-Simplex-Virus (HSV)	19
2.4.1.4	Adeno-assoziierte Viren (AAV)	20
2.4.2	Nicht-virale Transfektionssysteme	20
2.5	Polyomaviren	22
2.5.1	Genomaufbau der Polyomaviren	22
2.5.2	Die frühen Gene	22
2.5.3	Die späten Gene	23
2.5.4	Aufbau der Viruspartikel	25
2.5.5	Replikation der Polyomaviren	25
3	Grundlagen und Ziele der Arbeit	26
3.1	Grundlagen	26
3.2	Ziele der Arbeit	27
4	Ergebnisse	30
4.1	Untersuchungen zum Fusionsprotein VP1-DHFR	30
4.2	Untersuchungen zur funktionellen Modifikation von VP1 durch Insertion einer Proteindomäne	33
4.3	Zelltyp-spezifische Transduktion und Genexpression	36
4.4	Entwicklung alternativer Kopplungsmethoden an VP1	38
4.5	Untersuchungen zum spezifischen Gentransfer mit der Variante VP1-E ₈ C des Polyomahüllproteins VP1	40
5	Abkürzungen	42
6	Literaturverzeichnis	44
7	Veröffentlichungen	61
8	Anhang	146

1 Zusammenfassung

Die Entwicklung von Vektoren für die Gentherapie ist in den letzten Jahren sehr weit fortgeschritten. Dennoch ist bislang die klinische Anwendung dieser Vektoren stark limitiert. Unter anderem stellt die Zelltyp-spezifische Adressierung des Vektorsystems ein gravierendes Problem dar.

Ziel dieser Arbeit war die Entwicklung eines modularen, Zelltyp-spezifischen Vektorsystems für die Gentherapie, basierend auf dem murinen Polyomahüllprotein VP1.

Die Fähigkeit eukaryontische Zellen mit dem wtVP1-Protein zu transduzieren, beruht auf der Bindung an Sialinsäureresten und der Aufnahme der Kapside in Caveolae. Dieser Zelltyp-unspezifische Mechanismus der Zelladressierung muß verhindert und gleichzeitig ein Modul zur Zelltyp-spezifischen Adressierung eingeführt werden. Erste Untersuchungen befaßten sich mit der Insertion des Modellproteins DHFR in den HI-Loop von VP1. Die Insertion an dieser Stelle sollte zeigen, ob Proteindomänen funktionell auf der Kapsidoberfläche präsentiert werden können. Das durch die Insertion in den HI-Loop entstandene Fusionsprotein VP1-DHFR konnte isoliert und funktionell charakterisiert werden. Die enzymatischen Eigenschaften und die Stabilität der DHFR waren im Fusionsprotein zwar negativ beeinflusst, es konnten aber keine negativen Auswirkungen auf den VP1-Anteil im Protein beobachtet werden. So assemblierte VP1-DHFR zu Kapsiden. Außerdem konnte nachgewiesen werden, daß die Haemagglutination, ein Maß für die Bindung an Zellen, durch die Insertion unterdrückt wurde. Der Verlust der Zelltyp-unspezifischen Bindung an Sialinsäuren unter gleichzeitigem Beibehalt der Fähigkeit, zu Kapsiden zu assemblieren, waren die Grundvoraussetzung für die Insertion einer anderen funktionellen Proteindomäne in VP1.

Um eine Zelltyp-spezifische Adressierung zu erreichen, sollte die Wechselwirkung zwischen einem Zelltyp-spezifischen Antikörper und dem entsprechenden Antigen auf der Zelloberfläche genutzt werden. Die Bindung des Antikörpers an VP1 sollte dabei über das Antikörper-bindende Protein Z erfolgen. Die Insertion von Protein Z in den HI-Loop von VP1 führte zu dem funktionellen Fusionsprotein VP1-Z. Die biophysikalische Charakterisierung ergab, daß VP1-Z (i) eine vergleichbare Temperaturstabilität wie wtVP1 aufweist, (ii) Antikörper binden kann, (iii) keine Sialinsäurebindungseigenschaft mehr besitzt und (iv) die Kapsomere zu einer homogenen Spezies Kapside assembliert werden können.

Der Einsatz eines ternären Komplexes aus VP1-Z, Antikörper und DNS führte zur Zelltyp-spezifischen Adressierung des Vektorsystems in Zellkulturexperimenten. Unspezifische Wechselwirkungen mit Antigen-negativen Zelllinien blieben aus.

Weiterhin war es mit diesem Vektorsystem möglich, den funktionellen, spezifischen Gentransfer in Zellkultur zu demonstrieren. Die erreichten Transduktionsraten mit dem Vektorsystem VP1-Z/Herceptin lagen dabei im Bereich dessen, was bisher mit dem wtVP1 gemessen wurde. Unspezifische Transduktion von Antigen-negativen Zellen oder die Transduktion mit Einzelkomponenten konnte nicht beobachtet werden. Als limitierender Faktor für die Transduktion konnte die Freisetzung aus dem lysosomalen Kompartiment identifiziert werden.

Neben der Bindung ganzer Antikörper auf der Oberfläche von VP1-Z über eine nicht-kovalente Wechselwirkung, sollten auch die Eigenschaften der Variante VP1-E₈C untersucht werden. Mit dieser Variante ist die Bindung positiv geladener Peptide oder Proteine über polyionische Wechselwirkungen an der Oberfläche von VP1-E₈C und die kovalente Kopplung über eine Disulfidverbrückung möglich. Die Bindung des Zelltyp-spezifischen Antikörperfragmentes B3 an VP1-E₈C wurde durch eine polyionische Arg₈Cys-Sequenz an der V_H-Domäne vermittelt. Der Einsatz dieses Systems führte zur Zelltyp-spezifischen Transduktion von LeY-positiven Zellen. Neben dem Einsatz verschiedener LeY-positiver und -negativer Zelllinien konnte die Spezifität auch durch die Konkurrenz der Transduktion mit freiem LeY-Antigen gezeigt werden. Die Effektivität der Transduktion mit diesem Vektorsystem war allerdings sehr gering. Um die limitierenden Schritte zu identifizieren, wurden einzelne Stufen der Transduktion untersucht. Die Bindung an die Zelloberfläche und die Internalisierung sind sehr effektiv und stellen keine Limitation dar. Es wurde darüber hinaus nachgewiesen, daß auch dieses Vektorsystem durch Rezeptor-vermittelte Endozytose aufgenommen wird. Das bedeutet, wie auch bei VP1-Z/Herceptin, die intrazelluläre Überführung in das lysosomale Kompartiment und den Abbau des Transduktionssystems. Neben dieser Limitation konnte aber auch noch eine verringerte DNS-Bindung bei neutralem pH-Wert identifiziert werden. Durch die Absenkung des pH-Wertes während der Inkubation der DNS mit den Kapsiden konnte die DNS-Bindung etwas effizienter gestaltet werden. Diese Untersuchungen erlaubten es, das Transduktionsprotokoll für diese VP1-Variante sinnvoll zu verbessern und damit die Transduktionseffizienz zu steigern.

Mit der Entwicklung zweier verschiedener Kopplungsmethoden zur Bindung von Antikörpern bzw. Fv-Fragmenten auf der Oberfläche von VP1 konnte die systematische Entwicklung eines Zelltyp-spezifischen Vektorsystems für die Gentherapie dargestellt werden. Darüberhinaus wurden limitierende Schritte des funktionellen Gentransfers mit diesem System charakterisiert. Dadurch ergibt sich die Möglichkeit, das Transduktionssystem VP1 hinsichtlich der Transduktionseffizienz weiter zu verbessern. Der modulare Aufbau dieses

Vektorsystems erlaubt außerdem die einfache Kombination verschiedener VP1-Varianten, um neuentwickelte oder verbesserte Eigenschaften miteinander zu verbinden. Ein weiterer Vorteil besteht in der Flexibilität bei der Auswahl des zu transduzierenden Zelltyps. Der Austausch des Antikörpers oder Fv-Fragmentes auf der Oberfläche von VP1 vermittelt dem kompletten Vektorsystem eine neue Spezifität, ohne daß neue Varianten von VP1 konstruiert und charakterisiert werden müssen.

2 Einleitung

2.1 Molekulare Therapien

Die Entwicklung von Arzneistoffen beruhte in den vergangenen Jahrzehnten auf der Synthese von Substanzen durch die klassische organische Chemie, die als Agonisten oder Antagonisten zur Behandlung von Krankheiten eingesetzt wurden. Ebenso wurden eine große Anzahl Naturprodukte entweder direkt aus Wirtsorganismen gewonnen oder nach der Aufklärung ihrer Struktur chemisch hergestellt (z.B. Lactam-Antibiotika).

Mit der rasanten Entwicklung der Biotechnologie haben sich die Stoffklassen der Therapeutika erweitert. Das zunehmende Wissen über bestimmte Stoffwechselprozesse hat die prinzipielle Strategie zur Behandlung von Krankheiten verändert. So ist es heute vorstellbar, durch die Gentherapie nicht die Symptome einer genetisch bedingten Krankheit zu therapieren, um die Lebensqualität des Patienten zu verbessern, sondern die Voraussetzungen sind prinzipiell geschaffen, auf molekularer Ebene die genetische Information so zu verändern, daß die Krankheit therapiert werden kann.

Auch die Art der Therapeutika hat einen großen Wandel erfahren. Waren in den letzten 30 Jahren hauptsächlich niedermolekulare Substanzen im Einsatz, werden heute verstärkt die ersten Therapeutika auf Proteinbasis ebenso wie Nukleinsäuren eingesetzt.

2.2 Proteintherapie

Die Proteintherapie ist eine Therapieform, bei der ein Protein als Wirkstoff zum Einsatz kommt. Der große Vorteil beim Einsatz von Proteinen als Therapeutikum besteht in ihrer sehr spezifischen Funktion. Dies macht unerwartete Nebenwirkungen relativ unwahrscheinlich (Russell und Clarke, 1999).

Als therapeutische Einsatzgebiete für Proteine können in Frage kommen:

- direkter Ersatz eines defekten Proteins (A),
- Verstärkung eines vorhandenen Stoffwechselweges oder Prozesses, mit dem Ziel toxische Stoffwechselprodukte oder toxische Prozesse zu reduzieren (B),
- Einführung eines Enzyms mit einer Aktivität, die an dieser Stelle sonst nicht vorhanden ist (C).

Das wohl prominenteste Beispiel für den Einsatz rekombinanter Proteine ist die Anwendung rekombinanten Insulins zur Behandlung des *Diabetes mellitus* (Ahrens et al., 1986). Auch die klinische Anwendung verschiedener Kolonie-stimulierender Wachstumsfaktoren wird mit Erfolg durchgeführt (Holldack et al., 1992; Burdach, 1992; Metcalf, 1990; Begley, 1993).

Zur Verringerung von toxischen Stoffwechselprodukten wird bei der Therapie der zystischen Fibrose rekombinant hergestellte DNase eingesetzt. Das Medikament (Pulomozyme, Genentech Inc.) wird von den Patienten inhaliert und dadurch der DNS Gehalt im Sputum deutlich reduziert (Bates und Nahata, 1995). Die folgende Tabelle gibt eine Übersicht über Krankheiten, bei denen Proteine als Therapeutika eingesetzt wurden:

Krankheit	Protein
Einsatzgebiet A (siehe oben)	
<i>Severe combined immune deficiency</i> (SCID)	Bovine Adenosin-Desaminase PEG (ADA)
<i>Morbus Gaucher</i>	Glukozerebrosidase
<i>Morbus Hurler</i>	α -L-Iduronidase
α -Anti-Trypsin Mangel	α -Antitrypsin
<i>Diabetes mellitus</i>	Insulin
Wachstumshormon Mangel	Somatotropin (STH/hGH)
Haemophilie A	Faktor VIII
Einsatzgebiet B (siehe oben)	
Multiple Sklerose	Interferon β -1b
Zystische Fibrose	DNase
Glykogen Speicherkrankheiten	Granulozyten Kolonie-stimulierender Faktor (GCSF)
Einsatzgebiet C (siehe oben)	
Phenylketonurie (PKU)	Phenylalanin-Ammonium-Lyase
Akuter myocardialer Infarkt	Streptozym, tissue plasminogen aktivator (tPA)

Tabelle 1: Proteine als Therapeutika bei der Behandlung verschiedener Krankheiten, verändert nach Russell und Clarke (1999).

Therapeutischen Proteinen fehlen allerdings zwei entscheidende Eigenschaften, die ihren Einsatz in der Klinik stark limitieren. Zum einen fehlt ihnen die Fähigkeit, die Zellmembran zu überwinden, um im Zytosol oder Zellkern wirksam zu werden. Zum anderen ist es nicht generell möglich, Proteine zielgerichtet einzusetzen, d.h. daß nur bestimmte Zell- und Gewebearten das therapeutisch aktive Protein aufnehmen (Russell und Clarke, 1999). Erst mit der Aufklärung verschiedener zellulärer Oberflächenrezeptoren konnten Verfahren entwickelt werden, die eine Zelltyp-spezifische Adressierung bzw. eine Direktion der Proteine in bestimmte zelluläre Kompartimente ermöglichen. Durch Adressierung über den Mannose-6-Phosphat-Rezeptor oder Mannose-Rezeptor in Leukozyten konnte gezeigt werden, daß sich lysosomale Enzyme nach der Aufnahme in die Zellen im lysosomalen Kompartiment

befanden (Pfeffer, 1991). Ebenfalls eine Zelltyp-spezifische Adressierung eines Therapeutikums auf Proteinbasis gelang mit der Entwicklung des anti-ErbB2 spezifischen Antikörpers. Dieser Antikörper ist gegen die Rezeptor-Tyrosin-Kinase ErbB2 der EGF-Familie gerichtet. Dieser von Genentech hergestellte Antikörper ist der erste, der in klinischen Verfahren eingesetzt wird und sich als wirksames Therapeutikum zur Behandlung von Brustkrebs bewährt hat (Miles, 2001).

Ein neuer Aspekt zur Verbesserung der Aufnahme und der zielgerichteten Adressierung ist die Herstellung von Fusionsproteinen. Die Fusion des Nerven-Wachstumsfaktors (NGF) mit Transferrin führt zu einer verbesserten Aufnahme in Zellen des ZNS, vermutlich durch Rezeptor-vermittelte Endozytose (Park et al., 1998). Seit kurzem finden auch sogenannte Immunotoxine in der Krebstherapie Anwendung. Durch den Immunglobulin-Anteil ist eine Zelltyp-spezifische Adressierung möglich und Nebenwirkungen durch den Toxin-Anteil werden minimiert (Pastan et al., 1996; Pastan et al., 1995; Brinkmann et al., 1991). Diese hochspezifischen und hochpotenten Proteintherapeutika befinden sich in klinischen Phase II Studien. Andere Fusionsproteine bedienen sich nicht der Rezeptor-vermittelten Endozytose. Stattdessen nutzen sie die funktionellen Domänen von Translokationsproteinen aus pathogenen Organismen wie *Yersinia pseudotuberculosis*. Diese Proteindomäne dient der Überwindung der Zellmembran, der andere Teil des Fusionsproteins der DNS Bindung. Ein Beispiel hierfür ist das GAL4/Invasin Fusionsprotein (Paul et al., 1997). Interessant ist auch die Fusion mit einigen viralen Proteinen. So konnte gezeigt werden, daß Fusionsproteine mit dem HIV TAT Protein effektiv in verschiedenste Zelltypen und Gewebearten im Mausmodell transloziert wurden (Schwarze et al., 1999). Fusionsproteine mit dem Virushüllprotein VP22 des Herpes-Simplex-Virus konnten auch erfolgreich für die Transduktion eingesetzt werden. Dabei wurde nachgewiesen, daß die Fusionsproteine durch einen interzellulären Transport in benachbarte Zellen gelangen (Elliott und O'Hare, 1997). Mittels solcher Fusionsproteine konnte funktionelles p53 in Zellen eingeschleust werden (Phelan et al., 1998). Die Transfektion mittels viraler Proteine wird bisher allerdings noch nicht in klinischen Studien untersucht.

Die Therapie verschiedenster Krankheiten mittels rekombinant hergestellter Proteine wird in naher Zukunft noch deutlich an Bedeutung zunehmen. Die Herstellung großer Mengen an therapeutischem Protein ist heute durch biotechnologische Verfahren möglich. Durch die Sequenzierung des kompletten Humangenoms wird auch die Anzahl der therapeutisch relevanten Proteine und Zielstrukturen noch deutlich ansteigen.

2.3 Gen- und Krebstherapie

Ursprünglich wurde die Gentherapie als die Behandlung einer vorhandenen Erkrankung durch das Ersetzen einer fehlerhaften DNS Kopie mittels Gentransfer definiert. Diese Definition wurde in den letzten Jahren stark erweitert und umfaßt heute den Transfer von Nukleinsäuren, gleichwohl DNS als auch RNS, um Krankheiten zu verhüten oder zu therapieren (Crystal, 1995; Miller, 1992; Mulligan, 1993). Der Transfer von *antisense*-RNS oder -DNS oder Ribozymen gegen ein Onkogen, die Impfung gegen Hepatitis-Viren durch DNS, die das Hepatitis-S-Antigen codiert, die Behandlung zystischer Fibrose durch adenoviralen Gentransfer und die Implantation genetisch modifizierter haematopoetischer Stammzellen werden heutzutage aber ebenso als Beispiele für die Gentherapie angesehen. Gerade die Krebstherapie mit Vektoren für den Nukleinsäuretransfer hat in letzter Zeit mehr Aufmerksamkeit erregt. Die zellulären Grundlagen sind meist besser untersucht als bei Veränderungen in komplexen Stoffwechselvorgängen und oftmals stehen Tiermodelle für die Grundlagenforschung zur Verfügung.

Einen Überblick über Krankheiten, die potentiell mittels Gentherapie behandelt werden können gibt Tabelle 2:

Krankheit	Ursache	Inzidenz	Zielzellen
Genetische Krankheiten			
<i>Severe combined immunodeficiency</i> (SCID)	Adenosin-Desaminase (25% aller SCID Patienten)	selten	Knochenmarkzellen oder T-Zellen
Haemophilie A/B	Faktor VIII/IX	1:10 000 Männer 1:30 000 Männer	Leber, Muskeln, Fibroblasten oder Knochenmarkzellen
Familiäre Hypercholesterolaemie	Mangel an LDL Rezeptor	1:1 Million	Leber
Zystische Fibrose	Verlust/Mutation des CFTR Gens	1:3 000 Europäer	Lungenepithel
Thalassaemie / Sichelzellenanaemie	Defekte im α - oder β -Globin Gen	1:600 in bestimmten Bevölkerungsgruppen	Knochenmarkzellen
<i>Morbus Gaucher</i>	Mangel an Glukozerebrosidase	1:450 von Ashkenzai Juden	Knochenmarkzellen, Makrophagen
α -Antitrypsin Mangel, vererbte Emphyse	Mangel an α 1-Antitrypsin	1:3 500	Lunge oder Leberzellen
Erworbene Krankheiten			
Krebs	Verschiedene Gründe: Genetisch oder umweltbedingt	1 Millionen/Jahr in den USA	Verschiedene Krebszellarten: Leber, Gehirn, Pankreas, Brust, Niere
Neurologische Krankheiten	Parkinson, Alzheimer, Knochenmarkverletzungen	1 Millionen Parkinson und 4 Millionen Alzheimer Patienten in USA	Gehirn, neuronale Zellen, Gliazellen, Schwann'sche Zellen
Gefäßkrankheiten	Arteriosklerose, Restinosis	13 Millionen Patienten in USA	Vaskuläre Endothelzellen
Infektionskrankheiten	AIDS, Hepatitis	Steigende Patientenzahlen	T-Zellen, Leberzellen, Makrophagen

Tabelle 2: Krankheiten, die mittels Gentherapie behandelt werden könnten, verändert nach Verma und Somia (1997).

Eine aktuelle Übersicht über die bisher durchgeführten klinischen Studien kann unter folgender Internetadresse eingesehen werden: www.wiley.co.uk/genetherapy.

2.3.1 Krankheitsbilder

Krankheiten, die mittels Gen- oder Krebstherapie behandelt werden sollen, sind komplex. Dabei kann es sich um angeborene oder erworbene Krankheiten handeln, ebenso wie um Infektionskrankheiten. Kurz sollen hier einige Krankheitsbilder beschrieben werden, bei denen die Mittel der Gentherapie zum Einsatz kommen können.

2.3.1.1 Adenosin-Desaminase Mangel

Bei dieser Krankheit fehlt den Patienten die Adenosin-Desaminase, was zu einem schweren Immundefekt führt. Der Mangel an Adenosin-Desaminase führt zur Akkumulation von dATP. Dieses inhibiert die Ribonukleotid-Reduktase und damit die Synthese der anderen dNTPs. Damit kommt die DNS-Synthese und somit auch die Zellproliferation zum Erliegen. Durch diesen Effekt ist im Wesentlichen die Proliferation von Lymphozyten betroffen, da diese Zellen besonders aktiv Desoxyadenosin phosphorylieren. Diese erblich bedingte Krankheit war die erste, die mittels Gentherapie behandelt wurde (Ramsey et al., 1995; Blaese et al., 1995).

2.3.1.2 Zystische Fibrose

Die Zystische Fibrose (CF) wurde lange als Modellkrankheit für die Gentherapie angesehen, da ein monogener Defekt vorliegt. Bei 70% der Patienten handelt es sich um eine Deletion im CFTR-Gen (Koch und Hoiby, 2000), wobei auf Proteinebene die Aminosäure Phenylalanin an Position 508 deletiert ist. Das Genprodukt des CFTR-Gens ist ein Transmembranprotein, das einen Cl⁻-Ionenkanal bildet und gleichzeitig andere Ionenkanäle reguliert (Kunzelmann und Mall, 2001). Die Auswirkungen in der Lunge sind bei CF Patienten eine erhöhte Na⁺-Resorption und eine verminderte Cl⁻-Sekretion. Dadurch kommt es zu einer Verringerung der Flüssigkeit, die das Lungenepithel bedeckt. Das hat eine Verdickung des *Mucus* auf dem Lungenepithel und einen verminderten Abtransport aus der Lunge zur Folge. Diese *Mucus*-Ablagerung führt häufig auch zu chronischen Infekten mit *Pseudomonas aeruginosa*. Die bei der Immunreaktion lysierten Zellen in der Lunge setzen DNS und Actin frei, die wiederum zu einer weiteren Verdickung des *Mucus* führen (Zahm et al., 2001). Neben der Lunge sind auch noch andere Organe betroffen, wie Haut, Leber, Bauchspeicheldrüse und Nieren (Kunzelmann und Mall, 2001).

Versuche, das humane CFTR-Gen durch Adeno-assoziierten Viren zu transduzieren und damit eine normale CFTR-Funktion zu gewährleisten, führten in verschiedenen Modellen zu

vielversprechenden Ergebnissen (Carter und Flotte, 1996; Flotte et al., 1996; Conrad et al., 1996).

2.3.1.3 Duchenne Muskeldystrophie (DMD)

DMD ist eine X-chromosomal dominant vererbte Krankheit, die auf Mutationen oder Deletionen im Dystrophin-Gen beruht. Die Pathogenese der Krankheit beinhaltet Nekrosen der Skelett- und Herzmuskulatur und führt in der Regel zwischen dem 20. und 30. Lebensjahr zum Tod (Robbins und Ghivizzani, 1998). Obwohl sowohl die molekulare Ursache der Krankheit bekannt ist, als auch die Zielzellen für eine Therapie, ist die Behandlung der DMD bisher nicht erfolgreich. Ein Problem stellt vermutlich die große Menge des zu behandelnden Gewebes dar. Zusätzlich scheint die Basalmembran des Muskelgewebes eine physische Barriere zu sein, die von bisherigen Vektoren nicht ohne weiteres überwunden werden kann (Kakulas, 1997; Gussoni et al., 1997; Hauser et al., 1997; Douglas und Curiel, 1997).

2.3.1.4 Arthritis

Bei der Arthritis handelt es sich um ein Krankheitsbild, welches zwar nicht genetisch bedingt ist, dennoch durch Gentherapie behandelt werden kann.

Die rheumatoide Arthritis ist eine Autoimmunkrankheit, die sich in den Gelenken manifestiert. Klassische Therapieformen schließen die Gabe von Medikamenten auf Steroidbasis ein, die zwar entzündungshemmend wirken, aber die fortschreitende Gelenkszerstörung nicht aufhalten können. Versuche mit IL-1- und TNF α -Inhibitoren haben gezeigt, daß diese Proteine die Zerstörung der Gelenke verhindern (Otani et al., 1996).

In einem Versuch der klinischen Phase I werden deshalb synoviale Zellen der Gelenke entfernt und so transduziert, daß es zur Expression eines IL1-Inhibitors in den transduzierten Zellen kommt. Die modifizierten Zellen werden dann wieder implantiert (Evans et al., 1996).

2.3.1.5 Lysosomale Speicherkrankheiten

Die Gaucher Krankheit ist eine lysosomale Speicherkrankheit, die durch Mutationen im Gen für die Glukozerebrosidase verursacht wird. Obwohl das Gen in allen Zellen defekt ist, betrifft die Gaucher Krankheit vornehmlich die Makrophagen. Mittels einer Proteintherapie, d.h. der Gabe von modifizierter Glukozerebrosidase, wurde die Pathogenese vollständig verhindert (Robbins und Ghivizzani, 1998). Die retrovirale Transduktion haematopoetischer Stammzellen mit dem Glukozerebrosidase-Gen führte in Patienten zur Expression der

Glucozerebrosidase in allen abgeleiteten Zelllinien. Eine Langzeitexpression wurde in einer Populationen CD34⁺-Zellen aus periphärem Blut erreicht (Bahnson et al., 1994).

2.3.1.6 Krebs

Krebserkrankungen gehören zu einem Krankheitsbild, bei dem zwischen vererbt und erworben nicht immer genau unterschieden werden kann. Das fortschreitende Verständnis der molekularen Grundlagen von Krebserkrankungen führte auch zur Entwicklung von Behandlungsstrategien, die Vektoren aus der Gentherapie nutzen.

Eine wichtige Strategie zur Behandlung von Krebs durch die Gentherapie ist die Einschleusung von Tumorsuppressorgenen in Krebszellen (Tabelle 3). Dies führt in den Krebszellen, bei denen diese Gene defekt sind, zu Wachstumsstillstand oder Zelltod.

<i>Genprodukt</i>	<i>Funktion</i>	<i>Effekt in Zellkultur</i>	<i>Effekt im Mausmodell</i>
INK4A	Blockiert Zellzyklus durch CDK4 Inhibierung	Proliferationsstop	Tumorsuppression
INK4A-KIP1 Fusion	Inhibiert CDK4 und CDK2	Apoptose	Tumorregression
Rb	Zellzyklus unterbrochen durch E2F Repression	Proliferationsstop	Tumorsuppression
p130	Zellzyklus unterbrochen durch E2F Repression	Proliferationsstop	Tumorregression
ARF	Schützt p53, da MDM2 inhibiert wird	Proliferationsstop	Nicht durchgeführt
p53	Fördert Zellzyklus Arrest und Apoptose	Proliferationsstop und erhöhte Strahlungssensitivität	Tumorsuppression, verringerte Metastasenbildung
PTEN	Baut 3'-phosphorylierte Phosphoinosite ab, die Zellwachstum und Überlebens-Stoffwechselwege aktivieren	Proliferationsstop; Apoptose; erhöhte Strahlungssensitivität	Tumorsuppression oder kein Effekt
APC	Markiert β -Catenin zum Abbau	Apoptose	Nicht durchgeführt
BRCA1	Unversehrtheit des Genoms	Proliferationsstop oder Apoptose	Tumorsuppression

Tabelle 3: Expression von Tumorsuppressoren in Zellkultur und Mausmodell, nach McCormick (2001).

Für die bekannten Tumorsuppressorgene konnte gezeigt werden, daß ihre Expression *in vitro* zu einem Proliferationsstop führte. Auch Geneprodukte, die für die Integrität des Genoms verantwortlich sind, können zur Therapie eingesetzt werden (zB. BRCA1 und 2). So führte die Bildung von BRCA1 in Tumorzellen zum Proliferationsstop oder Apoptose bei Brust- oder Eierstockkrebs (Holt et al., 1996; Shao et al., 1996), obwohl wtBRCA1 ebenfalls von den Zellen gebildet wurde.

Ein wichtiger Aspekt beim Einsatz der Tumorsuppressorgene ist die sehr geringe Wahrscheinlichkeit von kollateralen Schäden in gesunden Zellen. Die eingeführten Gene sind in gesunden Zellen hochreguliert (McCormick, 2001). So konnte gezeigt werden, daß die Transduktion mit *tp53* keinen Effekt auf gesunde Zellen hatte, obwohl das bis zu 100-fache der therapeutisch relevanten Dosis angewendet wurde (Zhang et al., 1994). Eigentlich wurde nicht erwartet, daß die Expression von Tumorsuppressorgen einen sogenannten *Bystander*-Effekt auf umliegendes Krebsgewebe auslöst. Man ging vielmehr davon aus, daß jede einzelne Zelle transduziert werden muß. Erstaunlicherweise zeigt gerade TP53 einen solchen Effekt, vermutlich weil es die VEGF Expression herunterreguliert (Nishizaki et al., 1999; Bouvet et al., 1998) und außerdem die Expression von Thrombospondin fördert und damit die Angiogenese unterdrückt (Dameron et al., 1994).

Zur Zeit durchgeführte klinische Versuche nutzen Adenoviren zur Transduktion von Zellen mit *tp53* (Adp53). Dabei kann eine anti-Tumor Aktivität in einigen Patienten beobachtet werden, die wegen eines großzelligen (bzw. nicht-kleinzelligen) Bronchialkarzinoms oder Plattenepithelkarzinoms therapiert werden (Swisher et al., 1999; Clayman et al., 1999). Dieses Verfahren befindet sich derzeit in Kombination mit Chemotherapeutika in der klinischen Phase II und III (Nemunaitis et al., 2000a; Nemunaitis et al., 2000b).

Neben der Expression der Tumorsuppressorgene ist die Inhibierung spezifischer Onkogene auch Ziel der Krebstherapie. Tumore, die auf dem Onkogen *ras* beruhen, werden zerstört, wenn das *ras* Genprodukt auf RNS-Ebene genetisch (*antisense* Nukleotide oder Ribozyme) oder auf Protein-Ebene pharmakologisch eliminiert wird (Chin et al., 1999; Barrington et al., 1998). Chronische myelogene Leukämien zeigen einen deutlichen Rückgang, wenn das Onkoprotein p210-BCR-ABL durch den ABL Inhibitor STI-571 (Gleevec) inhibiert wird, obwohl es noch zahlreiche andere genetische Modifikationen in diesen Zellen gibt (Druker et al., 2001).

Erwähnt sei noch eine weitere Form der Krebstherapie, die sogenannte *suicide therapy*. Die sogenannten *suicide* Gene, die in die Krebszellen eingeschleust werden, codieren für Enzyme, die es der Krebszelle ermöglichen, eine nicht toxische Vorstufe eines Zellgiftes zu der aktiven

Form umzusetzen. Dieses kann dann nach dem Zelltod auch umliegende Zellen töten. Da diese Eigenschaft nur den Tumorzellen durch die Transfektion vermittelt wird, können gesunde Zellen die relativ harmlose Vorstufe des Giftes nicht metabolisieren. Beispiele für diese Art der Therapie sind: Herpes-Simplex-Virus Thymidin-Kinase (Moolten, 1986), Cytosin Deaminase (Trinh et al., 1995; Hanna et al., 1997), Nitroreduktase (Bridgewater et al., 1995), Carboxylesterase (Danks et al., 1998), Cytochrome P450 (Waxman et al., 1999), Purin-Nukleosid Phosphorylase (Sorscher et al., 1994).

Eine gänzlich andere Vorgehensweise zur Krebstherapie wird im Moment entwickelt. Dabei macht man sich replikationsdefiziente Adenoviren zu Nutze (McCormick, 2001). Diese Viren können sich nur in Zellen vermehren, die in dem Rb-Signaltransduktionsweg gestört sind, was für viele Krebszellen zutrifft. Diese Krebszellen sind dann potentielle Wirtszellen für die Virusreplikation, während gesunde Zellen die adenovirale Replikation nicht auslösen können (Heise et al., 2000; Heise und Kirn, 2000; Fueyo et al., 2000; Doronin et al., 2000). Die Replikation der Viren in der Tumorzelle führt zur Zellyse und zur Ausbreitung der Viren in weiteren Tumorzellen. Die Effektivität dieses Systems konnte durch Viren, die das Adenovirus Death Protein (ADP) überexprimieren, noch gesteigert werden. Dieses Protein steigert die Zellyse und die Freisetzung der Viren (Doronin et al., 2001).

Die Vielzahl der durchgeführten Studien und ihre Diversität zeigen deutlich, daß diese Therapieform ein großes Potential besitzt. Dennoch ist die Effektivität der Behandlung bislang noch äußerst gering. Zwar zeigen die eingesetzten viralen Systeme eine hohe Transduktions- und Expressionseffizienz (>90%) (Luo und Saltzman, 2000), aber dennoch konnte keines der angewendeten klinischen Protokolle einen durchschlagenden Erfolg erzielen (Anderson, 1998).

2.4 Vektoren für die Gen- und Krebstherapie

Wie bereits erwähnt, werden in der Gen- und Krebstherapie für die meisten klinischen Studien als Vektoren virale Systeme benutzt. Die natürliche Eigenschaft der Viren, Zellen zu befallen und sich unter Nutzung des Wirtsstoffwechsels zu vermehren, prädestinieren sie für gentherapeutische Anwendungen. Dennoch werden auch nicht-virale Systeme wie Liposomen für den Gentransfer entwickelt. Die Tabelle 4 gibt einen Überblick über verwendete Systeme und ihre Vor- und Nachteile:

Eigenschaft	Retrovirus	Lentivirus	Adenovirus	AAV	DNS/Lipid-DNS
Max. verpackbare DNS	7-7,5 kb	7-7,5 kb	ca. 30 kb	3,5-4,0 kb	Unbeschränkt
Max. erreichbarer Titer (Partikel/ml)	$>10^8$	$>10^8$	$>10^{11}$	$>10^{12}$	Unbeschränkt
Integration in das Genom	Ja	Ja	Nein	Ja, wenn <i>rep</i> Gen vorhanden	Gering
Expressionsdauer <i>in vivo</i>	Kurz	Lang	Kurz	Lang	Kurz
Stabilität	Gut	Nicht getestet	Gut	Gut	Sehr gut
Biotechnologische Produktion	Bis 25 l getestet	Nicht bekannt	Leichte Großproduktion	Schwer zu reinigen, schwere Großproduktion	Leichte Großproduktion
Immunologische Nebenreaktionen	Gering	Gering	Stark	Nicht bekannt	Keine
Empfänger Vorimmunität	Unwahrscheinlich	Unwahrscheinlich, außer AIDS Patienten	Vorhanden	Vorhanden	Keine
Sicherheitsrisiken	Insertionsmutationen	Insertionsmutationen	Entzündungsreaktionen, Toxizität	Entzündungsreaktionen, Toxizität	Keine

Tabelle 4: Eigenschaften verschiedener Vektoren in einer Gegenüberstellung, verändert nach Verma und Somia (1997).

2.4.1 Virale Vektoren

In der klinischen Forschung werden hauptsächlich 4 verschiedene virale Systeme eingesetzt, auf die im Folgenden näher eingegangen wird. Dennoch sollen auch alternative virale Systeme erwähnt werden. Hierbei handelt sich um +-Strang RNA-Viren wie Polio-, HepatitisB- oder Sindbis-Virus. Die therapeutische RNA wird nach dem Gentransfer in der Zelle autonom durch die virale Replikase vermehrt. Dadurch können sehr hohe Expressionsraten erreicht werden, was diese Systeme sehr interessant für die Vakzinierung macht (Robbins und Ghivizzani, 1998).

2.4.1.1 Retroviren

Retroviren werden aufgrund ihrer Genomstruktur in einfache und komplexe Retroviren eingeteilt (Coffin, 1996; Vogt, 1997). Beide Klassen enthalten die Gene *gag*, *pol*, *pro* und *env*. Die komplexen Retroviren enthalten darüberhinaus noch weitere Gene. Retroviren können neben dieser Klassifizierung auch noch in die Gruppen der Onkoviren, der Lentiviren und der Spumaviren eingeteilt werden (Coffin, 1996). Dabei zählen die meisten Onkoviren zu den einfachen, die Lenti- und Spumaviren zu den komplexen Viren.

Das Retrovirus infiziert die Zielzelle durch eine spezifische Bindung des env-Proteins mit einem zellulären Rezeptor. Das Virus wird dann internalisiert und die Membranhülle entfernt. Das RNS Genom wird *revers* transkribiert zu einer proviralen, doppelsträngigen DNS. Die dsDNS wird dann stabil in das Genom der Wirtszelle integriert. Für viele Viren ist der Zusammenbruch der Kernhülle während der Mitose der einzige Zeitpunkt, an dem sie in den Zellkern gelangen können. Eine Ausnahme stellen hier die Lentiviren dar (Robbins und Ghivizzani, 1998). Die Integration der proviralen DNS bedeutet eine Modifikation der Wirts-DNS über die gesamte Lebenszeit. Dies steht im Kontrast zur Gentherapie mittels anderer DNS-Viren wie Herpes-Simplex Virus oder Papilloma-Virus, deren genetische Information episomal verbleibt. Die Integration der proviralen DNS erfolgt über die vom Virus codierte Integrase. Nach der Integration erfolgt die Transkription der proviralen DNS. Dabei werden zum einen komplette provirale Sequenzen gebildet, die als neues virales RNS Genom dienen. Andere Sequenzen werden *spliced* und mit Hilfe der Wirtsproteine translatiert (*gag*, *pro*, *env*, *pol*). Da diese modifizierten RNS-Sequenzen keine Ψ Sequenz mehr enthalten, werden diese RNS Moleküle nicht in die virale Hülle verpackt. Das komplette Virus wird dann durch die mit env-Proteinen bestückte Membran ausgeschleust und erhält dadurch die Membranhülle.

Da Retroviren oftmals Säugerzellen als Wirtszellen haben, können sie nach Applikation Zellen infizieren und sich vermehren. In den meisten klinischen Anwendungen ist dieser Prozeß jedoch nicht erwünscht. Deshalb wurde das Konzept der replikationsdefizienten Retroviren entwickelt. Das Genom des Virus wird dabei auf ein sogenanntes Vektor- und ein Helfer-Konstrukt aufgeteilt (Miller, 1997). Das Helfer-Konstrukt (oder die Helfer-Zelllinie) produziert alle wichtigen Proteinkomponenten für die Herstellung eines Virus. Da nur die RNS, die vom Vektorkonstrukt gebildet wird, eine Verpackungssequenz besitzt, kann nur diese RNS verpackt werden. Allerdings codiert das Vektorkonstrukt nur das therapeutisch relevante Gen und keinerlei virale Proteine. Die so produzierten Viren sind replikationsdefizient (Hu und Pathak, 2000). Bisher sind von allen drei Klassen der Retroviren Vektoren für die Gentherapie entwickelt und beschrieben worden, der am meisten verbreitete Vektor ist allerdings abgeleitet vom MMLV, einem Onkovirus (Hu und Pathak, 2000). Die Sequenz des therapeutischen Gens wird dabei im Vektorkonstrukt zwischen die *LTRs* kloniert.

Der Vorteil der Retroviren, sich in ein Genom stabil zu integrieren und über Generationen vererbt zu werden, ist auch der Hauptgrund für die Sicherheitsbedenken, die es zu diesem System gibt. Es darf nicht zur Bildung replikationskompetenter Retroviren im Wirtsorganismus kommen und es darf keine Insertionsmutagenese bei der Genomintegration stattfinden. Insertionsmutagenese konnte mit replikationskompetenten Retroviren gezeigt werden (Coffin, 1996). Die Bildung replikationskompetenter Retroviren kann zum einen durch Rekombination während ihrer Herstellung stattfinden. Das kann zumindest statistisch minimiert werden, wenn Sequenzen in den Helfer- und Vektorkonstrukten nur geringe Homologien aufweisen (Hu und Pathak, 2000). Eine andere Möglichkeit der Entstehung replikationskompetenter Viren ist die homologe Rekombination mit verwandten Viren. Dadurch entstehen bei der Produktion von MMLV in Mäusezelllinien replikationskompetente Viren (Purcell et al., 1996). Abhilfe kann hier die Nutzung z.B. humaner Zelllinien bringen, von denen nicht bekannt ist, daß sie endogene, MMLV-verwandte Viren tragen (Ory et al., 1996). Darüberhinaus konnte gezeigt werden, daß diese replikationskompetenten MMLV in Primaten Lymphome hervorrufen können (Donahue et al., 1992; Vanin et al., 1994; Purcell et al., 1996).

Die Zelltyp-spezifische Adressierung viraler Partikel ist eine Voraussetzung für den Einsatz der Partikel *in vivo*. In verschiedenen Versuchen konnten durch Kombination von gag- und env-Proteinen unterschiedlicher viraler Stämme, sogenannte *pseudotyped* Viren konstruiert werden (Miller und Buttimore, 1986). Diese Strategie läßt sich allerdings nicht

verallgemeinern (Linial, 1999). Neben der Adressierung der viralen Partikel bekommt auch die Nutzung Zelltyp-spezifischer Promotoren immer mehr Bedeutung. Diese erlauben die Expression des transduzierten Gens nur in einer bestimmten Gewebeart. Ein ähnliches Prinzip verfolgen auch Vektorkonstrukte, bei denen die Expression durch bestimmte Stoffe, wie z.B. Tetracyclin, gesteuert wird. Die Expression des Transgens wird dann erst nach der Gabe dieses Induktors angeschaltet (Somia und Verma, 2000).

Die Produktion hoher Titer an retroviralen Vektoren stellt immer noch ein großes Problem dar. Zwar kann mittels *pingpong* Amplifikation (Kozak und Kabat, 1990) die *superinfection interference* vermindert werden. Allerdings entstehen bei dieser Methode oft replikationskompetente Viren. Physikalische Methoden, um hohe Titer zu erreichen, werden zwar verwendet (z.B. Ultrafiltration), allerdings scheinen Retroviren bezüglich verschiedener Arbeitsschritte labiler zu sein als andere virale Systeme (Hu und Pathak, 2000).

2.4.1.2 Adenovirale Vektoren

Adenoviren sind hüllenlose, dsDNS-Viren. Der virale Vermehrungszyklus beginnt mit der Bindung der *Fiber* Monomere an das sogenannte CAR Protein (*coxsackie virus and adenovirus receptor*) (Bergelson et al., 1997). Die darauffolgende Interaktion der *Penton* Basis mit Integrinen führt zur Rezeptor-vermittelten Endozytose. Adenoviren nutzen die Absenkung des pH-Wertes im späten Endosom zur Freisetzung aus dem endosomalen/lysosomalen Kompartiment. Das virale Partikel erreicht dann den Zellkern, in dem die Transkription stattfindet. Der virale Lebenszyklus endet mit dem Zelltod der Wirtszelle und der Freisetzung der Viren (Benihoud et al., 1999).

Adenoviren sind für den Einsatz als Vektoren interessant, da sie z.T. auch ruhende Zellen infizieren können. Es können zwischen 7,5 kb und 36 kb fremde genetische Information in das virale Genom integriert werden (Benihoud et al., 1999). Außerdem können hohe virale Titer produziert werden. Da das Genom der Adenoviren in den Zellen extrachromosomal verbleibt, ist die Gefahr von Insertionsmutationen gering. Ein großer Nachteil in der Anwendung ist bisher die geringe Expressionsdauer des durch adenovirale Vektoren transduzierten Gens *in vivo*. Um die Langzeitexpression in sich teilenden Zellen zu erhöhen, werden neue Wege beschritten. Dazu wird über ein *CRE/Lox* System ein zirkuläres Episom in der Wirtszelle geschaffen. Diese wird dann effizient über ein *EBNA/oriP* Replikationssystem des Epstein-Barr Virus repliziert (Benihoud et al., 1999).

Ein großes Problem bei der Anwendung der Adenoviren ist die Immunreaktion von Patienten auf die Gabe des Vektorsystems. Zwar gibt es Unterschiede in der Immunantwort, abhängig

von der Art der Verabreichung der Viren (Benihoud et al., 1999), die entstehende Immunität verhindert aber meist eine erneute Anwendung der Viren (Yang et al., 1996).

Um die Sicherheit der adenoviralen Vektoren zu erhöhen, wurden Vektoren geschaffen, bei denen die E1 Region im Genom entfernt ist. Helferzelllinien wie 293 liefern diese Genprodukte *in trans* (Graham und Prevec, 1995). Das statistische Auftreten von replikationskompetenten Viren, die eine vollständige E1 Sequenzen haben, macht allerdings die Produktion klinisch nutzbarer Viruschargen sehr aufwendig (Hehir et al., 1996).

Eine interessante Variante der Vektorproduktion sind die sogenannten *gutless* Viren. Die Expression viraler Gene in einem Wirtsorganismus ist *per se* zytotoxisch und ruft eine Immunantwort hervor (Dedieu et al., 1997; Dai et al., 1995). Je mehr Gene aus dem Genom entfernt werden, desto geringer sollte die Immunantwort ausfallen. Die ersten Ansätze zur Herstellung der *gutless* Viren nutzten dabei die Anwesenheit von Helferviren. Die Verringerung des ursprünglichen Genoms von Adenovirus führt dazu, daß die Klonierungskapazität deutlich steigt, auf bis zu 36 kb (Benihoud et al., 1999). Interessanterweise führt die Anwendung der *gutless* Viren oftmals auch zu einer verlängerten Expression des Transgens (Chen et al., 1997; Morsy et al., 1998; Schiedner et al., 1998). Die so hergestellten *gutless* Vektoren müssen noch aufwendig vom Helfervirus gereinigt werden, trotzdem bleibt eine Kontamination mit 1% Helfervirus bestehen (Kochanek et al., 1996; Lieber et al., 1996). Dennoch zeigen die so eingesetzten *gutless* Vektoren nur eine schwache Immunantwort (Chen et al., 1997; Morsy et al., 1998; Schiedner et al., 1998).

Mit der Anwendung in der Klinik wurden auch Strategien entwickelt, um die Zelltyp-spezifische Adressierung zu erreichen. Die Bindung des Virus erfolgt über das CAR-Protein an die Zelloberfläche verschiedenster Zelltypen. Die sekundäre Interaktion mit αV -Integrinen sorgt dann für die Rezeptor-vermittelte Endozytose. Versuche zur Änderung des Tropismus nutzen z.B. bispezifische Agenzien, wie Antikörperfragmente. Ein bispezifischer Antikörper gegen das *Fiber* Protein und den EGF-Rezeptor führt zur Verminderung des natürlichen Tropismus und zur Bindung an humane Gliomzellen über den EGF-Rezeptor (Miller et al., 1998). Eine weitere Alternative bietet die genetische Veränderung der Kapsidproteine. Insertionen in die *Fiber-Knob*-Proteine erweitern den Tropismus (Dmitriev et al., 1998) und die Modifikation des *Hexon* Protein ermöglicht einen alternativen Internalisierungsweg (Vigne et al., 1999). Die Modifikation der Zellbindung und der Internalisierung der veränderten Adenoviren führt allerdings sofort zu einem Produktionsproblem. Die Infektion von Produktionszelllinien kann nur erfolgen, wenn diese die neuen Rezeptoren aufweisen.

Dies bedeutet, daß für jede Variante des Adenovirus eine neue Produktionszelle geschaffen werden muß (Douglas et al., 1999).

2.4.1.3 Herpes-Simplex-Virus (HSV)

Herpes-Simplex Virus ist ein Virus mit einem linearen, doppelsträngigem DNS-Genom von ca. 150 kb. Das Genom von HSV codiert für über 70 Proteine. Mindestens zwei virale Glykoproteine, gB und gD, werden genutzt, um an Zelloberflächen zu binden. Dabei bindet das Virus an Heparinsulfat-Motive. Das Virus fusioniert danach mit der Zellmembran und die lineare DNS wird zu einem Ring geschlossen. Die virale Proteinsynthese wird durch das virale Protein VP16 initiiert, das Teil der viralen Hülle ist (Tegument). VP16 stimuliert die Expression von frühen viralen Genen, die zur Bildung der *immediate early* Proteine führt. Diese wiederum regen die Expression weiterer früher Gene für die DNS-Synthese an und es werden späte Gene exprimiert, die für die Verpackung der DNS zuständig sind. Die neu gebildeten Viren werden dann durch Zellyse freigesetzt (Robbins und Ghivizzani, 1998).

Der Vorteil bei HSV ist seine Eigenschaft, auch ruhende Zellen zu infizieren und dort als latentes Virus zu verbleiben. Die Eigenschaft auch Zellen des Nervensystems, die sich in der G0-Phase befinden, zu infizieren, macht HSV-Vektoren zu guten Kandidaten für die Therapie von neurodegenerativen Erkrankungen. Andererseits infiziert HSV auch andere Gewebe sehr effizient (Muskel und Leber) und könnte deshalb in einem breiten Applikationsrahmen eingesetzt werden (Robbins und Ghivizzani, 1998). Ein weiterer Vorteil ist die Größe an genetischer Information, die im Vektor verpackt werden kann. Da relativ viele Gene ohne Einfluß auf den Vektor entfernt werden können, ergibt sich eine theoretische Verpackungsgröße von bis zu 40 kb (Latchman, 2001). HSV-Vektoren wurden bereits für die Gentherapie eingesetzt (Fink und Glorioso, 1997; Fink et al., 1995; Geller, 1997; Marconi et al., 1996; Neve und Geller, 1995; Starr et al., 1996).

Der Nachteil der Anwendung bei HSV liegt auch hier im Bereich der Toxizität des Virus. Die Deletion früher Gene aus dem Genom, die *in trans* von einer Helferzelllinie zur Produktion zur Verfügung gestellt werden, hat sowohl zu einer besseren Sicherheit des Vektorsystems als auch zu einer geringeren Immunogenität geführt (Marconi et al., 1996; Wu et al., 1996; Zhu et al., 1996). Ein weiterführender Ansatz, um die Immunogenität zu verringern, ist der Einsatz von Amplikons. Die dabei entstehenden HSV-Partikel enthalten nur noch fremde DNS, die kein HSV-Gen mehr exprimiert. Obwohl durch dieses Verfahren ein Präparat ohne Helfervirus gewonnen werden kann, sind die viralen Titer für eine breite praktische Anwendung viel zu gering (Geller, 1997; Neve und Geller, 1995; Starr et al., 1996).

Außerdem wurde beobachtet, daß in den verschiedensten Geweben die transgene Expression nach einer Woche abgeschaltet wird.

2.4.1.4 Adeno-assoziierte Viren (AAV)

Die AAV gehören zu den Parvoviren. Es sind Viren mit einem einzelsträngigem DNS Genom, die zur Replikation die Anwesenheit von Adenoviren oder HSV brauchen. AAV können ruhende Zellen infizieren und sich stabil an einer bestimmten Stelle im Chromosom 19 integrieren (Samulski, 1993). Mit der Infektion und Integration der AAV konnte bisher noch keine Pathogenität in Verbindung gebracht werden.

Das AAV Genom codiert nur die Gene *rep* und *cap*. Um einen replikationsdefizienten Virus zu schaffen, können beide Gene deletiert und *in trans* angeboten werden. Die beiden *terminalen repeats*, die für Replikation und Integration verantwortlich sind, flankieren das inserierte Gen (Rolling und Samulski, 1995). Obwohl beide Gene entfernt werden können, kann AAV nur 5 kb genomische Information verpacken, eine relativ geringe Größe. Außerdem sorgt die Deletion der beiden Gene für den Verlust der spezifischen Integrationsfähigkeit bzw. führt zum episomalen Verbleib der DNS (Ferrari et al., 1996). Die klinische Forschung, die AAV benutzt, ist aus diesen Gründen auch noch nicht sehr weit fortgeschritten.

2.4.2 Nicht-virale Transfektionssysteme

Die einfachste Methode für den Gentransfer ist sicherlich die Injektion nackter DNS in ein Zielgewebe, wie für die Transfektion von Skelettmuskel, Leber oder Tumorgewebe gezeigt wurde (Wolff et al., 1990; Hickman et al., 1994; Yang und Huang, 1996). Eine weitaus effizientere Methode für den *ex vivo* Gentransfer ist die Manipulation einer einzelnen Zelle durch die Mikroinjektion von DNS in den Zellkern (Luo und Saltzman, 2000). Andere Verfahren bedienen sich hoher Drücke (Mann et al., 1999) oder Zellen werden mit DNS-beladenen Partikeln beschossen (Yang et al., 1990; Yang und Sun, 1995; Ye et al., 1990). Neben den mechanischen Methoden können eukaryontischer Zellen auch mit elektrischen Methoden transfiziert werden (Wong und Neumann, 1982; Neumann et al., 1982). Diese zählen zu den effektivsten Transfektionsmethoden *in vitro*, haben als Nebenwirkung aber eine hohe Mortalitätsrate. Obwohl die Anwendung *in vivo* schwierig ist, gibt es Beispiele der Transfektion von Haut (Banga und Prausnitz, 1998), Kornea (Oshima et al., 1998) und Muskelzellen (Aihara und Miyazaki, 1998).

Um die Effektivität der DNS Aufnahme zu steigern und gleichzeitig der DNS Schutz vor Abbau zu bieten, wurden schon vor über 30 Jahren chemische Substanzen benutzt (Vaheri und Pagano, 1965). Der Einsatz von chemischen Substanzen zum Schutz der DNS und zur Erhöhung der Transfektionseffizienz stellt auch bis heute noch den effektivsten und kostengünstigsten Weg dar. Das hier angewendete Prinzip beruht auf der Komplexbildung zwischen der DNS und Substanzen, wie z.B. DEAE-Dextran, Kalziumphosphat, Lipiden, Dendrimeren und Proteinen (Luo und Saltzman, 2000). Werden Zellen zusammen mit diesen Komplexen inkubiert, so können diese endozytiert werden (Luo und Saltzman, 2000). Eine Steigerung in der Transfektionseffizienz beim Einsatz chemischer Substanzen wurde durch die Entwicklung der Lipofektion erreicht (Felgner et al., 1987). Ein ebenfalls gut etabliertes System ist die Komplexbildung von DNS mittels Liposomen. Dabei werden anionische, kationische oder neutrale Liposomen eingesetzt. Der Einsatz von Liposomen als Vektor ist wahrscheinlich der am meisten genutzte (Luo und Saltzman, 2000) und wurde auch schon in klinischen Versuchen eingesetzt. Ein großer Nachteil ist allerdings das geringe Wissen um die Struktur der DNS-Liposomen Komplexe, das Fehlen von Adressierung und große Chargenunterschiede während der Herstellung (Luo und Saltzman, 2000). Den chemischen Methoden ist gemein, daß sie bei systemischer Gabe hochtoxisch sind (Filion und Phillips, 1998). Außerdem bleibt die Rolle der Chemikalien hinsichtlich der Transfektion noch relativ unklar, da auch die Injektion nackter DNS in Muskel oder die Inhalation nackter DNS zur Expression des Zielgens führen kann (Wolff et al., 1990; Zabner et al., 1997).

Neben chemischen Substanzen können auch Peptide oder Proteine zur Erhöhung der Transfektionsrate eingesetzt werden. Poly-L-Lysin kann DNS komplexieren und die Transfektionsrate deutlich erhöhen (Wagner et al., 1998). Durch chemische Modifikation oder Kopplung zellulärer Liganden kann eine gewisse Adressierung erreicht werden. Die Bildung eines Komplexes aus EGFP codierender DNS, gebunden an Streptavidin-Poly-L-Lysin, zusammen mit Biotin-gekoppeltem EGF führte zur Expression des GFP in EGF-Rezeptor positiven Zellen (Schaffer und Lauffenburger, 1998). All die artifiziellen Systeme beinhalten die Bildung von Komplexen zwischen einem Ligand und der DNS. Ein Punkt, der in der Entwicklung dieser Systeme allerdings noch kaum Berücksichtigung gefunden hat, ist die Freisetzung der DNS in den Zellen. Daß es in der Tat ein limitierender Schritt sein kann, wurde erst kürzlich gezeigt (Schaffer et al., 2000).

2.5 Polyomaviren

Polyomaviren gehören zur Familie der Papovaviren. Die Papovaviren werden nicht von einer Membran umhüllt und besitzen ein Genom aus doppelsträngiger DNS. Die Papovaviren lassen sich in zwei Gattungen, die Polyomaviren und die Papillomaviren unterteilen.

<i>Genus</i>	<i>Mensch</i>	<i>Tier</i>
Polyomavirus	BK-Virus	SV40
	JC-Virus	HaPV (Hamster-Papovavirus)
		Polyomavirus der Maus
		Polyomavirus des Wellensittich
		B-lymphotropes Papovavirus des Affen
Papillomavirus	HPV-1-75	CRPV (Shopes Kaninchenpapillomavirus)
	(humane Papillomaviren, Warzenviren)	BPV (Rinderpapillomavirus)

Tabelle 5: Vertreter der Papovaviren, nach Modrow und Falke (1997)

2.5.1 Genomaufbau der Polyomaviren

Das zirkuläre dsDNS-Genom der Polyomaviren besitzt eine Größe von ca. 5200 bp. Die DNS wird durch die Wirtshistone H2A, H2B, H3 und H4 zu einer Nukleosomenstruktur kondensiert (Cremisi et al., 1976).

Das Genom der Polyomaviren wird in einen frühen und einen späten Bereich aufgeteilt. Der frühe Bereich codiert für die sogenannten T-Antigene, die vor allem regulatorische Funktionen ausüben. So bindet das große T-Antigen an den Replikationsursprung und initiiert die Replikation. Die Virushülle wird von den Strukturproteinen VP1, VP2 und VP3 gebildet, die von den späten Genen codiert werden. Sowohl bei den frühen als auch bei den späten Genen werden alternative *splice*-Mechanismen angewendet um die verschiedenen Proteine zu bilden. Zwischen den frühen und den späten Genen befindet sich ein regulatorischer Bereich, der Replikationsursprung, Promotoren und *Enhancer*-Elemente enthält (Modrow und Falke, 1997).

2.5.2 Die frühen Gene

Die frühen Gene bei Polyomaviren sind das große, mittlere und kleine T-Antigen. Die durch die frühen Gene exprimierten Proteine interagieren mit zellulären Faktoren und spielen eine Rolle bei der Regulation der Expression der späten Gene und der Regulation der Replikation des viralen Genoms. Das große T-Antigen bindet an die zellulären Proteine Rb und p53 und

inaktiviert so die entsprechenden Signaltransduktionswege (Sachsenmeier und Pipas, 2001; Zhu et al., 1991).

Das mittlere T-Antigen bindet und aktiviert Src-Tyrosinkinasen (Dilworth, 1995; Dunant und Ballmer-Hofer, 1997). Die Bindung aktiviert dann einen anderen, Src-abhängigen Signaltransduktionsweg. Das mittlere als auch das kleine T-Antigen binden an Phosphatase-2 und verändern deren Substratspezifität und zelluläre Lokalisation (Walter et al., 1990; Messerschmitt et al., 1996; Cayla et al., 1993) und werden mit der Transformation der Zellen in Verbindung gebracht (Pallas et al., 1990).

Die Bindung des großen T-Antigens an den Replikationsursprung führt zur Replikation des viralen Genoms (Modrow und Falke, 1997). Dabei erhöht die zusätzliche Bindung des kleinen T-Antigens die Replikationsgeschwindigkeit deutlich (Berger und Wintersberger, 1986). Die frühen Genprodukte induzieren dann die Expression der späten Gene.

2.5.3 Die späten Gene

Die späten Gene codieren für die drei Strukturproteine VP1, VP2 und VP3, wobei die Gene für VP2 und VP3 überlappen. Dies erklärt, warum VP3 eine verkürzte Variante von VP2 darstellt.

Das murine VP1 ist ein pentameres Protein und besteht aus 384 Aminosäuren pro Monomer (M_r 42.5 kDa). Außerdem weist das VP1-Protein einen hohen Phosphorylierungs- und Acetylierungsgrad auf (Bolen et al., 1981; Ponder et al., 1977; Garcea et al., 1985).

VP1 läßt sich in 3 strukturelle Bereiche aufteilen:

- ein flexibler N-terminaler Bereich, der in der Kristallstruktur nicht aufgelöst ist und vermutlich zur DNS-Bindung beiträgt. Der N-Terminus befindet sich nach der Assemblierung der Kapsomeren im Inneren der Kapside (Liddington et al., 1991).
- die *core* Struktur, die ein *b-barrel* mit *jelly-roll* Topologie besitzt (Stehle und Harrison, 1997). Hier sind die Zellbindungseigenschaften zu finden (Liddington et al., 1991)
- ein C-terminaler Bereich, der ebenfalls nicht komplett in der Struktur aufgelöst ist. Dieser Teil dient der Wechselwirkung mit anderen Pentameren (Stehle und Harrison, 1997).

Bei den murinen Polyomaviren bindet pro Pentamer entweder eine Kopie von VP2 oder VP3 (Barouch und Harrison, 1994). Dabei ragen ca. 40 Aminosäuren des C-Terminus von VP2/VP3 in die zentrale Kavität eines VP1-Pentamers (Barouch und Harrison, 1994). Eine vergleichbare Wechselwirkung wurde auch für das SV40 Virus beschrieben (Gharakhanian et al., 1988; Gharakhanian und Kasamatsu, 1990). Die genaue Funktion von VP2 und VP3 ist bislang nicht bekannt. Es wird allerdings vermutet, daß die Myristilierung am N-Terminus

von VP2 (Streuli und Griffin, 1987) mit dem Eindringen der Viren in die Zelle in Verbindung steht (Chen et al., 1998a). Die Oberfläche der viralen Hülle definiert auch die Bindungseigenschaften des Polyomavirus an Zelloberflächen. Alle bisher beschriebenen Stämme des Polyomavirus binden Zuckerreste, die auf (α -2,3)-verbundene, α -5-N-acetyl-Neuraminsäure enden (Sialinsäurerest). Während alle Stämme geradkettige Liganden erkennen (NeuNAc-([α]-2,3)-Gal-([β]-1,3)-GalNAc), erkennen einzelne Stämme auch verzweigtkettige Liganden (NeuNAc-([α]-2,3)-Gal-([β]-1,3)-[([α]-2,6)-NeuNAc]-GalNAc) (Fried et al., 1981; Cahan et al., 1983). Hochgradig tumorigene Stämme binden nur geradkettige Zuckerreste und besitzen an Position 91 einen Glutaminsäurerest. Die gering tumorigenen Stämme können beide Zuckerreste binden und besitzen ein Glycin an Position 91 (Freund et al., 1991). Die Aminosäure 91 befindet sich im BC2-Loop der VP1 Struktur (Stehle und Harrison, 1997). Dieser Loop, zusammen mit dem EF-Loop und der Spitze des BC2-Loops und des DE-Loops des benachbarten VP1, bilden die Bindungstasche für die Zuckermotive (Stehle und Harrison, 1996; Stehle und Harrison, 1997).

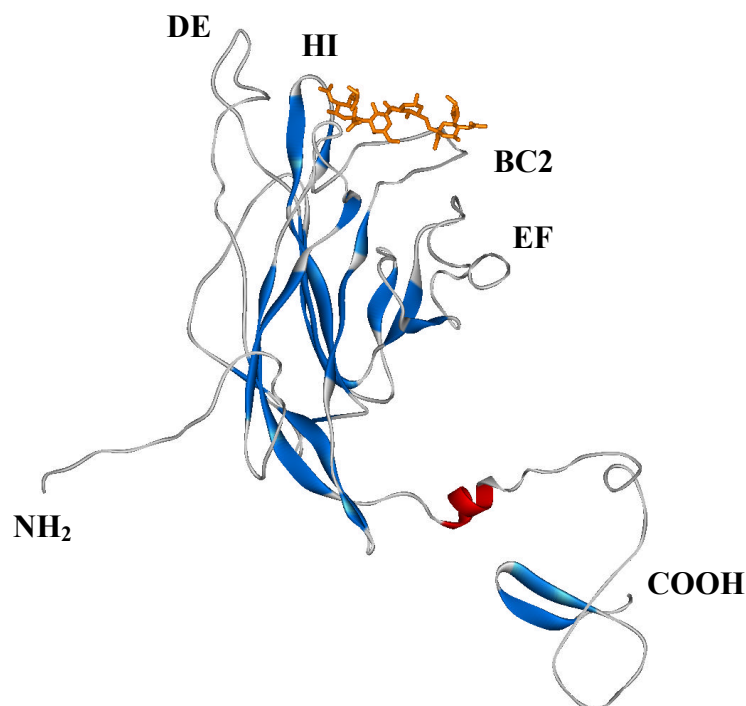


Abbildung 1: Struktur eines VP1 Monomers zusammen mit dem Tetrasaccharid NeuNAc-(α 2,3)-Gal-(β 1,3)-GlcNAc-(α 6,2)-NeuNAc. Die Loops DE, HI, BC2 und EF sind beschriftet. Das Oligosaccharid liegt zwischen HI- und BC2-Loop. Die Auflösung der Kristallstruktur beträgt 3.65 Å. PDB Code: 1SIE, verändert nach Stehle und Harrison (1996).

2.5.4 Aufbau der Viruspartikel

Die Struktur des SV40 wurde 1991 (Liddington et al., 1991), die des murinen Polyomavirus 1994 (Stehle et al., 1994) aufgeklärt. Die Polyomaviren besitzen eine T=7 ikosaedrische Geometrie und die Kapside der Polyomaviren haben einen Durchmesser von ca. 50 nm. Das Kapsid wird von 72 Pentameren gebildet, die aus VP1 bestehen. Dieser Aufbau führt zu einer hexa- und pentavalenten Anordnung der Pentamere im Kapsid (60 x hexa- und 12 x pentavalent). Das bedeutet, daß VP1 neben den Wechselwirkungen innerhalb des Pentamers auch Wechselwirkungen mit VP1-Molekülen der benachbarten Pentamere eingeht. Die Strukturproteine VP2 und VP3 befinden sich im Inneren des Kapsids.

2.5.5 Replikation der Polyomaviren

Das Wirtsspektrum der Polyomaviren ist sehr umfangreich. Die Bindung an eukaryontische Zellen erfolgt über die Wechselwirkung mit Zuckerresten auf deren Oberfläche. Diese Interaktion an der Zelloberfläche scheint aber nicht in einer Rezeptor-vermittelten Endozytose zu münden. Durch die Bindung an Caveolin wird die Internalisierung in Caveolae ausgelöst (Richterova et al., 2001). Der Weg der kompletten Partikel führt dann zum Kern der Wirtszelle (Richterova et al., 2001; Mackay und Consigli, 1976; An et al., 2000a; An et al., 2000b). Für einen produktiven Infektionszyklus muß die infizierte Zelle in der S-Phase sein, damit das Polyomavirus die notwendigen zellulären Faktoren nutzen kann (Modrow und Falke, 1997). Nachdem in der Wirtszelle das virale Genom repliziert wurde, werden auch die späten Gene exprimiert (Modrow und Falke, 1997). Da diese Genprodukte eine Kernlokalisationssequenz besitzen, werden die Strukturproteine in den Kern transportiert (Moreland und Garcea, 1991; Chang et al., 1992), wo das virale Genom, welches in einer Nukleosomenstruktur mit den Wirtshistonen vorliegt, verpackt wird. Die Assemblierung zu viralen Partikeln ist dabei von der Phosphorylierung des VP1 Proteins abhängig (Garcea et al., 1985; Li und Garcea, 1994; Garcea und Benjamin, 1983). Die gebildeten Viren werden durch Zellyse freigesetzt (Modrow und Falke, 1997).

3 Grundlagen und Ziele der Arbeit

3.1 Grundlagen

Der Einsatz viraler Systeme zum Gentransfer erweist sich trotz einer inzwischen vieljährigen Entwicklung als äußerst problematisch. Hauptsächlich die Sicherheitsaspekte sind beim Einsatz vollständiger Viren nicht überschaubar. Hingegen bietet der Einsatz der synthetischen Transfektionsmittel zwar einen höheren Sicherheitsstandard, allerdings ist die Transfektionseffizienz nicht mit biologischen Systemen vergleichbar, bzw. der Einsatz hoher Konzentrationen ist oft toxisch.

Eine Alternative dazu sind von Viren abgeleitete Transfektionssysteme, die nur *in vitro* rekonstituierte virale Partikel darstellen. Diese besitzen keinerlei virale genetische Information mehr.

Die Rekonstitution von viralen Partikeln *in vitro* wurde mit verschiedenen viralen Hüllproteinen gezeigt:

- AAV rep- und Kapsid-Proteine (Zhou und Muzyczka, 1998)
- Rous Sarcoma gag-Proteine (Campbell und Vogt, 1997)
- HIV p6 Domäne (Campbell und Vogt, 1995)
- HIV gag-Protein (Morikawa et al., 1999)
- Papillomavirus L1 Protein (Zhang et al., 1998)
- Bovine Papillomavirus L1, L2 Protein (Zhou et al., 1993)
- Murines Polyomavirus Protein VP1 (Salunke et al., 1986)

Diese virusanalogen Partikel können prinzipiell für den Gentransfer genutzt werden, wenn rekombinante DNS in die Partikel eingeschlossen wird. Die so entstehenden Partikel bestehen aus leicht zu reinigenden Einzelkomponenten, die in großem Maßstab hergestellt werden können. Für das murine Polyomavirus wurde gezeigt, daß leere, virale Partikel, die aus Mauszellen gewonnen wurden, DNS aufnehmen können (Slilaty et al., 1982). Ein 1.6 kp großes Fragment des Polyomagenoms konnte in diese Partikel verpackt werden. Dieser Vektor konnte genutzt werden, Ratten F111 Zellen zu transformieren (Slilaty und Aposhian, 1983). Auch die Herstellung virusanaloger Partikel, die nur aus dem Polyoma-Hüllprotein VP1 bestehen, ist möglich (Salunke et al., 1986). Die so hergestellten Partikel können heterologe DNS binden und Zellen funktionell transfizieren (Forstova et al., 1995). Neben der Verpackung heterologer DNS, die zur Expression von Reportergenen genutzt wird, wurde auch die Verpackung kurzer, funktioneller Oligonukleotide oder die Verpackung von Proteinen beschrieben (Braun et al., 1999; Schmidt et al., 2001a). Um die Ausbeute und Reinheit von VP1 zu erhöhen, wurden Strategien zur rekombinanten Herstellung entwickelt

(Leavitt et al., 1985). Das gereinigte VP1-Pentamer ist in der Lage, unter verschiedenen Bedingungen zu virusanalogen Partikeln (VLP) zu assemblieren. Die gewählten Bedingungen haben dabei einen deutlichen Einfluß auf die Morphologie der Kapside (Salunke et al., 1986; Salunke et al., 1989). Bei den bisherigen Versuchen zur Transfektion in Zellkultur und im Tiermodell wurden VLPs von wtVP1 benutzt (Krauzewicz et al., 2000a; Krauzewicz et al., 2000b; Heidari et al., 2000). Dabei machte man sich die zwar spezifische, aber Zelltyp-unabhängige Bindung der VLPs an Sialinsäuren zu Nutze (Stehle und Harrison, 1996; Stehle und Harrison, 1997). Im Tiermodell wurden deshalb auch sehr unterschiedliche Gewebe transfiziert. Die dabei erzielte Expressionsdauer war ebenfalls, je nach Gewebeart, unterschiedlich (Heidari et al., 2000).

Allerdings ist auch hier, wie bei den viralen und nicht-viralen Systemen, die nicht vorhandene Selektivität des Transfektionssystems eine große Einschränkung.

3.2 Ziele der Arbeit

Die Entwicklung der verschiedenen Vektorsysteme, ob viral oder synthetisch, hat gezeigt, daß der zielgerichteten Adressierung des Vektorsystems eine große Bedeutung zukommt. Die Zelltyp-spezifische Adressierung hilft, Nebenwirkungen bei der Gentherapie zu verringern und den Therapieerfolg zu steigern (Schaffer und Lauffenburger, 2000).

Bei den synthetischen Systemen ermöglicht die Konjugation von Zelltyp-spezifischen Liganden die Adressierung des Transfersystems. Beispiele sind die Konjugation von RGD-Peptiden (Harbottle et al., 1998; Erbacher et al., 1999; Colin et al., 1998; Schneider et al., 1999), Wachstumsfaktoren wie EGF und TGF- α (Chen et al., 1998b; Fominaya et al., 1998; Schaffer und Lauffenburger, 1998; Xu et al., 1998; Schaffer et al., 2000) oder VEGF (Li et al., 1999). Mit einer anderen Ligandengruppe, den Oligosacchariden, konnte die erste Zelltyp-spezifische Adressierung mittels gekoppelter Galaktose an Zellen mit dem Asialoglykoprotein Rezeptor gezeigt werden (Wu und Wu, 1987). Adressierung konnte auch durch die Vernetzung mit Mannose erreicht werden (Ferkol et al., 1996). Generell muß allerdings neben der Zelltyp-spezifischen Aufnahme auch die unspezifische Aufnahme unterbunden werden, da sonst immer als Nebenreaktion eine Zelltyp-unspezifische Transfektion zu beobachten ist. Eine sehr vielversprechende Möglichkeit zur Adressierung ist die Nutzung Zelltyp-spezifischer Antikörper (Chen et al., 1998b; Chapel et al., 1999; Uhrek et al., 1998; Mohr et al., 1999). Inzwischen können über verschiedene Techniken Antikörper für verschiedene Antigene entwickelt oder bestehende Antikörper verbessert werden (Stemmer, 1994; Boder und Wittrup, 1997).

Neben der Frage der Adressierung muß auch die Frage nach der Effizienz der verwendeten Systeme gestellt werden. Inzwischen sind einige der limitierenden Schritte des Gentransfers aufgeklärt:

- Überwindung des Blutstroms (Barron et al., 1998; Zelphati et al., 1998; Ogris et al., 1999)
- Bindung an die Zielzelle (Schaffer und Lauffenburger, 1998),
- Freisetzung aus den Endosomen (Wagner et al., 1992; Boussif et al., 1995),
- Transport zum Kern (Subramanian et al., 1999),
- Dissemblierung des Vektors im Kern (Schaffer et al., 2000).

Auch das sind Parameter, die für jedes System bestimmt und ggf. optimiert werden müssen.

Bei den viralen Systemen wurden ähnliche Wege beschritten. Allerdings können bei den viralen Systemen Modifikationen auf genetischer Basis eingeführt werden, die z.B. Fusionen der Hüllproteine mit zellulären Liganden beinhalten. Solche Modifikationen wurden für die Insertion hochaffiner Liganden in Retroviren, Adenoviren, Herpes-Simplex-Virus und Adeno-assoziierte Viren (Valsesia-Wittmann et al., 1996; Ager et al., 1996; Watkins et al., 1997; Laquerre et al., 1998; Yang et al., 1998; Bartlett et al., 1999; Fielding et al., 1998) oder die sterische Blockade der natürlichen Bindungsstelle durch den neuen Liganden beschrieben (Miller et al., 1998; Snitkovsky und Young, 1998). Eine sehr elegante Methode ist die Insertion einer Domäne, die die natürliche Bindungsstelle verdeckt, aber durch eine Protease spaltbar ist. Nach dem Proteaseverdau wird die Bindungsstelle wieder zugänglich. Wird die entsprechende Protease nur lokal gebildet, erreicht man damit eine ortsspezifische Infektiosität der Partikel (Peng et al., 1999; Buchholz et al., 1998). In eine ähnliche Richtung gehen auch die Ansätze der translationellen Adressierung, bzw. der replikationskompetenten Viren. Bei beiden Ansätzen ergibt sich die Spezifität des Vektors durch das intrazelluläre Milieu nach der Infektion (Peng und Russell, 1999).

Eine Alternative zur direkten Insertion von Liganden oder Zelltyp-spezifischen Antikörperfragmenten in die virale Oberfläche bietet die Insertion eines IgG-bindenden Proteins. Damit können verschiedene Antikörper auf der Oberfläche des Systems gebunden werden, die eine Zelltyp-Spezifität vermitteln (Ohno et al., 1997; Ohno und Meruelo, 1997; Morizono et al., 2001).

All diese Ansätze lassen sich zu verschiedenen Strategien der Adressierung zusammenfassen:

- Erweiterung des Wirtsbereiches: Der natürliche Wirtsbereich schließt die Bindung an humane Zellen nicht ein und wird dahingehend erweitert (Jiang et al., 1998).

- Einschränkung des Wirtsbereiches: Gerade für die klinische Anwendung ist es enorm wichtig, daß bei einer Therapie möglichst nur Zielzellen therapiert werden, um keine Nebenwirkungen zu erhalten.

Um sowohl die optimierten, natürlichen Transfektionseigenschaften viraler Proteine zu nutzen, als auch den Sicherheitsaspekt zu berücksichtigen, wurde das virale Hüllprotein VP1 des murinen Polyomavirus ausgewählt.

Basierend auf der Kristallstruktur von wtVP1 soll durch rationales Design eine Loop-Struktur identifiziert werden, die es erlaubt, Peptide oder Proteine in die VP1-Sequenz zu inserieren und so funktionell auf den virusanalogen Partikeln zu präsentieren. Die Insertion liefert die Grundlage, um ein Zelltyp-spezifisches Transfektionssystem zu entwickeln. Die inserierten Sequenzen ermöglichen es dann, verschiedene Module zur Zelltyp-spezifischen Adressierung zu koppeln. Sowohl die Kopplung über polyionische Wechselwirkungen, als auch die Kopplung über Protein-Protein Wechselwirkung sollen untersucht und eingesetzt werden. Ein wichtiger Punkt stellt dabei das modulare Prinzip dar. Das einmal charakterisierte Grundgerüst, hier eine bestimmte Variante von VP1, soll für alle weiteren Anwendungen genutzt werden können, ohne daß für jede neue Fragestellung ein neuer Zelltyp-spezifischer Vektor entwickelt und charakterisiert werden muß.

Die entwickelten Fusionsproteine sollen biophysikalisch charakterisiert und ihr Einsatz als Zelltyp-spezifischer Vektor in Zellkultur untersucht werden. Außerdem sollen die so entwickelten Transduktionssysteme Einblick in zellbiologische Fragestellungen wie Aufnahmeweg und intrazelluläres Schicksal der Partikel geben, um mögliche limitierende Schritte zu identifizieren.

4 Ergebnisse

4.1 Untersuchungen zum Fusionsprotein VP1-DHFR

(Gleiter et al., 1999)

Das Hüllprotein VP1 des murinen Polyomavirus kann *in vitro* assembliert und für die Transduktion in Zellkultur und im Tiermodell eingesetzt werden. Die Bindung des Partikels erfolgt dabei an Sialinsäuremoleküle auf der Zelloberfläche. Diese Bindung ist zwar spezifisch, aber durch die weite Verbreitung der Sialinsäuren im Organismus zeigt sich keine ausgeprägte Zelltyp-Spezifität. Für den Einsatz als Vektor in der Gen- oder Krebstherapie muß also der Tropismus dieses Partikels verändert werden. Eine Möglichkeit zur Veränderung liefert die Fusion mit Proteinen oder Peptiden, die eine neue Spezifität vermitteln (Konishi et al., 1998; Martin et al., 1998; Bothmann und Pluckthun, 1998; Clackson et al., 1991). Dabei muß allerdings berücksichtigt werden, daß die ursprüngliche Bindungsaffinität komplett unterdrückt wird.

Um zu zeigen, daß auch VP1 dahingehend modifiziert werden kann, soll ein Modellprotein ausgewählt und in den HI-Loop inseriert werden. Die Insertion in diesen Oberflächenloop ist auf mehreren Überlegungen begründet: (i) Der C- und N-Terminus von VP1 stehen für eine Fusion nicht zur Verfügung, da sie an Protein-Protein, bzw. Protein-DNS Wechselwirkungen beteiligt sind. Außerdem sind sie im Kapsid nach innen gerichtet. Die Fusion mit den Termini würde also nicht zur Veränderung der Oberfläche von VP1 Kapsiden führen. (ii) An der β -Glucanase konnte gezeigt werden, daß Änderungen in den Oberflächenloops keinen Einfluß auf die Gesamtstruktur haben (Ay et al., 1998a). Da die Glucanase ebenfalls eine *jelly-roll* Topologie besitzt, können diese Ergebnisse mit hoher Wahrscheinlichkeit auf VP1 übertragen werden. Zirkuläre Permutationen, auch bei anderen Proteinen, haben weiterhin gezeigt, daß Veränderungen an C- und N-Terminus nicht unbedingt großen Einfluß auf die Tertiärstruktur haben (Buchwalder et al., 1992; Ay et al., 1998b; Hennecke et al., 1999). Dies ist vor allem für das zu inserierende Protein wichtig, das über Aminosäurelinkersequenzen im HI-Loop fixiert wird. Das Modellprotein muß mehrere Eigenschaften in sich vereinen:

- biochemisch gut charakterisiert
- die Kristallstruktur sollte aufgeklärt sein
- N- und C-Terminus sollten zugänglich sein
- die Funktionalität des Proteins sollte leicht meßbar sein
- es sollte löslich in *E.coli* herzustellen sein

- die Größe des Proteins sollte im Bereich anderer therapeutisch relevanter Proteine liegen, z.B. Fv-Fragmente.

Ein Protein, welches diese Voraussetzung erfüllt, ist die Dihydrofolat-Reduktase (DHFR) aus *E.coli*. Die DHFR ist ein Enzym aus der Klasse der Oxidoreduktasen. Untersuchungen durch zirkuläre Permutation haben außerdem gezeigt, daß voll aktive Varianten erzeugt werden können, wenn N- und C-Terminus ständig miteinander verknüpft sind (Buchwalder et al., 1992).

Die Konstruktion und biochemische Charakterisierung des Fusionsproteins aus VP1 und DHFR sollte es ermöglichen, Sequenz- bzw. Strukturbereiche in VP1 zu identifizieren, deren Modifikation die Präsentation eines funktionellen Proteins auf der Oberfläche der VLPs erlauben.

Das Fusionsprotein VP1-DHFR ließ sich löslich in *E.coli* herstellen und bis zur Homogenität reinigen. Die enzymatische Aktivität der DHFR war auch im Fusionsprotein vorhanden, was auf eine korrekte Tertiärstruktur schließen ließ. Dennoch waren die enzymatischen Parameter im Vergleich zur wtDHFR verändert. Die K_M -Werte für Dihydrofolat und NADPH waren um Faktor 50 bzw. 8 erhöht. Diese Erhöhung der K_M -Werte kann strukturell begründet sein, da die DHFR für einen katalytischen Zyklus eine große Flexibilität einzelner Abschnitte des Proteins benötigt (Sawaya und Kraut, 1997). Durch die Fixierung der Termini der DHFR in der Insertionsmutante kann es zu einer Einschränkung dieser Flexibilität kommen. Unter Umständen kann auch die räumliche Nähe der 5 DHFR Moleküle im pentameren Fusionsprotein zu einem eingeschränkten Zugang von Substrat und Kofaktor zum aktiven Zentrum führen. Daß das Enzym im Fusionsprotein veränderte Eigenschaften aufweist, kann auch an der thermischen Stabilität nachgewiesen werden. Ohne Zugabe von Kofaktoren denaturierte die DHFR innerhalb der Fusion schon unterhalb von 10°C. Die Stabilität konnte aber durch die Zugabe von NADPH auf ca. 30°C erhöht werden. Trotz dieser Unterschiede von isolierter DHFR und VP1-DHFR hinsichtlich Stabilität und Enzymaktivität zeigen diese Ergebnisse eindeutig, daß ein Protein funktionell in den HI-Loop von VP1 inseriert werden kann.

Wichtiger als die Eigenschaften der DHFR im Fusionsprotein sind allerdings die Eigenschaften von VP1, die das Grundgerüst für die VLPs darstellen. Die Stabilität von VP1 im Fusionsprotein scheint durch die Insertion nicht beeinflusst zu sein. So ist die Stabilität von wtVP1 und VP1 in der Fusion identisch. In beiden Fällen weist der Temperaturübergang einen Mittelpunkt der Denaturierung von 50°C auf. Die einzelnen Partner im Fusionsprotein verhalten sich also unabhängig voneinander. Die pentamere Grundstruktur von VP1 innerhalb

des Fusionsproteins konnte mittels analytischer Ultrazentrifugation und Gelfiltration nachgewiesen werden. Das Pentamer wies dabei einen s -Wert von $s_{w,20} = 7.8$ S auf. Das Molekulargewicht wurde im Gleichgewichtslauf mit 288 kDa bestimmt, was mit dem theoretischen Wert von 308 kDa gut übereinstimmt. Neben der Fähigkeit, die Grundstruktur der Kapsomere auszubilden, ist auch entscheidend, ob diese Kapsomere zu Kapsiden assemblieren können. Die Assemblierung zu Kapsiden ist von der Zugabe von Ca^{2+} -Ionen und der Ausbildung von Disulfidbrücken abhängig. Durch Dialyse gegen einen entsprechenden Assemblierungspuffer kann die Bildung der Kapside induziert werden. Wird das wtVP1 Protein eingesetzt, können sich je nach Assemblierungsbedingung Kapside unterschiedlicher Form ausbilden (Salunke et al., 1989). Innerhalb eines Ansatzes sind die gebildeten Kapside jedoch relativ homogen. VP1-DHFR hingegen assemblierte sehr inhomogen. Eine elektronenmikroskopische Analyse zeigte, daß die betrachteten Kapside Durchmesser zwischen 20 und 50nm aufwiesen. Diese Heterogenität konnte mittels analytischer Ultrazentrifugation bestätigt werden. Darüberhinaus zeigten die Daten, daß unter diesen Bedingungen etwa 20 Prozent der Kapsomere nicht assemblierten.

Wahrscheinlich führt die Präsentation der 18 kDa großen DHFR auf VP1 bei der Assemblierung zu einer hohen Packungsdichte der Proteine, die die Assemblierung beeinträchtigt. Die beobachteten, kleineren Partikel weisen eine größere Oberflächenkrümmung auf, so daß sterische Effekte auf der Oberfläche der Partikel minimiert werden.

Um VP1 als Vektor einsetzen zu können, müssen auch die natürlichen Zellbindungseigenschaften verändert werden. Die Bindung von VP1 an Sialinsäuren kann durch die Haemagglutination von Erythrozyten nachgewiesen werden. Die Insertion der DHFR in den HI-Loop führt zum kompletten Verlust dieser Eigenschaft. Die Fusion hat entweder Auswirkung auf die Struktur der Bindungstasche und verändert somit das Bindungsverhalten, oder die DHFR blockiert sterisch die Bindungstasche auf der Oberfläche, so daß die Bindung der Zuckermoleküle sterisch nicht mehr möglich ist.

Die Insertion in den HI-Loop bietet somit die Möglichkeit, funktionelle Domänen in die VP1-Struktur zu inserieren. Dabei bleiben die biophysikalischen Eigenschaften von VP1 weitgehend erhalten, jedoch wird die Zelltyp-unabhängige Bindung unterbunden.

4.2 Untersuchungen zur funktionellen Modifikation von VP1 durch Insertion einer Proteindomäne

(Gleiter und Lilie, 2001)

Die Untersuchungen zur Insertion des Modellproteins DHFR in den HI-Loop von VP1 lassen den Schluß zu, daß die Insertion einzelner strukturierter Proteindomänen in VP1 möglich ist. Ähnliches wurde auch für den DE-Loop gezeigt (Schmidt et al., 2001b).

Für eine Zelltyp-spezifische Adressierung von VP1 Kapsiden müssen zwei Dinge erreicht werden: (i) die Zelltyp-unabhängige Bindung von VP1 an Sialinsäurereste muß unterbunden werden. (ii) Es muß ein Modul auf der Oberfläche der VLPs gekoppelt werden, das eine Zelltyp-spezifische Bindung vermittelt. Eine ideale Proteinklasse zur Nutzung als Zelltyp-spezifisches Modul stellt die Familie der Immunoglobuline dar. Für Antikörper konnte vielfach gezeigt werden, daß sie hochspezifisch mit niedrigen K_D -Werten an Antigene binden. Die Herstellung großer Antikörperbibliotheken und die biotechnologische Produktion sind heute Stand der Technik. Die Entwicklung tumorspezifischer Antikörper stellt derzeit einen Schwerpunkt in der medizinischen Forschung dar.

Da Antikörper bei der rekombinanten Herstellung im Zytoplasma von *E.coli*, dem für VP1 optimierten Expressionssystem, nicht löslich exprimiert werden können, mußte eine andere Herstellungsstrategie entwickelt werden. Die direkte Insertion eines Antikörpers oder eines Fv-Fragmentes in den HI-Loop von VP1 wäre zwar prinzipiell möglich, die direkte Modifikation hätte aber zur Folge, daß für jede neue Fragestellung eine neue Variante kloniert, exprimiert, gereinigt und charakterisiert werden muß. Ein modulares System würde die Anwendung eines solchen Vektorsystems sehr viel einfacher gestalten. Daher sollte kein Antikörpermolekül in VP1 inseriert werden, sondern ein Adaptermolekül, um je nach Zielzelle Antikörper auf der Oberfläche verankern zu können.

Dieses Adaptermolekül muß in der Lage sein, Antikörper spezifisch und mit hoher Affinität zu binden. Ein natürlich vorkommendes Antikörper-bindendes Protein ist Protein A aus *Staphylococcus aureus*. Aus diesem Multidomänen-Protein wurde die Antikörper-bindende Domäne B, das Protein Z, isoliert (Moks et al., 1986), löslich produziert und die Struktur bestimmt. Protein Z, mit einem Molekulargewicht von 6,8 kDa, besitzt 3 α -Helices als einziges Strukturmerkmal (Deisenhofer, 1981). Die Bindung von Antikörpern führt nicht zu einer Strukturänderung (Jendeberg et al., 1996) und weist einen K_D -Wert von etwa 15 nM auf (Starovasnik et al., 1997). Protein Z wurde bereits in anderen Projekten als Fusionspartner genutzt, so z.B. zur Proteinreinigung (Larsson et al., 1996) oder zur Insertion in das Sindbis-

Virus- oder Retrovirus-Hüllprotein (Ohno et al., 1997; Chu et al., 1994). In all diesen Fällen lag das Protein funktionell vor. Außerdem konnte gezeigt werden, daß Protein Z manchmal die Löslichkeit und Stabilität des Fusionsproteins erhöhen kann (Samuelsson et al., 1994; Samuelsson und Uhlen, 1996).

Wie bei dem Fusionsprotein VP1-DHFR wurde Protein Z mittels N- und C-terminaler Serin-Glyzin-Linker in den HI-Loop von VP1 inseriert. Nach der Expression und Reinigung wurde das Fusionsprotein VP1-Z biophysikalisch und funktionell charakterisiert.

Die Funktionalität von VP1-Z hängt von der Integrität der Struktur ab. Diese wurde durch CD-Spektroskopie nachgewiesen. So konnte demonstriert werden, daß der Sekundärstrukturgehalt beider Proteine im Fusionsprotein vollständig erhalten bleibt. Die pentamere Grundstruktur von VP1-Z konnte durch analytische Ultrazentrifugation nachgewiesen werden. Die Assemblierung unter geeigneten Bedingungen führte zur Bildung von Kapsiden. Die elektronenmikroskopische Analyse zeigte, daß bei diesem Fusionsprotein eine sehr homogene Spezies an Kapsiden entsteht, die mit einem Durchmesser von ca. 45 nm den wtVP1 Kapsiden sehr ähnlich ist. Die Funktionalität des Protein Z im Fusionsprotein wurde über die Bindung eines humanisierten Antikörpers nachgewiesen. Die Analyse der Bindungsstöchiometrie ergab, daß statistisch 0.8 Antikörpermoleküle pro VP1-Z-Monomer gebunden werden, was einer 1 zu 1 Stöchiometrie entspricht. Ebenso wie bei der Insertion der DHFR in den HI-Loop, führte die Insertion von Protein Z zum Verlust der Sialinsäurebindungsfähigkeit von VP1, die Grundvoraussetzung, um die Zelltyp-spezifische Adressierung zu entwickeln. Die thermische Stabilität des pentameren Fusionsproteins war mit 38°C etwas geringer, als die des wtVP1 Pentamers. Allerdings konnte bei der Analyse der Kapside zwischen VP1-Z und wtVP1 kein Unterschied detektiert werden. Bei beiden Proteinen zeigte der Übergang der Denaturierung einen Mittelpunkt von 52°C. Eine Stabilisierung durch die Bildung der Kapside kann mit dem Auftreten neuer Strukturmerkmale im Kapsid und der Disulfidverbrückung im Zusammenhang stehen (Schmidt et al., 2000).

Die biophysikalische Untersuchung von VP1-Z zeigt deutlich, daß VP1-Z in der Lage ist, Kapside auszubilden, die Antikörper auf der Oberfläche präsentieren können. Der Verlust der Sialinsäurebindung und die ausreichende Stabilität der Kapside ermöglichen es, das entwickelte System in der Zellkultur zu testen und weiterzuentwickeln.

Die Spezifität des neuentwickelten Systems konnte in Zellkulturexperimenten dargestellt werden. Die VP1-Z Kapside konnten mit fluoreszenzmarkierter DNS und Zelltyp-spezifischem Antikörper beladen werden. Der ternären Komplex wurde dann zusammen mit

Zellen inkubiert. Nur die Zellen, die positiv für das Antigen waren, zeigten eine deutliche Fluoreszenz. Negative Zelllinien zeigten keine Färbung. Durch LSM und Schnitte durch einzelne Zellen konnte außerdem nachgewiesen werden, daß die fluoreszenzmarkierte DNS nach der Bindung auch aufgenommen wird und in intrazellulären Vesikeln zu finden ist.

4.3 Zelltyp-spezifische Transduktion und Genexpression

(Gleiter und Lilie, 2002)

Die Charakterisierung der VP1-Z Kapside hatte gezeigt, daß sie mit Hilfe Zelltyp-spezifischer Antikörper spezifisch mit entsprechenden Zielzellen interagieren und von diesen aufgenommen werden können. Damit war die Voraussetzung geschaffen, dieses Vektorsystem für einen funktionellen Gentransfer in Zielzellen einzusetzen. Um ein System mit medizinischem Potential zu testen, wurde der Antikörper Herceptin ausgewählt. Herceptin erkennt den zellulären Oberflächenrezeptor ErbB2. Die Überexpression von ErbB2 ist ein Merkmal bei bestimmten Formen des Brustkrebs. Herceptin wird deshalb bei Brustkrebs als erfolgversprechendes Therapeutikum eingesetzt (Miles, 2001).

In einem ersten experimentellen Ansatz wurde der *in vitro* hergestellte ternäre Komplex, bestehend aus den VLPs, Herceptin und DNS, mit ErbB2-positiven Zellen inkubiert. Dieser Versuch führte allerdings nicht zu einer Transduktion der Zellen, unabhängig von der Art der ErbB2-positiven Zellen (MCF7 oder SK-BR3), als auch den verwendeten Reportergenen (GFP, Luciferase, β -Galaktosidase). Die Ursache hierfür ergibt sich aus dem Aufnahmemechanismus der Partikel in die Zellen. Während wtVP1 über Caveolae internalisiert wird, führt die Bindung von Herceptin an ErbB2 zur Rezeptor-vermittelten Endozytose der VLPs. Anschließend gelangen die VLPs über die späten Endosomen in die Lysosomen, wo sie abgebaut werden.

Eine Möglichkeit, das lysosomale System zu stören, ist der Einsatz des lysosomotropen Agens Chloroquin. Wurde Chloroquin während der Transduktion dem Medium zugesetzt, so konnte eine Genexpression von Markergenen sowohl mit GFP, als auch β -Galaktosidase nachgewiesen werden. Der die Transduktion limitierende Schritt ist also in einer fehlenden Freisetzung aus dem lysosomalen Kompartiment zu sehen. Der Einsatz von Chloroquin kann natürlich nur ein analytisches Hilfsmittel im Zellkulturexperiment sein. Zur Quantifizierung der Transduktionsrate unter diesen Bedingungen wurden GFP-exprimierende Zellen mittels FACS-Analyse ausgewertet. Damit wurde eine Transduktionseffizienz von 0,45 % aller Zellen bestimmt. Eine ähnliche Effizienz wurde auch für wtVP1 beschrieben (Krauzewicz et al., 2000a; Krauzewicz et al., 2000b). Obwohl die Transduktionseffizienzen vergleichbar sind, liegen sie noch weit unter einer Effizienz, die eine Anwendung sinnvoll erscheinen läßt.

Neben der Transduktionseffizienz ist aber die Zelltyp-Spezifität ein entscheidender Parameter.

So mußte nachgewiesen werden, ob VP1-Z noch zu einer Zelltyp-unspezifischen Transduktion befähigt ist. Die Transduktion von ErbB2-positiven Zelllinien erwies sich als strikt abhängig von der Anwesenheit des Antikörpers im Transduktionskomplex. Nur der Einsatz des ternären Komplexes führte zur Transduktion der Zellen. Alle anderen Kombinationen der drei Komponenten erwiesen sich bezüglich des funktionellen Gentransfers als inaktiv. Darüberhinaus konnte gezeigt werden, daß nur Zelllinien transduziert wurden, die ErbB2 überexprimierten.

Die Entwicklung eines Zelltyp-spezifischen Transduktionssystems konnte also in vollem Umfang dargestellt werden. Der noch zu klärende Punkt ist natürlich das intrazelluläre Schicksal der Kapside. Hier müssen Strategien entwickelt werden, die eine Freisetzung aus Endosomen ermöglichen, bevor ein Großteil der Partikel degradiert wird. Möglichkeiten sind z.B. der Einsatz fusogener Peptide wie das HA-2 Peptid oder Lysteriolysin O. Diese erfahren bei der Absenkung des pH-Wertes im lysosomalen Kompartiment eine Strukturänderung. Diese Form der Peptide ist in der Lage, sich in die Membran der Lysosomen einzulagern und dort Poren zu bilden (Garipey und Kawamura, 2001; Walton et al., 1999). Über die gebildeten Poren könnten die VLPs dann in das Cytoplasma gelangen. Dadurch könnte auf den Einsatz von Chloroquin verzichtet und die Transduktionseffizienz deutlich erhöht werden.

4.4 Entwicklung alternativer Kopplungsmethoden an VP1

(Stubenrauch et al., 2001)

Die Kopplung Zelltyp-spezifischer Antikörper an die Oberfläche von VP1-Z beruht auf einer nicht-kovalenten Bindung der Antikörper an Protein Z. Durch SPR-Messungen konnte zwar für diese Wechselwirkung eine hohe Affinität im Bereich einer nanomolaren Dissoziationskonstante ermittelt werden, dennoch ist die Dissoziation des Antikörpers von der Kapsidoberfläche möglich. Daraus resultiert natürlich die Gefahr, daß es bei der natürlichen Konzentration von 10 mg/ml Antikörper im Blut von Patienten zu einem Austausch von Antikörpern auf der Oberfläche kommt und die VLPs einen neuen Tropismus erhalten. Mittels einer kovalenten Kopplung des Adressierungsmoduls auf der Oberfläche kann dieses Problem umgangen werden.

Von Stubenrauch et al. wurde die VP1-Variante VP1-E₈C entwickelt (Stubenrauch et al., 2000). Die dabei in den HI-Loop inserierte Sequenz aus acht Glutamaten und einem Zystein wurden als *Reinigungstag* benutzt. Neben der Reinigung kann dieser *tag* auch zur Kopplung polyionischer Peptide oder Fusionsproteine mit polyionischer Sequenz genutzt werden.

Um ein solches System zu entwickeln, wurde das Fv-Fragment des Antikörpers B3 gewählt. Dieser Antikörper erkennt das Tumor-spezifische Kohlenhydratmotiv LewisY (LeY) als Antigen. Die V_H-Domäne des Fv-Fragmentes wurde um die Sequenz Arg₈CysPro verlängert. Durch die komplementär geladenen Aminosäuren ist eine polyionische Wechselwirkung zwischen VP1-E₈C und dem modifizierten Fv-Fragment möglich. Durch die Wahl eines geeigneten Redoxsystems kann dann eine Disulfidbrücke zwischen den beiden Proteinen ausgebildet werden. Damit ist eine kovalente Kopplung des Fv-Fragmentes an VP1-E₈C möglich.

Wie auch bei den Varianten VP1-DHFR und VP1-Z konnte durch den Haemagglutinationstest nachgewiesen werden, daß VP1-E₈C keine Bindungsaffinität mehr zu Sialinsäureresten besitzt. Damit war eine Grundvoraussetzung für eine Zelltyp-spezifische Adressierung gegeben.

Die Assoziation von VP1-E₈C und Fv-B3 konnte mittels Gelfiltration und SDS-PAGE eindeutig nachgewiesen werden. Die optimale Kopplungsrate betrug 30-40 Fv-Fragmente pro VLP. Bei höheren Beladungszahlen kam es zur Aggregation, ein Effekt, der nicht nur bei der Kopplung von Antikörperfragmenten auftrat, sondern auch bei der Bindung von isolierten Arg₁₀Cys-Peptiden. Mit der Bindung von DNS konnte somit auch für dieses Konstrukt ein

Vektorsystem erstellt werden, welches zur Zelltyp-spezifischen Adressierung und Transduktion befähigt sein sollte.

Die Anwendung dieses Transduktionssystems in der Zellkultur führte zur Transduktion von LeY-positiven Zellen (MCF 7 und A431). Dabei lag das Expressionslevel eines Markergens ungefähr 2.5-5fach höher als bei den Kontrolltransduktionen. Wie auch der funktionelle Gentransfer des VP1-Z/Herceptin-Systems von dem gebundenen Antikörper abhängig war, so war auch der funktionelle Gentransfer in die LeY-positiven Zellen abhängig von dem gekoppelten Fv-B3-Fragment. LeY-negative Zelllinien konnten nicht transduziert werden. Durch die Konkurrenz mit freiem Antigen ließ sich zeigen, daß die Transduktion hochspezifisch war. Wurde dem Medium Sialyllaktose, der natürliche Bindungspartner von wtVP1, zugegeben, konnte die Transduktion nicht inhibiert werden.

Die Transduktion von LeY-positiven Zellen mit dem Vektorsystem erwies sich zwar spezifisch, allerdings war die Transduktionseffizienz sehr gering. Daher war die Analyse der limitierenden Schritte der Transduktion mit diesem System Gegenstand weiterer Untersuchungen.

4.5 Untersuchungen zum spezifischen Gentransfer mit der Variante VP1-E₈C des Polyomahüllproteins VP1

(May et al., 2001)

Das Transduktionssystem, das aus VP1-E₈C und dem gekoppelten Fv-Fragment B3 besteht (VP1-B3), zeigte nicht die erwartete Effizienz, die man aufgrund der sehr effizienten Zelladressierung erwarten würde. Nutzt man fluoreszenzmarkiertes VP1-E₈C im Komplex mit dem Fv-Fragment B3, so war zu sehen, daß fast alle LeY-positiven Zellen eine deutliche Fluoreszenzfärbung zeigten. Neben der Oberflächenassoziation des Vektorsystems war dabei auch eine intrazelluläre Lokalisation zu beobachten. Wenn das Antikörperfragment nicht gekoppelt wurde, konnte keine Bindung des markierten VP1-E₈C beobachtet werden. Damit konnte die Bindung des Vektors an die Zelloberfläche und die Aufnahme in die Zellen als limitierender Schritt ausgeschlossen werden. Um den Mechanismus der Aufnahme der VLPs aufzuklären, wurden Kolokalisationsstudien von VP1-B3 und Transferrin durchgeführt. Transferrin ist ein Ligand, der nach Bindung an den Rezeptor mittels Clathrin-abhängiger Endozytose internalisiert wird. Durch fluoreszenzmikroskopische Aufnahmen konnte die Kolokalisation der fluoreszenzmarkierten Komponenten VP1-B3 und Transferrin gezeigt werden. Zudem konnte die Aufnahme von VP1-B3 durch Chlorpromazin, einen spezifischen Inhibitor der Endozytose, verhindert werden. Die Aufnahme der Partikel erfolgt demzufolge nach der Bindung an das Oberflächenantigen durch Endozytose. Wie bei dem Transduktionssystem VP1-Z/Herceptin, gibt es auch hier keinen Mechanismus, um VP1-B3 effektiv aus dem lysosomalen Kompartiment freizusetzen. Deshalb stellt der lysosomale Abbau der Komponenten auch bei diesem System einen limitierenden Faktor für die Transduktion dar.

Neben der Internalisierung ist die DNS-Bindung als limitierender Schritt des Gentransfers denkbar. Zwar besitzt VP1 bei neutralem pH-Wert eine leicht negative Nettoladung, es kann aber in diesem Zustand immer noch DNS binden und zur Transduktion eingesetzt werden. Durch die Insertion von 8 Glutamaten in den HI-Loop wird die negative Ladungsdichte auf der Kapsidoberfläche drastisch erhöht. Die Bindung von 30-40 Fv-Fragmenten über polyionische Wechselwirkungen ändert an der Nettoladung nicht viel. Da das Phosphatrückgrat der DNS auch negativ geladen ist, kann es zu einer elektrostatischen Abstoßung und verminderten Bindung kommen. Die DNS-Bindung wurde mittels analytischer Ultrazentrifugation und Gelshift-Assays untersucht. Erst bei einem pH-Wert von 5, wenn also die negative Ladung einiger Glutamatreste titriert wurde, konnten ca. 40% der

eingesetzten DNS an VP1-E₈C Kapside gebunden werden. Bei neutralem pH-Wert war mit diesen Analysenmethoden keine signifikante DNS-Bindung nachzuweisen. Diese veränderte DNS Bindungseigenschaft bei neutralem pH-Wert steht im Kontrast zu der sehr guten DNS Bindungseigenschaft des wt Proteins oder VP1-Z unter diesen Bedingungen.

Die DNS Bindung konnte somit als ein weiterer limitierender Faktor bei der Transduktion mit VP1-E₈C Kapsiden identifiziert werden. Unter Berücksichtigung der verbesserten DNS-Bindung bei pH 5 und dem Einsatz von Chloroquin in der Zellkultur, um eine partielle Freisetzung der Partikel aus den Lysosomen zu gewährleisten, konnte die Transduktion der LeY-positiven Zellen deutlich gesteigert werden. Eine Transduktionseffizienz von unter 1% zeigt aber deutlich, daß noch weitere Optimierungsschritte notwendig sind, bzw. daß noch andere limitierende Faktoren vorliegen.

Das vorgestellte Vektorsystem, bestehend aus VP1-E₈C und dem Antikörperfragment B3, kann zwar Zelltyp-spezifisch Zellen transduzieren, allerdings mit einer sehr geringen Effektivität. Um die Transduktionsrate zu steigern, muß die im Vergleich zum wtVP1 verminderte DNS-Bindung deutlich gesteigert werden. Möglichkeiten dazu bietet z.B. die gemischte Assemblierung mit anderen Varianten des VP1 Proteins, z.B. VP1-Z. Dadurch kann die Anzahl der auf der Oberfläche präsentierten negativen Ladungen verringert und die DNS-Bindung verbessert werden. Dies eröffnet auch die Möglichkeit, die gemischte Assemblierung mit VP1-Varianten durchzuführen, die eine spezifische DNS-Bindungssequenz am N-Terminus besitzen. Dadurch könnte DNS spezifisch gebunden und in die Kapside verpackt werden. Der Einsatz kürzerer, funktioneller DNS-Fragmente anstatt ganzer Plasmide sollte ebenfalls die Verpackung erleichtern, da diese Fragmente nach der Bindung an den N-Terminus während der Assemblierung im Inneren der Kapside Platz finden und verpackt werden können.

5 Abkürzungen

AAV	Adeno-assoziierte Viren
ADP	<i>Adenovirus death protein</i>
AIDS	<i>Aquired immunodeficiency syndrom</i>
Arg ₈ CysPro	Polyionisches Peptid mit der Sequenz Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Cys-Pro
bp	Basenpaare
BRCA1	<i>Breast cancer associated protein 1</i>
CAR	<i>Coxsackie Virus and Adenovirus Receptor</i>
CD34	<i>Cluster of differentiation</i>
CF	<i>Cystic fibrosis</i>
CFTR	<i>Cystic fibrosis transmembrane conductance regulator</i>
DHFR	Dihydrofolat-Reduktase
DMD	Duchenne Muskeldystrophy
DNS	Desoxyribonukleinsäure
dsDNS	Doppelsträngige Desoxyribonukleinsäure
EGF	<i>Epidermal growth factor</i>
EGFP	<i>Enhanced green fluorescent protein</i>
FACS	<i>Fluorescence assisted cell sorting</i>
Fv-B3	Antikörperbindendes Fragment des Antikörpers B3
Gal	Galaktosidase
GalNAc	N-acetyl-Galaktosidase
GAL4	Hefespezifischer Transkriptionsfaktor GAL4
GCSF	<i>Granulocyte colony-stimulating factor</i>
HIV	<i>Human Immunodeficiency Virus</i>
HIV TAT	<i>Human Immunodeficiency Virus Transactivator Protein</i>
HSV	Herpes-Simplex-Virus
IL-1	Interleukin 1
kb	Kilobasenpaare
kDa	Kilodalton
K _M	Michaelis-Menten Konstante
LeY	LewisY
LSM	<i>Laser scanning microscopy</i>
LTR	<i>Long terminal repeats</i>

MMLV	<i>Moloney Murine Leukemia Virus</i>
NeuNAc	Sialinsäurerest
NGF	<i>Nerve growth factor</i>
<i>ras</i>	<i>Rat Sarcoma</i>
RGD	Tripeptid mit der Sequenz Arg-Gly-Asp
RNS	Ribonukleinsäure
SCID	<i>Severe combined immunodeficiency</i>
SPR	<i>Surface plasmon resonance</i>
SV 40	<i>Simian Vaculating Virus 40</i>
TGF- α	<i>Transforming growth factor alpha</i>
TNF- α	<i>Tumor-Nekrosisfaktor α</i>
tPA	<i>Tissue plasminogen activator</i>
VLP	<i>Virus like particles</i>
VEGF	<i>Vascular endothelial growth factor</i>
VP1	Polyomavirushüllprotein VP1
VP1-B3	Assoziat aus VP1-E ₈ C und dem Fv-Fragment des Antikörpers B3
VP1-DHFR	VP1-Variante mit der inserierten DHFR im HI-Loop
VP1-E8C	VP1-Variante mit dem Insertionspeptid Glu ₈ Cys im HI-Loop von VP1
VP1-Z	VP1-Variante mit inseriertem Protein Z im HI-Loop
VP2	Polyomavirushüllprotein VP2
VP3	Polyomavirushüllprotein VP3
VP16	Virales Hüllprotein 16 des Herpes-Simplex-Virus
V _H	Schwere Kette der variablen Antikörperdomänen
wt	Wildtyp
wtDHFR	Wildtyp DHFR
ZNS	Zentrales Nervensystem

6 Literaturverzeichnis

- Ager,S., Nilson,B.H., Morling,F.J., Peng,K.W., Cosset,F.L., and Russell,S.J. (1996). Retroviral display of antibody fragments; interdomain spacing strongly influences vector infectivity. *Hum. Gene Ther.* 7 , 2157-2164.
- Ahrens,E.R., Gossain,V.V., and Rovner,D.R. (1986). Human insulin. Its development and clinical use. *Postgrad. Med* 80, 181-4, 187.
- Aihara,H. and Miyazaki,J. (1998). Gene transfer into muscle by electroporation in vivo. *Nat. Biotechnol* 16, 867-870.
- An,K., Lovgren,T.R., Tilley,M.B., and Consigli,R.A. (2000a). Use of the confocal microscope to determine polyomavirus recombinant capsid-like particle entry into mouse 3T6 cells. *J Virol. Methods* 84, 153-159.
- An,K., Paulsen,A.Q., Tilley,M.B., and Consigli,R.A. (2000b). Use of electron microscopic and immunogold labeling techniques to determine polyomavirus recombinant VP1 capsid-like particles entry into mouse 3T6 cell nucleus. *J Virol. Methods* 90, 91-97.
- Anderson,W.F. (1998). Human gene therapy. *Nature* 392, 25-30.
- Ay,J., Gotz,F., Borriss,R., and Heinemann,U. (1998a). Structure and function of the Bacillus hybrid enzyme GluXyn-1: native-like jellyroll fold preserved after insertion of autonomous globular domain. *Proc Natl Acad Sci U S A* 95, 6613-6618.
- Ay,J., Hahn,M., Decanniere,K., Piotukh,K., Borriss,R., and Heinemann,U. (1998b). Crystal structures and properties of de novo circularly permuted 1,3-1,4-beta-glucanases. *Proteins* 30, 155-167.
- Bahnson,A.B., Nimgaonkar,M., Fei,Y., Boggs,S.S., Robbins,P.D., Ohashi,T., Dunigan,J., Li,J., Ball,E.D., and Barranger,J.A. (1994). Transduction of CD34+ enriched cord blood and Gaucher bone marrow cells by a retroviral vector carrying the glucocerebrosidase gene. *Gene Ther.* 1, 176-184.
- Banga,A.K. and Prausnitz,M.R. (1998). Assessing the potential of skin electroporation for the delivery of pro. *Trends Biotechnol* 16, 408-412.
- Barouch,D.H. and Harrison,S.C. (1994). Interactions among the major and minor coat proteins of polyomavirus. *J Virol.* 68, 3982-3989.
- Barrington,R.E., Subler,M.A., Rands,E., Omer,C.A., Miller,P.J., Hundley,J.E., Koester,S.K., Troyer,D.A., Bearss,D.J., Conner,M.W., Gibbs,J.B., Hamilton,K., Koblan,K.S., Mosser,S.D., O'Neill,T.J., Schaber,M.D., Senderak,E.T., Windle,J.J., Oliff,A., and Kohl,N.E. (1998). A farnesyltransferase inhibitor induces tumor regression in transgenic mice harboring multiple oncogenic mutations by mediating alterations in both cell cycle control and apoptosis. *Mol. Cell Biol* 18, 85-92.
- Barron,L.G., Meyer,K.B., and Szoka,F.C., Jr. (1998). Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid-DNA complexes. *Hum. Gene Ther.* 9, 315-323.

- Bartlett, J.S., Kleinschmidt, J., Boucher, R.C., and Samulski, R.J. (1999). Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'gamma)2 antibody. *Nat. Biotechnol* 17, 181-186.
- Bates, R.D. and Nahata, M.C. (1995). Aerosolized dornase alpha (rhDNase) in cystic fibrosis. *J Clin Pharm. Ther.* 20, 313-315.
- Begley, C.G. (1993). Haemopoietic growth factors--from discovery to clinical application. *Med J Malaysia* 48, 3-8.
- Benihoud, K., Yeh, P., and Perricaudet, M. (1999). Adenovirus vectors for gene delivery. *Curr. Opin. Biotechnol* 10, 440-447.
- Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L., and Finberg, R.W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 275, 1320-1323.
- Berger, H. and Wintersberger, E. (1986). Polyomavirus small T antigen enhances replication of viral genomes in 3T6 mouse fibroblasts. *J Virol.* 60, 768-770.
- Blaese, R.M., Culver, K.W., Miller, A.D., Carter, C.S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., and Tolstoshev, P. (1995). T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* 270, 475-480.
- Boder, E.T. and Wittrup, K.D. (1997). Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol* 15, 553-557.
- Bolen, J.B., Anders, D.G., Trempey, J., and Consigli, R.A. (1981). Differences in the subpopulations of the structural proteins of polyoma virions and capsids: biological functions of the multiple VP1 species. *J Virol.* 37, 80-91.
- Bothmann, H. and Pluckthun, A. (1998). Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nat. Biotechnol.* 16, 376-380.
- Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., and Behr, J.P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* 92, 7297-7301.
- Bouvet, M., Ellis, L.M., Nishizaki, M., Fujiwara, T., Liu, W., Bucana, C.D., Fang, B., Lee, J.J., and Roth, J.A. (1998). Adenovirus-mediated wild-type p53 gene transfer down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer. *Cancer Res.* 58, 2288-2292.
- Braun, H., Boller, K., Lower, J., Bertling, W.M., and Zimmer, A. (1999). Oligonucleotide and plasmid DNA packaging into polyoma VP1 virus-like particles expressed in *Escherichia coli*. *Biotechnol Appl. Biochem* 29 (Pt 1), 31-43.
- Bridgewater, J.A., Springer, C.J., Knox, R.J., Minton, N.P., Michael, N.P., and Collins, M.K. (1995). Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. *Eur J Cancer* 31A, 2362-2370.

- Brinkmann,U., Pai,L.H., Fitzgerald,D.J., Willingham,M., and Pastan,I. (1991). B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. *Proc Natl Acad Sci U S A* 88, 8616-8620.
- Buchholz,C.J., Peng,K.W., Morling,F.J., Zhang,J., Cosset,F.L., and Russell,S.J. (1998). In vivo selection of protease cleavage sites from retrovirus display libraries. *Nat. Biotechnol* 16, 951-954.
- Buchwalder,A., Szadkowski,H., and Kirschner,K. (1992). A fully active variant of dihydrofolate reductase with a circularly permuted sequence. *Biochemistry* 31, 1621-1630.
- Burdach,S. (1992). Molecular regulation of hematopoietic cytokines: implications and indications for clinical use in pediatric oncology. *Med Pediatr. Oncol. Suppl* 2, 10-17.
- Cahan,L.D., Singh,R., and Paulson,J.C. (1983). Sialyloligosaccharide receptors of binding variants of polyoma virus. *Virology* 130, 281-289.
- Campbell,S. and Vogt,V.M. (1995). Self-assembly in vitro of purified CA-NC proteins from Rous sarcoma virus and human immunodeficiency virus type 1. *J Virol.* 69, 6487-6497.
- Campbell,S. and Vogt,V.M. (1997). In vitro assembly of virus-like particles with Rous sarcoma virus Gag deletion mutants: identification of the p10 domain as a morphological determinant in the formation of spherical particles. *J Virol.* 71, 4425-4435.
- Carter,B.J. and Flotte,T.R. (1996). Development of adeno-associated virus vectors for gene therapy of cystic fibrosis. *Curr. Top. Microbiol. Immunol.* 218, 119-144.
- Cayla,X., Ballmer-Hofer,K., Merlevede,W., and Goris,J. (1993). Phosphatase 2A associated with polyomavirus small-T or middle-T antigen is an okadaic acid-sensitive tyrosyl phosphatase. *Eur J Biochem* 214, 281-286.
- Chang,D., Haynes,J.I., Brady,J.N., and Consigli,R.A. (1992). Identification of a nuclear localization sequence in the polyomavirus capsid protein VP2. *Virology* 191, 978-983.
- Chapel,A., Poncet,P., Neildez-Nguyen,T.M., Vetillard,J., Brouard,N., Goupy,C., Chavanel,G., Hirsch,F., and Thierry,D. (1999). Targeted transfection of the IL-3 gene into primary human hematopoietic progenitor cells through the c-kit receptor. *Exp. Hematol.* 27, 250-258.
- Chen,H.H., Mack,L.M., Kelly,R., Ontell,M., Kochanek,S., and Clemens,P.R. (1997). Persistence in muscle of an adenoviral vector that lacks all viral genes. *Proc Natl Acad Sci U S A* 94, 1645-1650.
- Chen,J., Gamou,S., Takayanagi,A., Ohtake,Y., Ohtsubo,M., and Shimizu,N. (1998b). Targeted in vivo delivery of therapeutic gene into experimental squamous cell carcinomas using anti-epidermal growth factor receptor antibody: immunogene approach. *Hum. Gene Ther.* 9, 2673-2681.
- Chen,X.S., Stehle,T., and Harrison,S.C. (1998a). Interaction of polyomavirus internal protein VP2 with the major capsid protein VP1 and implications for participation of VP2 in viral entry. *EMBO J* 17, 3233-3240.

Chin,L., Tam,A., Pomerantz,J., Wong,M., Holash,J., Bardeesy,N., Shen,Q., O'Hagan,R., Pantginis,J., Zhou,H., Horner,J.W., Cordon-Cardo,C., Yancopoulos,G.D., and DePinho,R.A. (1999). Essential role for oncogenic Ras in tumour maintenance. *Nature* 400, 468-472.

Chu,T.H., Martinez,I., Sheay,W.C., and Dornburg,R. (1994). Cell targeting with retroviral vector particles containing antibody- envelope fusion proteins. *Gene Ther.* 1, 292-299.

Clackson,T., Hoogenboom,H.R., Griffiths,A.D., and Winter,G. (1991). Making antibody fragments using phage display libraries. *Nature* 352, 624-628.

Clayman,G.L., Frank,D.K., Bruso,P.A., and Goepfert,H. (1999). Adenovirus-mediated wild-type p53 gene transfer as a surgical adjuvant in advanced head and neck cancers. *Clin Cancer Res.* 5, 1715-1722.

Coffin,JM. (1996). Retroviridae: The viruses and their replication. In Fields Virology, B.N.Fields, D.M.Knipe, and P.M.Howley, eds. (New York: Lippincott-Raven Publishers), pp. 1767-1847.

Colin,M., Harbottle,R.P., Knight,A., Kornprobst,M., Cooper,R.G., Miller,A.D., Trugnan,G., Capeau,J., Coutelle,C., and Brahimi-Horn,M.C. (1998). Liposomes enhance delivery and expression of an RGD-oligolysine gene transfer vector in human tracheal cells. *Gene Ther.* 5, 1488-1498.

Conrad,C.K., Allen,S.S., Afione,S.A., Reynolds,T.C., Beck,S.E., Fee-Maki,M., Barraza-Ortiz,X., Adams,R., Askin,F.B., Carter,B.J., Guggino,W.B., and Flotte,T.R. (1996). Safety of single-dose administration of an adeno-associated virus (AAV)- CFTR vector in the primate lung. *Gene Ther.* 3, 658-668.

Cremisi,C., Pignatti,P.F., and Yaniv,M. (1976). Random location and absence of movement of the nucleosomes on SV 40 nucleoprotein complex isolated from infected cells. *Biochem Biophys Res. Commun.* 73, 548-554.

Crystal,R.G. (1995). Transfer of genes to humans: early lessons and obstacles to success. *Science* 270, 404-410.

Dai,Y., Schwarz,E.M., Gu,D., Zhang,W.W., Sarvetnick,N., and Verma,I.M. (1995). Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. *Proc Natl Acad Sci U S A* 92, 1401-1405.

Dameron,K.M., Volpert,O.V., Tainsky,M.A., and Bouck,N. (1994). The p53 tumor suppressor gene inhibits angiogenesis by stimulating the production of thrombospondin. *Cold Spring Harb. Symp. Quant. Biol* 59, 483-489.

Danks,M.K., Morton,C.L., Pawlik,C.A., and Potter,P.M. (1998). Overexpression of a rabbit liver carboxylesterase sensitizes human tumor cells to CPT-11. *Cancer Res.* 58, 20-22.

Dedieu,J.F., Vigne,E., Torrent,C., Jullien,C., Mahfouz,I., Caillaud,J.M., Aubailly,N., Orsini,C., Guillaume,J.M., Opolon,P., Delaere,P., Perricaudet,M., and Yeh,P. (1997). Long-term gene delivery into the livers of immunocompetent mice with E1/E4-defective adenoviruses. *J Virol.* 71, 4626-4637.

- Deisenhofer, J. (1981). Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry* 20, 2361-2370.
- Dilworth, S.M. (1995). Polyoma virus middle T antigen: meddler or mimic? *Trends Microbiol.* 3, 31-35.
- Dmitriev, I., Krasnykh, V., Miller, C.R., Wang, M., Kashentseva, E., Mikheeva, G., Belousova, N., and Curiel, D.T. (1998). 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, 9706-9713.
- Donahue, R.E., Kessler, S.W., Bodine, D., McDonagh, K., Dunbar, C., Goodman, S., Agricola, B., Byrne, E., Raffeld, M., and Moen, R. (1992). Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. *J Exp. Med* 176, 1125-1135.
- Doronin, K., Kuppuswamy, M., Toth, K., Tollefson, A.E., Krajcsi, P., Krougliak, V., and Wold, W.S. (2001). Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. *J Virol.* 75, 3314-3324.
- Doronin, K., Toth, K., Kuppuswamy, M., Ward, P., Tollefson, A.E., and Wold, W.S. (2000). Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. *J Virol.* 74, 6147-6155.
- Douglas, J.T. and Curiel, D.T. (1997). Strategies to accomplish targeted gene delivery to muscle cells employing tropism-modified adenoviral vectors. *Neuromuscul. Disord.* 7, 284-298.
- Douglas, J.T., Miller, C.R., Kim, M., Dmitriev, I., Mikheeva, G., Krasnykh, V., and Curiel, D.T. (1999). A system for the propagation of adenoviral vectors with genetically modified receptor specificities. *Nat. Biotechnol* 17, 470-475.
- Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., Lydon, N.B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C.L. (2001). Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N. Engl. J Med* 344, 1031-1037.
- Dunant, N. and Ballmer-Hofer, K. (1997). Signalling by Src family kinases: lessons learnt from DNA tumour viruses. *Cell Signal.* 9, 385-393.
- Elliott, G. and O'Hare, P. (1997). Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* 88, 223-233.
- Erbacher, P., Remy, J.S., and Behr, J.P. (1999). Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. *Gene Ther.* 6, 138-145.
- Evans, C.H., Robbins, P.D., Ghivizzani, S.C., Herndon, J.H., Kang, R., Bahnson, A.B., Barranger, J.A., Elders, E.M., Gay, S., Tomaino, M.M., Wasko, M.C., Watkins, S.C., Whiteside, T.L., Glorioso, J.C., Lotze, M.T., and Wright, T.M. (1996). Clinical trial to assess the safety, feasibility, and efficacy of transferring a potentially anti-arthritic cytokine gene to human joints with rheumatoid arthritis. *Hum. Gene Ther.* 7, 1261-1280.

- Felgner,P.L., Gadek,T.R., Holm,M., Roman,R., Chan,H.W., Wenz,M., Northrop,J.P., Ringold,G.M., and Danielsen,M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* 84, 7413-7417.
- Ferkol,T., Perales,J.C., Mularo,F., and Hanson,R.W. (1996). Receptor-mediated gene transfer into macrophages. *Proc Natl Acad Sci U S A* 93, 101-105.
- Ferrari,F.K., Samulski,T., Shenk,T., and Samulski,R.J. (1996). Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol.* 70, 3227-3234.
- Fielding,A.K., Maurice,M., Morling,F.J., Cosset,F.L., and Russell,S.J. (1998). Inverse targeting of retroviral vectors: selective gene transfer in a mixed population of hematopoietic and nonhematopoietic cells. *Blood* 91, 1802-1809.
- Filion,F.C. and Phillips,N.C. (1998). Major limitations in the use of cationic liposomes for DNA delivery. *Int. J. Pharmaceut.* 162, 159-170.
- Fink,D.J. and Glorioso,J.C. (1997). Herpes simplex virus-based vectors: problems and some solutions. *Adv. Neurol.* 72, 149-156.
- Fink,D.J., Ramakrishnan,R., Marconi,P., Goins,W.F., Holland,T.C., and Glorioso,J.C. (1995). Advances in the development of herpes simplex virus-based gene transfer vectors for the nervous system. *Clin Neurosci.* 3, 284-291.
- Flotte,T., Carter,B., Conrad,C., Guggino,W., Reynolds,T., Rosenstein,B., Taylor,G., Walden,S., and Wetzel,R. (1996). A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. *Hum. Gene Ther.* 7, 1145-1159.
- Fominaya,J., Uherek,C., and Wels,W. (1998). A chimeric fusion protein containing transforming growth factor- α mediates gene transfer via binding to the EGF receptor. *Gene Ther.* 5, 521-530.
- Forstova,J., Krauzewicz,N., Sandig,V., Elliott,J., Palkova,Z., Strauss,M., and Griffin,B.E. (1995). Polyoma virus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells. *Hum. Gene Ther.* 6, 297-306.
- Freund,R., Garcea,R.L., Sahli,R., and Benjamin,T.L. (1991). A single-amino-acid substitution in polyomavirus VP1 correlates with plaque size and hemagglutination behavior. *J Virol.* 65, 350-355.
- Fried,H., Cahan,L.D., and Paulson,J.C. (1981). Polyoma virus recognizes specific sialyligosaccharide receptors on host cells. *Virology* 109, 188-192.
- Fueyo,J., Gomez-Manzano,C., Alemany,R., Lee,P.S., McDonnell,T.J., Mitlianga,P., Shi,Y.X., Levin,V.A., Yung,W.K., and Kyritsis,A.P. (2000). A mutant oncolytic adenovirus targeting the Rb pathway produces anti- glioma effect in vivo. *Oncogene* 19, 2-12.
- Garcea,R.L., Ballmer-Hofer,K., and Benjamin,T.L. (1985). Virion assembly defect of polyomavirus hr-t mutants: underphosphorylation of major capsid protein VP1 before viral DNA encapsidation. *J Virol.* 54, 311-316.

- Garcea,R.L. and Benjamin,T.L. (1983). Host range transforming gene of polyoma virus plays a role in virus assembly. *Proc Natl Acad Sci U S A* 80, 3613-3617.
- Garipey,J. and Kawamura,K. (2001). Vectorial delivery of macromolecules into cells using peptide-based vehicles. *Trends Biotechnol* 19, 21-28.
- Geller,A.I. (1997). Herpes simplex virus-1 plasmid vectors for gene transfer into neurons. *Adv. Neurol.* 72, 143-148.
- Gharakhanian,E. and Kasamatsu,H. (1990). Two independent signals, a nuclear localization signal and a Vp1- interactive signal, reside within the carboxy-35 amino acids of SV40 Vp3. *Virology* 178, 62-71.
- Gharakhanian,E., Takahashi,J., Clever,J., and Kasamatsu,H. (1988). In vitro assay for protein-protein interaction: carboxyl-terminal 40 residues of simian virus 40 structural protein VP3 contain a determinant for interaction with VP1. *Proc Natl Acad Sci U S A* 85, 6607-6611.
- Gleiter,S. and Lilie,H. (2001). Coupling of antibodies via protein Z on modified polyoma virus-like particles. *Protein Sci* 10, 434-444.
- Gleiter, S. and Lilie, H. (2002). Cell-type specific targeting and gene expression using a variant of polyoma VP1 virus like particles. *Eingereicht*.
- Gleiter,S., Stubenrauch,K., and Lilie,H. (1999). Changing the surface of a virus shell fusion of an enzyme to polyoma VP1. *Protein Sci* 8, 2562-2569.
- Graham,F.L. and Prevec,L. (1995). Methods for construction of adenovirus vectors. *Mol. Biotechnol* 3, 207-220.
- Gussoni,E., Blau,H.M., and Kunkel,L.M. (1997). The fate of individual myoblasts after transplantation into muscles of DMD patients. *Nat. Med* 3, 970-977.
- Hanna,N.N., Mauceri,H.J., Wayne,J.D., Hallahan,D.E., Kufe,D.W., and Weichselbaum,R.R. (1997). Virally directed cytosine deaminase/5-fluorocytosine gene therapy enhances radiation response in human cancer xenografts. *Cancer Res.* 57, 4205-4209.
- Harbottle,R.P., Cooper,R.G., Hart,S.L., Ladhoff,A., McKay,T., Knight,A.M., Wagner,E., Miller,A.D., and Coutelle,C. (1998). An RGD-oligolysine peptide: a prototype construct for integrin-mediated gene delivery. *Hum. Gene Ther.* 9, 1037-1047.
- Hauser,M.A., Amalfitano,A., Kumar-Singh,R., Hauschka,S.D., and Chamberlain,J.S. (1997). Improved adenoviral vectors for gene therapy of Duchenne muscular dystrophy. *Neuromuscul. Disord.* 7, 277-283.
- Hehir,K.M., Armentano,D., Cardoza,L.M., Choquette,T.L., Berthelette,P.B., White,G.A., Couture,L.A., Everton,M.B., Keegan,J., Martin,J.M., Pratt,D.A., Smith,M.P., Smith,A.E., and Wadsworth,S.C. (1996). Molecular characterization of replication-competent variants of adenovirus vectors and genome modifications to prevent their occurrence. *J Virol.* 70, 8459-8467.
- Heidari,S., Krauzewicz,N., Kalantari,M., Vlastos,A., Griffin,B.E., and Dalianis,T. (2000). Persistence and tissue distribution of DNA in normal and immunodeficient mice inoculated with polyomavirus VP1 pseudocapsid complexes or polyomavirus. *J Virol.* 74, 11963-11965.

- Heise,C., Hermiston,T., Johnson,L., Brooks,G., Sampson-Johannes,A., Williams,A., Hawkins,L., and Kirn,D. (2000). An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat. Med* 6, 1134-1139.
- Heise,C. and Kirn,D.H. (2000). Replication-selective adenoviruses as oncolytic agents. *J Clin Invest* 105, 847-851.
- Hennecke,J., Sebbel,P., and Glockshuber,R. (1999). Random circular permutation of DsbA reveals segments that are essential for protein folding and stability. *J Mol. Biol.* 286, 1197-1215.
- Hickman,M.A., Malone,R.W., Lehmann-Bruinsma,K., Sih,T.R., Knoell,D., Szoka,F.C., Walzem,R., Carlson,D.M., and Powell,J.S. (1994). Gene expression following direct injection of DNA into liver. *Hum. Gene Ther.* 5, 1477-1483.
- Holldack,J., Burdach,S., Eisberg,A., Frisch,J., and Schulz,G. (1992). Biology and pharmacology of hematopoietic growth factors. *Med Pediatr. Oncol. Suppl* 2, 2-9.
- Holt,J.T., Thompson,M.E., Szabo,C., Robinson-Benion,C., Arteaga,C.L., King,M.C., and Jensen,R.A. (1996). Growth retardation and tumour inhibition by BRCA1. *Nat. Genet.* 12, 298-302.
- Hu,W.S. and Pathak,V.K. (2000). Design of retroviral vectors and helper cells for gene therapy. *Pharmacol. Rev.* 52, 493-511.
- Jendeberg,L., Tashiro,M., Tejero,R., Lyons,B.A., Uhlen,M., Montelione,G.T., and Nilsson,B. (1996). The mechanism of binding staphylococcal protein A to immunoglobulin G does not involve helix unwinding. *Biochemistry* 35, 22-31.
- Jiang,A., Chu,T.H., Nocken,F., Cichutek,K., and Dornburg,R. (1998). Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies. *J Virol.* 72, 10148-10156.
- Kakulas,B.A. (1997). Problems and potential for gene therapy in Duchenne muscular dystrophy. *Neuromuscul. Disord.* 7, 319-324.
- Koch,C. and Hoiby,N. (2000). Diagnosis and treatment of cystic fibrosis. *Respiration* 67, 239-247.
- Kochanek,S., Clemens,P.R., Mitani,K., Chen,H.H., Chan,S., and Caskey,C.T. (1996). A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc Natl Acad Sci U S A* 93, 5731-5736.
- Konishi,H., Ochiya,T., Chester,K.A., Begent,R.H., Muto,T., Sugimura,T., and Terada,M. (1998). Targeting strategy for gene delivery to carcinoembryonic antigen-producing cancer cells by retrovirus displaying a single-chain variable fragment antibody [published erratum appears in *Hum Gene Ther* 1998 May 20;9(8):1257]. *Hum Gene Ther* 9, 235-248.
- Kozak,S.L. and Kabat,D. (1990). Ping-pong amplification of a retroviral vector achieves high-level gene expression: human growth hormone production. *J Virol.* 64, 3500-3508.

- Krauzewicz,N., Cox,C., Soeda,E., Clark,B., Rayner,S., and Griffin,B.E. (2000a). Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids. *Gene Ther.* 7, 1094-1102.
- Krauzewicz,N., Stokrova,J., Jenkins,C., Elliott,M., Higgins,C.F., and Griffin,B.E. (2000b). Virus-like gene transfer into cells mediated by polyoma virus pseudocapsids. *Gene Ther.* 7, 2122-2131.
- Kunzelmann,K. and Mall,M. (2001). Pharmacotherapy of the ion transport defect in cystic fibrosis. *Clin Exp. Pharmacol. Physiol* 28, 857-867.
- Laquerre,S., Anderson,D.B., Stolz,D.B., and Glorioso,J.C. (1998). Recombinant herpes simplex virus type 1 engineered for targeted binding to erythropoietin receptor-bearing cells. *J Virol.* 72, 9683-9697.
- Larsson,M., Brundell,E., Nordfors,L., Hoog,C., Uhlen,M., and Stahl,S. (1996). A general bacterial expression system for functional analysis of cDNA- encoded proteins. *Protein Expr Purif* 7, 447-457.
- Latchman,D.S. (2001). Gene delivery and gene therapy with herpes simplex virus-based vectors. *Gene* 264, 1-9.
- Leavitt,A.D., Roberts,T.M., and Garcea,R.L. (1985). Polyoma virus major capsid protein, VP1. Purification after high level expression in *Escherichia coli*. *J Biol Chem* 260, 12803-12809.
- Li,J.M., Han,J.S., Huang,Y., Tain,P.K., Qu,S.M., Yao,M., Jiang,H.Q., Wan,D.F., Luo,J.C., Gu,C.X., and Gu,J.R. (1999). A novel gene delivery system targeting cells expressing VEGF receptors. *Cell Res.* 9, 11-25.
- Li,M. and Garcea,R.L. (1994). Identification of the threonine phosphorylation sites on the polyomavirus major capsid protein VP1: relationship to the activity of middle T antigen. *J Virol.* 68, 320-327.
- Liddington,R.C., Yan,Y., Moulai,J., Sahli,R., Benjamin,T.L., and Harrison,S.C. (1991). Structure of simian virus 40 at 3.8-Å resolution. *Nature* 354, 278-284.
- Lieber,A., He,C.Y., Kirillova,I., and Kay,M.A. (1996). Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first- generation vectors in vitro and in vivo. *J Virol.* 70, 8944-8960.
- Linial,M.L. (1999). Foamy viruses are unconventional retroviruses. *J Virol.* 73, 1747-1755.
- Luo,D. and Saltzman,W.M. (2000). Synthetic DNA delivery systems. *Nat. Biotechnol* 18, 33-37.
- Mackay,R.L. and Consigli,R.A. (1976). Early events in polyoma virus infection: attachment, penetration, and nuclear entry. *J Virol.* 19, 620-636.
- Mann,M.J., Gibbons,G.H., Hutchinson,H., Poston,R.S., Hoyt,E.G., Robbins,R.C., and Dzau,V.J. (1999). Pressure-mediated oligonucleotide transfection of rat and human cardiovascular tissues. *Proc Natl Acad Sci U S A* 96, 6411-6416.

- Marconi,P., Krisky,D., Oligino,T., Poliani,P.L., Ramakrishnan,R., Goins,W.F., Fink,D.J., and Glorioso,J.C. (1996). Replication-defective herpes simplex virus vectors for gene transfer in vivo. *Proc Natl Acad Sci U S A* 93, 11319-11320.
- Martin,F., Kupsch,J., Takeuchi,Y., Russell,S., Cosset,F.L., and Collins,M. (1998). Retroviral vector targeting to melanoma cells by single-chain antibody incorporation in envelope. *Hum. Gene Ther.* 9, 737-746.
- May, T., Gleiter, S., Rudolph, R., and Lilie, H. (2001). Assessment of cell type specific gene transfer of polyoma virus like particles presenting a tumor specific antibody Fv-fragment. Eingereicht.
- McCormick,F. (2001). Cancer gene therapy: Fringe or cutting edge? *Nature Reviews Cancer* 1, 130-141.
- Messerschmitt,A., Disela,C., Dilworth,S., Marti,A.G., and Ballmer-Hofer,K. (1996). Polyomavirus middle-T antigen lacking a membrane anchor sequence accumulates in the nucleus. *J Gen. Virol.* 77 (*Pt 1*), 17-26.
- Metcalf,D. (1990). The colony stimulating factors. Discovery, development, and clinical applications. *Cancer* 65, 2185-2195.
- Miles,D.W. (2001). Update on HER-2 as a target for cancer therapy: Herceptin in the clinical setting. *Breast Cancer Res.* 3, 380-384.
- Miller,A.D. (1992). Human gene therapy comes of age. *Nature* 357, 455-460.
- Miller,A.D. (1997). Development and applications of retroviral vectors. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).
- Miller,A.D. and Buttimore,C. (1986). Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell Biol* 6, 2895-2902.
- Miller,C.R., Buchsbaum,D.J., Reynolds,P.N., Douglas,J.T., Gillespie,G.Y., Mayo,M.S., Raben,D., and Curiel,D.T. (1998). Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res.* 58, 5738-5748.
- Modrow,S. and Falke,D. (1997). Papovaviren. In *Molekulare Virologie*, (Heidelberg: Spektrum Akademischer Verlag), pp. 358-391.
- Mohr,L., Schauer,J.I., Boutin,R.H., Moradpour,D., and Wands,J.R. (1999). Targeted gene transfer to hepatocellular carcinoma cells in vitro using a novel monoclonal antibody-based gene delivery system. *Hepatology* 29, 82-89.
- Moks,T., Abrahmsen,L., Nilsson,B., Hellman,U., Sjoquist,J., and Uhlen,M. (1986). Staphylococcal protein A consists of five IgG-binding domains. *Eur. J Biochem.* 156, 637-643.
- Moolten,F.L. (1986). Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res.* 46, 5276-5281.

- Moreland,R.B. and Garcea,R.L. (1991). Characterization of a nuclear localization sequence in the polyomavirus capsid protein VP1. *Virology* 185, 513-518.
- Morikawa,Y., Goto,T., and Sano,K. (1999). In vitro assembly of human immunodeficiency virus type 1 Gag protein. *J Biol Chem* 274, 27997-28002.
- Morizono,K., Bristol,G., Xie,Y.M., Kung,S.K., and Chen,I.S. (2001). Antibody-directed targeting of retroviral vectors via cell surface antigens. *J Virol.* 75, 8016-8020.
- Morsy,M.A., Gu,M., Motzel,S., Zhao,J., Lin,J., Su,Q., Allen,H., Franlin,L., Parks,R.J., Graham,F.L., Kochanek,S., Bett,A.J., and Caskey,C.T. (1998). An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. *Proc Natl Acad Sci U S A* 95, 7866-7871.
- Mulligan,R.C. (1993). The basic science of gene therapy. *Science* 260, 926-932.
- Nemunaitis,J., Ganly,I., Khuri,F., Arseneau,J., Kuhn,J., McCarty,T., Landers,S., Maples,P., Romel,L., Randlev,B., Reid,T., Kaye,S., and Kim,D. (2000a). Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. *Cancer Res.* 60, 6359-6366.
- Nemunaitis,J., Swisher,S.G., Timmons,T., Connors,D., Mack,M., Doerksen,L., Weill,D., Wait,J., Lawrence,D.D., Kemp,B.L., Fossella,F., Glisson,B.S., Hong,W.K., Khuri,F.R., Kurie,J.M., Lee,J.J., Lee,J.S., Nguyen,D.M., Nesbitt,J.C., Perez-Soler,R., Pisters,K.M., Putnam,J.B., Richli,W.R., Shin,D.M., and Walsh,G.L. (2000b). Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J Clin Oncol.* 18, 609-622.
- Neumann,E., Schaefer-Ridder,M., Wang,Y., and Hofschneider,P.H. (1982). Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J* 1, 841-845.
- Neve,R.L. and Geller,A.I. (1995). A defective herpes simplex virus vector system for gene delivery into the brain: comparison with alternative gene delivery systems and usefulness for gene therapy. *Clin Neurosci.* 3, 262-267.
- Nishizaki,M., Fujiwara,T., Tanida,T., Hizuta,A., Nishimori,H., Tokino,T., Nakamura,Y., Bouvet,M., Roth,J.A., and Tanaka,N. (1999). Recombinant adenovirus expressing wild-type p53 is antiangiogenic: a proposed mechanism for bystander effect. *Clin Cancer Res.* 5, 1015-1023.
- Ogris,M., Brunner,S., Schuller,S., Kircheis,R., and Wagner,E. (1999). PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* 6, 595-605.
- Ohno,K. and Meruelo,D. (1997). Retrovirus vectors displaying the IgG-binding domain of protein A. *Biochem Mol. Med* 62, 123-127.
- Ohno,K., Sawai,K., Iijima,Y., Levin,B., and Meruelo,D. (1997). Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A. *Nat. Biotechnol* 15, 763-767.

- Ory,D.S., Neugeboren,B.A., and Mulligan,R.C. (1996). A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc Natl Acad Sci U S A* 93, 11400-11406.
- Oshima,Y., Sakamoto,T., Yamanaka,I., Nishi,T., Ishibashi,T., and Inomata,H. (1998). Targeted gene transfer to corneal endothelium in vivo by electric pulse. *Gene Ther.* 5, 1347-1354.
- Otani,K., Nita,I., Macaulay,W., Georgescu,H.I., Robbins,P.D., and Evans,C.H. (1996). Suppression of antigen-induced arthritis in rabbits by ex vivo gene therapy. *J Immunol.* 156, 3558-3562.
- Pallas,D.C., Shahrik,L.K., Martin,B.L., Jaspers,S., Miller,T.B., Brautigan,D.L., and Roberts,T.M. (1990). Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. *Cell* 60, 167-176.
- Park,E., Starzyk,R.M., McGrath,J.P., Lee,T., George,J., Schutz,A.J., Lynch,P., and Putney,S.D. (1998). Production and characterization of fusion proteins containing transferrin and nerve growth factor. *J Drug Target* 6, 53-64.
- Pastan,I., Pai,L.H., Brinkmann,U., and FitzGerald,D. (1996). Recombinant immunotoxins. *Breast Cancer Res. Treat.* 38, 3-9.
- Pastan,I.H., Pai,L.H., Brinkmann,U., and Fitzgerald,D.J. (1995). Recombinant toxins: new therapeutic agents for cancer. *Ann. N. Y. Acad Sci* 758, 345-354.
- Paul,R.W., Weisser,K.E., Loomis,A., Sloane,D.L., LaFoe,D., Atkinson,E.M., and Overell,R.W. (1997). Gene transfer using a novel fusion protein, GAL4/invasin. *Hum. Gene Ther.* 8, 1253-1262.
- Peng,K.W. and Russell,S.J. (1999). Viral vector targeting. *Curr. Opin. Biotechnol* 10, 454-457.
- Peng,K.W., Vile,R., Cosset,F.L., and Russell,S. (1999). Selective transduction of protease-rich tumors by matrix- metalloproteinase-targeted retroviral vectors. *Gene Ther.* 6, 1552-1557.
- Pfeffer,S.R. (1991). Targeting of proteins to the lysosome. *Curr. Top. Microbiol. Immunol.* 170, 43-65.
- Phelan,A., Elliott,G., and O'Hare,P. (1998). Intercellular delivery of functional p53 by the herpesvirus protein VP22. *Nat. Biotechnol* 16, 440-443.
- Ponder,B.A., Robbins,A.K., and Crawford,L.V. (1977). Phophorylation of polyoma and SV40 virus proteins. *J Gen. Virol.* 37, 75-83.
- Purcell,D.F., Broscius,C.M., Vanin,E.F., Buckler,C.E., Nienhuis,A.W., and Martin,M.A. (1996). An array of murine leukemia virus-related elements is transmitted and expressed in a primate recipient of retroviral gene transfer. *J Virol.* 70, 887-897.
- Ramsey,W.J., Mullen,C.A., and Blaese,R.M. (1995). Retrovirus mediated gene transfer as therapy for adenosine deaminase (ADA) deficiency. *Leukemia* 9 Suppl 1, S70.

- Richterova,Z., Liebl,D., Horak,M., Palkova,Z., Stokrova,J., Hozak,P., Korb,J., and Forstova,J. (2001). Caveolae are involved in the trafficking of mouse polyomavirus virions and artificial VP1 pseudocapsids toward cell nuclei. *J Virol.* 75, 10880-10891.
- Robbins,P.D. and Ghivizzani,S.C. (1998). Viral vectors for gene therapy. *Pharmacol. Ther.* 80, 35-47.
- Rolling,F. and Samulski,R.J. (1995). AAV as a viral vector for human gene therapy. Generation of recombinant virus. *Mol. Biotechnol* 3, 9-15.
- Russell,C.S. and Clarke,L.A. (1999). Recombinant proteins for genetic disease. *Clin Genet.* 55, 389-394.
- Sachsenmeier,K.F. and Pipas,J.M. (2001). Inhibition of Rb and p53 is insufficient for SV40 T-antigen transformation. *Virology* 283, 40-48.
- Salunke,D.M., Caspar,D.L., and Garcea,R.L. (1986). Self-assembly of purified polyomavirus capsid protein VP1. *Cell* 46, 895-904.
- Salunke,D.M., Caspar,D.L., and Garcea,R.L. (1989). Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophys J* 56, 887-900.
- Samuelsson,E., Moks,T., Nilsson,B., and Uhlen,M. (1994). Enhanced in vitro refolding of insulin-like growth factor I using a solubilizing fusion partner. *Biochemistry* 33, 4207-4211.
- Samuelsson,E. and Uhlen,M. (1996). Chaperone-like effect during in vitro refolding of insulin-like growth factor I using a solubilizing fusion partner. *Ann. N. Y. Acad Sci* 782, 486-494.
- Samulski,R.J. (1993). Adeno-associated virus: integration at a specific chromosomal locus. *Curr. Opin. Genet. Dev.* 3, 74-80.
- Sawaya,M.R. and Kraut,J. (1997). Loop and subdomain movements in the mechanism of Escherichia coli dihydrofolate reductase: crystallographic evidence. *Biochemistry* 36, 586-603.
- Schaffer,D.V., Fidelman,N.A., Dan,N., and Lauffenburger,D.A. (2000). Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. *Biotechnol Bioeng.* 67, 598-606.
- Schaffer,D.V. and Lauffenburger,D.A. (1998). Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. *J Biol Chem* 273, 28004-28009.
- Schaffer,D.V. and Lauffenburger,D.A. (2000). Targeted synthetic gene delivery vectors. *Curr. Opin. Mol. Ther.* 2, 155-161.
- Schiedner,G., Morral,N., Parks,R.J., Wu,Y., Koopmans,S.C., Langston,C., Graham,F.L., Beaudet,A.L., and Kochanek,S. (1998). Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat. Genet.* 18, 180-183.

- Schmidt,U., Gunther,C., Rudolph,R., and Bohm,G. (2001a). Protein and peptide delivery via engineered polyomavirus-like particles. *FASEB J* 15, 1646-1648.
- Schmidt,U., Rudolph,R., and Bohm,G. (2000). Mechanism of assembly of recombinant murine polyomavirus-like particles. *J Virol.* 74, 1658-1662.
- Schmidt,U., Rudolph,R., and Bohm,G. (2001b). Binding of external ligands onto an engineered virus capsid. *Protein Eng* 14, 769-774.
- Schneider,H., Harbottle,R.P., Yokosaki,Y., Jost,P., and Coutelle,C. (1999). Targeted gene delivery into alpha9beta1-integrin-displaying cells by a synthetic peptide. *FEBS Lett.* 458, 329-332.
- Schwarze,S.R., Ho,A., Vocero-Akbani,A., and Dowdy,S.F. (1999). In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 285, 1569-1572.
- Shao,N., Chai,Y.L., Shyam,E., Reddy,P., and Rao,V.N. (1996). Induction of apoptosis by the tumor suppressor protein BRCA1. *Oncogene* 13, 1-7.
- Slilaty,S.N. and Aposhian,H.V. (1983). Gene transfer by polyoma-like particles assembled in a cell-free system. *Science* 220, 725-727.
- Slilaty,S.N., Berns,K.I., and Aposhian,H.V. (1982). Polyoma-like particle: characterization of the DNA encapsidated in vitro by polyoma empty capsids. *J Biol Chem* 257, 6571-6575.
- Snitkovsky,S. and Young,J.A. (1998). Cell-specific viral targeting mediated by a soluble retroviral receptor- ligand fusion protein. *Proc Natl Acad Sci U S A* 95, 7063-7068.
- Somia,N. and Verma,I.M. (2000). Gene therapy: trials and tribulations. *Nat. Rev. Genet.* 1, 91-99.
- Sorscher,E.J., Peng,S., Bebok,Z., Allan,P.W., Bennett,L.L., Jr., and Parker,W.B. (1994). Tumor cell bystander killing in colonic carcinoma utilizing the Escherichia coli DeoD gene to generate toxic purines. *Gene Ther.* 1, 233-238.
- Starovasnik,M.A., Braisted,A.C., and Wells,J.A. (1997). Structural mimicry of a native protein by a minimized binding domain. *Proc Natl Acad Sci U S A* 94, 10080-10085.
- Starr,P.A., Lim,F., Grant,F.D., Trask,L., Lang,P., Yu,L., and Geller,A.I. (1996). Long-term persistence of defective HSV-1 vectors in the rat brain is demonstrated by reactivation of vector gene expression. *Gene Ther.* 3, 615-623.
- Stehle,T. and Harrison,S.C. (1996). Crystal structures of murine polyomavirus in complex with straight- chain and branched-chain sialyloligosaccharide receptor fragments. *Structure.* 4, 183-194.
- Stehle,T. and Harrison,S.C. (1997). High-resolution structure of a polyomavirus VP1-oligosaccharide complex: implications for assembly and receptor binding. *EMBO J* 16, 5139-5148.
- Stehle,T., Yan,Y., Benjamin,T.L., and Harrison,S.C. (1994). Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* 369, 160-163.

- Stemmer, W.P. (1994). Rapid evolution of a protein in vitro by DNA shuffling. *Nature* 370, 389-391.
- Streuli, C.H. and Griffin, B.E. (1987). Myristic acid is coupled to a structural protein of polyoma virus and SV40. *Nature* 326, 619-622.
- Stubenrauch, K., Bachmann, A., Rudolph, R., and Lilie, H. (2000). Purification of a viral coat protein by an engineered polyionic sequence. *J Chromatogr B Biomed. Sci Appl.* 737, 77-84.
- Stubenrauch, K., Gleiter, S., Brinkmann, U., Rudolph, R., and Lilie, H. (2001). Conjugation of an antibody Fv fragment to a virus coat protein: cell-specific targeting of recombinant polyoma-virus-like particles. *Biochem J* 356, 867-873.
- Subramanian, A., Ranganathan, P., and Diamond, S.L. (1999). Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nat. Biotechnol* 17, 873-877.
- Swisher, S.G., Roth, J.A., Nemunaitis, J., Lawrence, D.D., Kemp, B.L., Carrasco, C.H., Connors, D.G., El Naggar, A.K., Fossella, F., Glisson, B.S., Hong, W.K., Khuri, F.R., Kurie, J.M., Lee, J.J., Lee, J.S., Mack, M., Merritt, J.A., Nguyen, D.M., Nesbitt, J.C., Perez-Soler, R., Pisters, K.M., Putnam, J.B., Jr., Richli, W.R., Savin, M., and Waugh, M.K. (1999). Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer. *J Natl Cancer Inst.* 91, 763-771.
- Trinh, Q.T., Austin, E.A., Murray, D.M., Knick, V.C., and Huber, B.E. (1995). Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. *Cancer Res.* 55, 4808-4812.
- Uherek, C., Fominaya, J., and Wels, W. (1998). A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery. *J Biol Chem* 273, 8835-8841.
- Vaheri, A. and Pagano, J.S. (1965). Infectious poliovirus RNA: a sensitive method of assay. *Virology* 27, 434-436.
- Valsesia-Wittmann, S., Morling, F.J., Nilson, B.H., Takeuchi, Y., Russell, S.J., and Cosset, F.L. (1996). Improvement of retroviral retargeting by using amino acid spacers between an additional binding domain and the N terminus of Moloney murine leukemia virus SU. *J Virol.* 70, 2059-2064.
- Vanin, E.F., Kaloss, M., Brosius, C., and Nienhuis, A.W. (1994). Characterization of replication-competent retroviruses from nonhuman primates with virus-induced T-cell lymphomas and observations regarding the mechanism of oncogenesis. *J Virol.* 68, 4241-4250.
- Verma, I.M. and Somia, N. (1997). Gene therapy -- promises, problems and prospects. *Nature* 389, 239-242.
- Vigne, E., Mahfouz, I., Dedieu, J.F., Brie, A., Perricaudet, M., and Yeh, P. (1999). RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J Virol.* 73, 5156-5161.

- Vogt,V.M. (1997). Retroviral virions and genomes. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).
- Wagner,E., Ogris,M., and Zauner,W. (1998). Polylysine-based transfection systems utilizing receptor-mediated delivery. *Adv. Drug Deliv. Rev.* 30, 97-113.
- Wagner,E., Plank,C., Zatloukal,K., Cotten,M., and Birnstiel,M.L. (1992). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc Natl Acad Sci U S A* 89, 7934-7938.
- Walter,G., Ruediger,R., Slaughter,C., and Mumby,M. (1990). Association of protein phosphatase 2A with polyoma virus medium tumor antigen. *Proc Natl Acad Sci U S A* 87, 2521-2525.
- Walton,C.M., Wu,C.H., and Wu,G.Y. (1999). A DNA delivery system containing listeriolysin O results in enhanced hepatocyte-directed gene expression. *World J Gastroenterol.* 5, 465-469.
- Watkins,S.J., Mesyanzhinov,V.V., Kurochkina,L.P., and Hawkins,R.E. (1997). The 'adenobody' approach to viral targeting: specific and enhanced adenoviral gene delivery. *Gene Ther.* 4, 1004-1012.
- Waxman,D.J., Chen,L., Hecht,J.E., and Jounaidi,Y. (1999). Cytochrome P450-based cancer gene therapy: recent advances and future prospects. *Drug Metab Rev.* 31, 503-522.
- Wolff,J.A., Malone,R.W., Williams,P., Chong,W., Acsadi,G., Jani,A., and Felgner,P.L. (1990). Direct gene transfer into mouse muscle in vivo. *Science* 247, 1465-1468.
- Wong,T.K. and Neumann,E. (1982). Electric field mediated gene transfer. *Biochem Biophys Res. Commun.* 107, 584-587.
- Wu,G.Y. and Wu,C.H. (1987). Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J Biol Chem* 262, 4429-4432.
- Wu,N., Watkins,S.C., Schaffer,P.A., and DeLuca,N.A. (1996). Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate-early genes encoding ICP4, ICP27, and ICP22. *J Virol.* 70, 6358-6369.
- Xu,B., Wiehle,S., Roth,J.A., and Cristiano,R.J. (1998). The contribution of poly-L-lysine, epidermal growth factor and streptavidin to EGF/PLL/DNA polyplex formation. *Gene Ther.* 5, 1235-1243.
- Yang,J.P. and Huang,L. (1996). Direct gene transfer to mouse melanoma by intratumor injection of free DNA. *Gene Ther.* 3, 542-548.
- Yang,N.S., Burkholder,J., Roberts,B., Martinell,B., and McCabe,D. (1990). In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. *Proc Natl Acad Sci U S A* 87, 9568-9572.
- Yang,N.S. and Sun,W.H. (1995). Gene gun and other non-viral approaches for cancer gene therapy. *Nat. Med* 1, 481-483.

- Yang, Q., Mamounas, M., Yu, G., Kennedy, S., Leaker, B., Merson, J., Wong-Staal, F., Yu, M., and Barber, J.R. (1998). Development of novel cell surface CD34-targeted recombinant adenoassociated virus vectors for gene therapy. *Hum. Gene Ther.* 9, 1929-1937.
- Yang, Y., Su, Q., Grewal, I.S., Schilz, R., Flavell, R.A., and Wilson, J.M. (1996). Transient subversion of CD40 ligand function diminishes immune responses to adenovirus vectors in mouse liver and lung tissues. *J Virol.* 70, 6370-6377.
- Ye, G.N., Daniell, H., and Sanford, J.C. (1990). Optimization of delivery of foreign DNA into higher-plant chloroplasts. *Plant Mol. Biol.* 15, 809-819.
- Zabner, J., Cheng, S.H., Meeker, D., Launspach, J., Balfour, R., Perricone, M.A., Morris, J.E., Marshall, J., Fasbender, A., Smith, A.E., and Welsh, M.J. (1997). Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia in vivo. *J Clin Invest* 100, 1529-1537.
- Zahm, J.M., Debordeaux, C., Maurer, C., Hubert, D., Dusser, D., Bonnet, N., Lazarus, R.A., and Puchelle, E. (2001). Improved activity of an actin-resistant DNase I variant on the cystic fibrosis airway secretions. *Am J Respir. Crit Care Med* 163, 1153-1157.
- Zelphati, O., Uyechi, L.S., Barron, L.G., and Szoka, F.C., Jr. (1998). Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. *Biochim. Biophys Acta* 1390, 119-133.
- Zhang, W., Carmichael, J., Ferguson, J., Inglis, S., Ashrafian, H., and Stanley, M. (1998). Expression of human papillomavirus type 16 L1 protein in *Escherichia coli*: denaturation, renaturation, and self-assembly of virus-like particles in vitro. *Virology* 243, 423-431.
- Zhang, W.W., Fang, X., Mazur, W., French, B.A., Georges, R.N., and Roth, J.A. (1994). High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Ther.* 1, 5-13.
- Zhou, J., Stenzel, D.J., Sun, X.Y., and Frazer, I.H. (1993). Synthesis and assembly of infectious bovine papillomavirus particles in vitro. *J Gen. Virol.* 74 (Pt 4), 763-768.
- Zhou, X. and Muzyczka, N. (1998). In vitro packaging of adeno-associated virus DNA. *J Virol.* 72, 3241-3247.
- Zhu, J.Y., Abate, M., Rice, P.W., and Cole, C.N. (1991). The ability of simian virus 40 large T antigen to immortalize primary mouse embryo fibroblasts cosegregates with its ability to bind to p53. *J Virol.* 65, 6872-6880.
- Zhu, Z., DeLuca, N.A., and Schaffer, P.A. (1996). Overexpression of the herpes simplex virus type 1 immediate-early regulatory protein, ICP27, is responsible for the aberrant localization of ICP0 and mutant forms of ICP4 in ICP4 mutant virus-infected cells. *J Virol.* 70, 5346-5356.

7 Veröffentlichungen

Protein Science (1999), 8:2562–2569. Cambridge University Press. Printed in the USA.
Copyright © 1999 The Protein Society

Changing the surface of a virus shell fusion of an enzyme to polyoma VP1

STEFAN GLEITER, KAY STUBENRAUCH, AND HAUKE LILIE

Martin-Luther-Universität Halle, Institut für Biotechnologie, Kurt-Mothes Straße 3, D-06120 Halle, Germany

(RECEIVED June 21, 1999; ACCEPTED October 6, 1999)

Abstract

Recent developments on virus-like particles have demonstrated their potential in transfecting eucaryotic cells. In the case of particles based on the major coat protein VP1 of polyoma virus, transfection occurs via binding of VP1 to sialic acids. Since sialic acid is present on almost every eucaryotic cell line, this results in an unspecific cell targeting. Generation of a cell-type specificity of this system would imply the presentation of a new function on the surface of VP1. To analyze whether a new functional protein can be placed on VP1, we inserted dihydrofolate reductase from *Escherichia coli* as a model protein. The effect of such an insertion on both VP1 and the inserted protein was investigated, respectively. The function of VP1, like the formation of pentameric capsomers and its ability to assemble into capsids, was not influenced by the insertion. The inserted dihydrofolate reductase showed major changes when compared to the wild-type form. The thermal stability of the enzyme was dramatically reduced in the fusion protein; nevertheless, the dihydrofolate reductase proved to be a fully active enzyme with only slightly increased K_M values for its substrates. This model system provides the basis for further modifications of the VP1 protein to achieve an altered surface of VP1 with new properties.

Keywords: assembly; dihydrofolate reductase; fusion protein; polyoma coat protein VP1; virus-like particle

Murine polyoma virus is a nonenveloped DNA virus belonging to the papovaviridae. The virus coat consists of three coat proteins VP1, VP2, and VP3 (Eckhart, 1990). However, the formation of virus-like particles (VLPs) does not require all three proteins. The isolated major coat protein VP1 was shown to form VLPs itself in vitro (Salunke et al., 1986, 1989) and in vivo (Montross et al., 1991; Forstova et al., 1993).

The VLPs with a diameter of about 50 nm are formed of 360 molecules of VP1, arranged in 72 pentamers. These VLPs are suitable for packaging of DNA, which leads to its use as a transfection agent for eucaryotic cells (Slitay et al., 1982; Forstova et al., 1995; Braun et al., 1999). For this reason, VP1 potentially could be used as a carrier for DNA, RNA, or proteins in gene and cancer therapy. In the case of cell transfection, the adhesion of VLPs to the cells is directed by the ability of VP1 to bind to sialic acid residues, present on almost all cells of higher eucaryotes. For cell-type specific targeting, new functional domains have to be established on the surface of VP1 to mediate a specific targeting

function. Cell-type specific binding of engineered viruses has been shown in case of retroviruses, where the N-terminal fusion of a scFv-fragment mediated the specific binding of the retrovirus to cells in cell culture (Konishi et al., 1998; Martin et al., 1998).

To analyze whether a functional domain can be placed on the surface of VLPs of polyoma VP1, we constructed a fusion protein of VP1 with dihydrofolate reductase (DHFR) as an enzymatic active model system. N- and C-terminus of VP1 are of functional importance and are located in the interior of the VLPs (Garcea et al., 1987; Moreland et al., 1991; Moreland & Garcea, 1991). Therefore, fusions with the termini would not allow presenting a fusion partner on the surface of the VLPs. We decided to insert an amino acid sequence into the surface structure of VP1.

The secondary and tertiary structures of VP1 are mainly characterized by β -sheets, which are arranged in a jelly-roll topology (Stehle et al., 1994; Stehle & Harrison, 1996; Ay et al., 1998a). The pentameric VP1 forms a cylindric structure with an inner and outer site, due to its assembly into VLPs. On the outside, VP1 possesses three large protruding loops, each connecting two strands of a β -sheet. These three loops are termed DE-, HI-, and BC2-loop (Stehle et al., 1994; Stehle & Harrison, 1997). Structural analyses prove that the DE-loop is involved in monomer-monomer interactions within the pentamer (Stehle & Harrison, 1997). The other two loops seem to allow an insertion of an amino acid sequence without strongly disturbing the VP1 structure. We decided to use the position between Asn293 and Tyr294 in the HI-loop for an insertion, because this loop is rather separated within the structure.

Reprint requests to: Hauke Lilie, Institut für Biotechnologie, Martin-Luther-Universität Halle, Kurt-Mothes Straße 3, D-06120 Halle, Germany; e-mail: lilie@biochemtech.uni-halle.de.

Abbreviations: DHFR, hydrofolate reductase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxy-ethyl)piperazine-*N'*-(2-ethanesulfonic acid); IPTG, isopropyl- β -D-thiogalactopyranoside; NADPH, b-Nicotinamide adenine dinucleotide phosphate; PDB, Protein Data Bank; wt, wild-type.

Furthermore, an insertion of another protein would place the protein upright from the VP1 pentamer. This may also sterically block the sialic acid binding site of VP1. Thus, the insertion of a protein at this site should not only place a new function on the surface of VLPs but also diminish the ability of VP1 to bind to sialic acids, necessary for polyomavirus to enter most eucaryotic cells. Hence, this might abolish the natural cell targeting of the VLPs.

The model protein used for the insertion needs to fulfill several prerequisites. The size of the insert should be similar to proteins that might be used later on for cell-type specific binding, which could be Fv-fragments with an average size of about 25 kDa. A second parameter is the access of a crystal structure for modeling studies of the complete fusion protein and the linker design. In a first approach, N- and C-termini of the inserted protein should be close to each other, thus enabling the use of two linker peptides of similar length to connect both N- and C-terminus of the insert with VP1, respectively. Furthermore, expression of the protein has to be possible in a soluble form in the cytosol of *Escherichia coli*. The functional analysis should be feasible by a simple, rapid, and sensitive assay. Based on these prerequisites we chose dihydrofolate reductase from *E. coli*, which is characterized in detail with respect to structure, enzymatic mechanism, folding, and stability (Penner & Frieden, 1987; Leontiev et al., 1993; Sawaya & Kraut, 1997). Because DHFR's N- and C-termini are close to each other on the surface of the protein, we designed Ser-Gly-linker sequences with about the same length (eight and nine amino acids, respectively) to connect VP1 with DHFR. The linker peptides were constructed to enable a flexible layout of five DHFRs on the surface of pentameric VP1 (Fig. 1).

Here we show that it is possible to insert a functional protein into the sequence of VP1. The new fusion protein presents the engineered sequence on the outside of VP1-VLPs. Further, we describe the characteristics of the fusion protein, which could be the basis for future vector systems constructed with multiple functions for different applications.

Results

Expression and purification

For the production of recombinant protein, the cells were cultivated at 15 °C overnight following induction. The first purification step of the soluble fusion protein VP1-DHFR was an affinity chromatography on a chitin column. For further purification, a fractionated ammonium sulfate precipitation and gel filtration were performed (Fig. 2), yielding VP1-DHFR with about 80% purity. The major contaminant (~36 kDa, see Fig. 2) represented a proteolytic cleaved fragment of VP1-DHFR, which was also incorporated in the pentameric protein. Such proteolytic degradation was also shown for wt-VP1 (Leavitt et al., 1985).

One crucial factor for the purification of the fusion protein was the presence of NADPH during chromatography or dialysis. Attempts of purification without the cofactor of DHFR showed a rapid loss of DHFR activity. This was a first indication of a significant destabilization of DHFR in the fusion protein.

Stability of the VP1-DHFR

The enzymatic activity of DHFR in the fusion protein was dependent on the presence of the cofactor during purification. This suggested that DHFR as part of the fusion protein denatured at

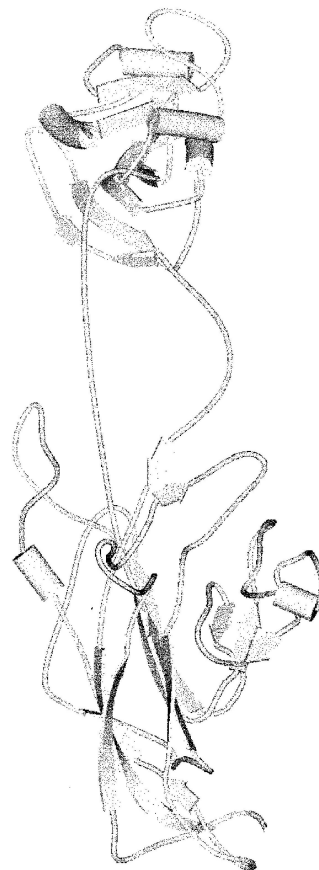


Fig. 1. Model of the fusion protein VP1-DHFR (schematic drawing). The model is composed of the structures of polyoma VP1 (PDB entry 1VPS) and *E. coli* DHFR (PDB entry 4dfr). The Serine-Glycine Linkers were inserted using the program SYBELL. No extensive attempts were made to energy minimize the structure of the fusion protein.

temperatures below 10 °C during purification in the absence of NADPH. To further analyze the stability of the fusion protein, temperature denaturation measurements were performed. As shown in Figure 3, in the absence of NADPH two transitions could be detected. One showed a midpoint of $T_m = 49\text{--}50$ °C, which is similar to that of wild-type VP1 (data not shown; Stubenrauch et al., 1999). The other transition was in the range of 4–7 °C, and belonged to the denaturation of DHFR. This finding coincides with the fact that purified VP1-DHFR lost its activity even below 10 °C (data not shown). The addition of 12 μM NADPH shifted this transition from 4 °C to about 30 °C (see Fig. 3). Further increasing the NADPH concentration 10-fold did not result in an apparent further stabilization of the DHFR part (data not shown). The denaturation of VP1 ($T_m = 50$ °C) was not affected by the presence of NADPH, indicating that the effect of NADPH as cofactor of DHFR was rather specific. These data indicated that the stability of VP1 seemed not to be influenced. Thermodynamic parameters could not be calculated from the transitions because in both cases the temperature denaturation was irreversible (data not shown).

2564

S. Gleiter et al.

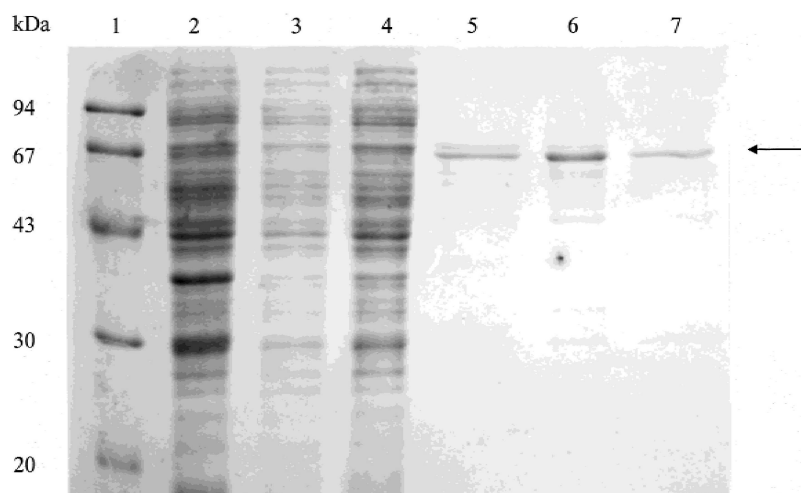


Fig. 2. Purification of VP1-DHFR. *E. coli* producing VP1-DHFR was treated as described. Along the purification scheme aliquots were taken and analyzed by 12% SDS-PAGE. VP1-DHFR is marked by an arrow. Lane 1, marker; Lane 2, cell lysate; Lane 3, supernatant after pelleting cell debris; Lane 4, flow through from intein beads; Lane 5, elution from intein beads after cleavage with DTT; Lane 6, resuspended ammonium sulfate precipitate of VP1-DHFR; Lane 7, major fraction of VP1-DHFR after gel filtration.

Activity and assembly

Besides the stability of the fusion protein, the functional properties were most important. The stabilization of DHFR by addition of NADPH was already the first indication that the active site of DHFR was functional. For a more detailed characterization, the K_M values for dihydrofolate and NADPH were determined. Since several different conditions were described in the literature to measure enzymatic activity of DHFR, especially with respect to tem-

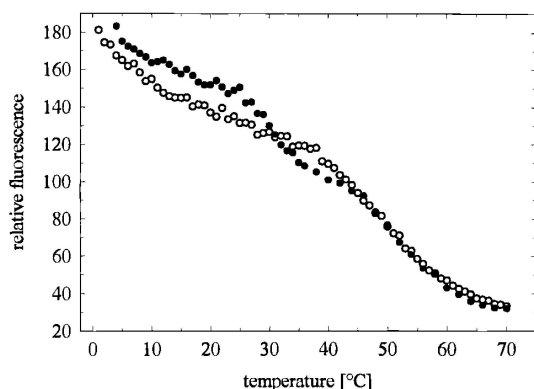


Fig. 3. Temperature denaturation of VP1-DHFR. VP1-DHFR at a protein concentration of 50 $\mu\text{g/mL}$ in buffer 100 was incubated at a heating rate of 0.3 $^{\circ}\text{C}/\text{min}$ with or without 12 μM NADPH, respectively. The change of fluorescence during heating was monitored using 280 nm for excitation and 340 nm for emission. In the absence of NADPH (○), two transitions at 4 and 50 $^{\circ}\text{C}$ could be observed, respectively. In the presence of 12 μM NADPH (●), the first transition shifted to a temperature of about 30 $^{\circ}\text{C}$, whereas the second transition was not affected.

perature and assay buffer composition, we first analyzed which conditions would be suitable to characterize the strongly destabilized DHFR within the fusion protein.

In Figure 4A, the incubation of the DHFR fusion protein at two different temperatures, normally used in DHFR assays, is shown. Incubation of VP1-DHFR at 37 $^{\circ}\text{C}$ in the presence of 10 μM NADPH resulted in an inactivation of DHFR proceeding with a half time of about $t_{1/2} = 10$ min. This denaturation process is in good agreement with the temperature transition of the DHFR moiety within the fusion protein (see Fig. 3). Due to this fast denaturation, these assay conditions proved not to be suitable for the enzymatic characterization of VP1-DHFR. In contrast, at 20 $^{\circ}\text{C}$ and 12 μM NADPH no inactivation could be observed in a time range of about 1 h. Therefore, the K_M for dihydrofolate was determined at 20 $^{\circ}\text{C}$, conditions very similar to that used by Penner and Frieden (1987). The apparent K_M value of dihydrofolate was $K_M = 14$ μM (Fig. 4B), which is 50-fold higher than that for wild-type DHFR (wtDHFR). The apparent K_M value for NADPH was determined to be $K_M = 8$ μM (data not shown). This is about eight times higher than that of wtDHFR (Penner & Frieden, 1987). It has to be noted that these are only average values, reflecting the crowded appearance of five DHFR molecules on one VP1 pentamer. That not all DHFR molecules act identical under these conditions is also suggested by a residual activity after one hour of incubation at 37 $^{\circ}\text{C}$ (see Fig. 4A). Due to this apparent inhomogeneity of DHFR, a more detailed enzymatic characterization was not possible in this context. Although the analysis of enzymatic activity of DHFR within the fusion protein was hampered by this complexity, the data clearly demonstrated that a functional enzyme could be inserted in the HI-loop of VP1.

For the VP1 part of the fusion protein, two parameters are crucial as indicators of functionality. One is the ability to form pentamers. This specific association requires a highly ordered tertiary structure, especially because a kind of domain swapping has

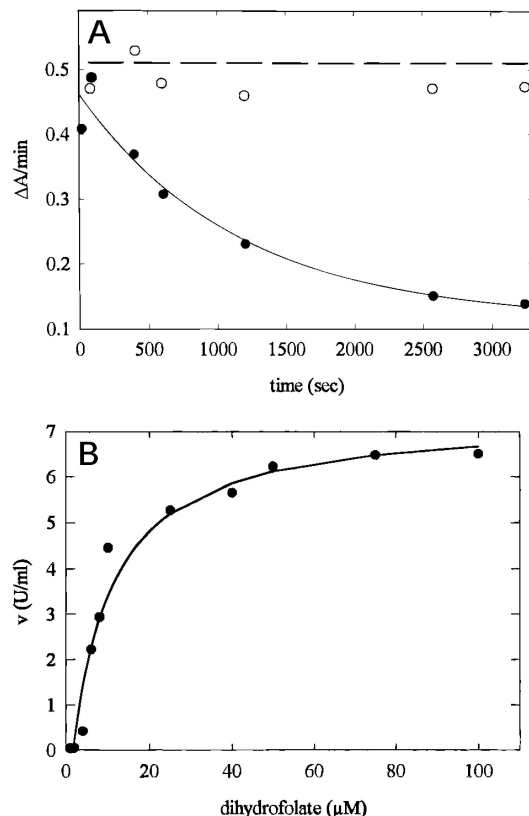


Fig. 4. Enzymatic activity of VP1-DHFR. **A:** For inactivation kinetics VP1-DHFR was incubated in 50 mM potassium phosphate, 1 mM EDTA, 10 mM β -mercaptoethanol, 10 μM NADPH, pH 7.0 at 20 °C (○) and 37 °C (●). At different time points aliquots were assayed on enzymatic activity of DHFR. **B:** To determine v/S -characteristics of VP1-DHFR, the fusion protein was preincubated in 50 mM potassium phosphate, 1 mM EDTA, 10 mM β -mercaptoethanol, 10 μM NADPH, pH 7.0 at 20 °C for 3 min. The turn over was started by adding dihydrofolate at different concentrations. From the data an apparent K_M value of $K_M = 14 \mu\text{M}$ was calculated.

to occur between neighboring monomers within the pentamer. One strand of a β -sheet of one monomer is generated by a neighboring monomer within the pentamer (Stehle & Harrison, 1997). Analytical gel filtration of the purified VP1-DHFR showed that the fusion protein indeed associated to pentamers (data not shown). Using analytical ultracentrifugation as an additional technique, an s -value of $s_{w,20} = 7.8 \text{ S}$ could be calculated (Fig. 5A), similar to that of wt VP1 with $s_{app} = 7.2 \text{ S}$ (Salunke et al., 1986) and a molecular weight of 288 kDa was determined very similar to the calculated mass of the pentameric fusion protein of $M_r = 308 \text{ kDa}$ (Fig. 5B). These results clearly showed that the insertion of the DHFR did not affect the interaction of VP1 monomers within the pentameric state.

VP1 possesses a binding affinity to sialic acid residues. The respective binding site lies beneath the HI-loop. To investigate whether the insertion of DHFR in the HI-loop blocks the binding site, a haemagglutination assay was performed. Haemagglutina-

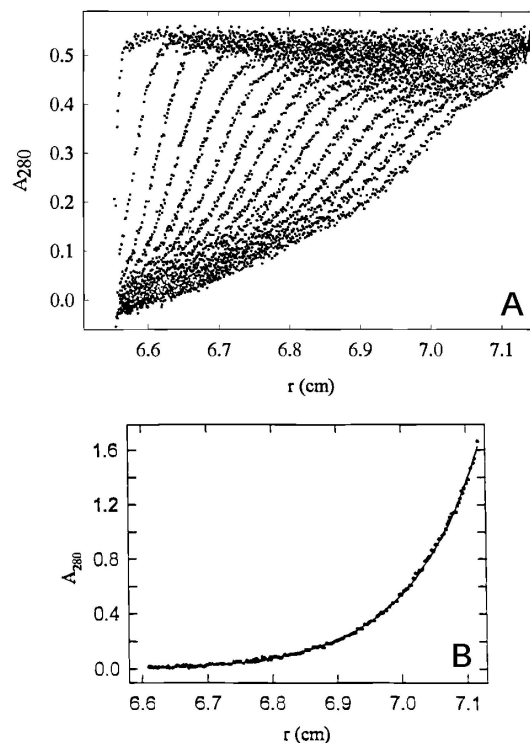


Fig. 5. Analytical ultracentrifugation of the VP1-DHFR pentamer. **A:** The sedimentation velocity of VP1-DHFR was determined at 30,000 rpm, 10 °C. Radial scans at 280 nm were taken every 10 min. From the experimental data an s -value of $s_{w,20} = 7.8 \text{ S}$ was calculated. **B:** Sedimentation equilibrium was achieved at 10,000 rpm, 10 °C. The fit of the experimental data, corrected for temperature and viscosity effects resulted in a molecular mass of $M_r = 288,000$.

tion was previously used to investigate the interaction of different polyoma strains with cell surfaces (Freund et al., 1991). As shown in Figure 6, wtVP1 haemagglutinated sheep erythrocytes at a protein concentration of at least 50 ng/mL. For the fusion protein VP1-DHFR, however, even at concentration of 50 $\mu\text{g/mL}$ no haemagglutination activity could be observed. Most likely DHFR sterically blocks the sialic acid binding site of VP1.

Another functional parameter of VP1 is its ability to assemble into VLPs. To analyze whether VP1-DHFR pentamers were still

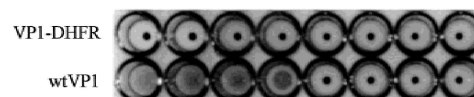


Fig. 6. Haemagglutination assay of VP1-DHFR. Haemagglutination of VP1-DHFR and wtVP1 was determined with sheep erythrocytes. Protein concentrations ranged from 0.05 mg/mL to $0.5 \times 10^{-6} \text{ mg/mL}$ (10-fold dilution steps from left to right). VP1-DHFR showed no haemagglutination whereas wtVP1 haemagglutinated erythrocytes at a protein concentration of at least 50 ng/mL.

2566

S. Gleiter et al.

able to form VLPs, the fusion protein was dialyzed against assembly buffer. Protein concentration, temperature, and time of dialysis were chosen as described by Salunke et al. (1989). The presence of VLPs could be detected by analytical gel filtration with a specific elution comparable to that of wtVP1 (data not shown). Sedimentation velocity analysis of the VLPs revealed an apparent s -value of $s_{app} = 56$ S (Fig. 7), clearly indicating an assembly process has taken place. However, the s -value is smaller than that for VLPs formed of wtVP1 under identical conditions ($s = 144$ S (Crawford & Crawford, 1963; Salunke et al., 1986; Kosukegawa et al., 1996)), suggesting overall smaller particles in the case of VP1-DHFR. Additionally, about 20% of VP1-DHFR did not assemble (cf. Fig. 7). To analyze the size of the VLPs in more detail, electron microscopy was applied. Negative stained VLPs with a size between 20 and 50 nm in diameter could be observed (Fig. 8). Although under the conditions used, wtVP1 predominantly assemble into 50 nm particles, containing 72 pentamers, smaller VLPs consisting only of 24 or 18 pentamers are also known (Salunke et al., 1989). It appears that the presentation of DHFR on the surface of the VP1-VLPs shifted the equilibrium between the different forms of assembly toward the smaller species. This might be explained by steric hindrance of DHFR on the surface of the VLPs. Smaller particles have a higher curvature and, therefore, DHFR on those particles would be not as tightly packed as on bigger particles with a smaller curvature. Nevertheless, the data presented clearly showed that the formation of VLPs is possible with the fusion protein.

Discussion

The modification of the surface of viruses is widely used for research and medical purposes. In the last years, different virus coat proteins from retroviruses or polio virus, for example, were modified to develop new vector systems for gene therapy or vaccination (Tang et al., 1997; Martin et al., 1998). The type of

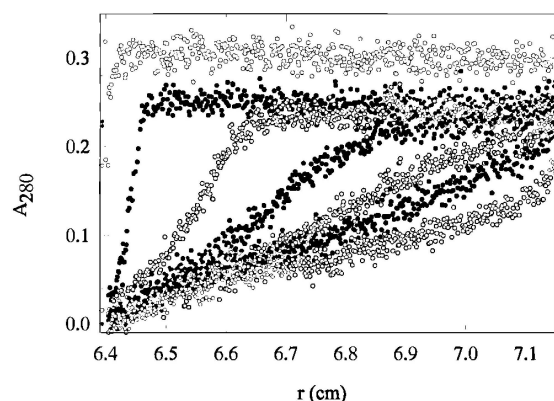


Fig. 7. Analytical ultracentrifugation of VLPs of VP1-DHFR. VP1-DHFR at a concentration of 0.25 mg/mL was assembled by dialysis against 20 mM Tris, 0.75 M ammonium sulfate, 1 mM CaCl_2 , 5% (w/v) glycerol, 10 μM NADPH, pH 7.3 at 20 °C. The VLPs were centrifugated at 10,000 rpm. Sedimentation scans at 280 nm were measured every 10 min. For cleanness only every fifth scan is displayed. The apparent sedimentation velocity of the VLPs was calculated with $s_{app} = 56$ S. About 20% of VP1-DHFR did not assemble into VLPs.

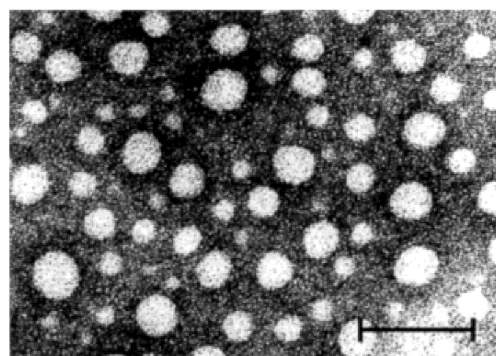


Fig. 8. Electron micrograph of VLPs of VP1-DHFR. VLPs were adsorbed on a formvar coated copper grid. Negative staining of the protein was performed using uranylacetate. Electron micrographs were taken at a LEO EM 920 Omega electron microscope at a 100,000-fold magnification. The bar corresponds to 145 nm.

modifications ranges from fusions of small peptide fragments up to scFv fragments. In another approach, fusion of phage proteins with other proteins is used for phage display systems (Clackson et al., 1991; Bothmann & Pluckthun, 1998). In the case of polyoma virus, recombinantly produced VLPs of the major coat protein VP1 were recently used for transfection of eucaryotic cells (Forstova et al., 1995; Soeda et al., 1998). For these studies, wtVP1, with its ability to bind sialic acids on cell surfaces, was employed. To further analyze the potential of polyoma VP1 in constructing vectors for gene therapy, we investigated the possibility of changing the surface of the VLPs by insertion of a protein with a distinct function into the sequence of VP1.

Here, the ecDHFR was inserted as a model protein. It is a small protein of about 18 kDa and belongs to the class of oxidoreductases whose characteristics are well studied. Analyses of the fusion protein comprises of the enzymatic characteristics of DHFR and structural and functional parameters of VP1. The crucial feature of VP1 is the association into pentamers thus forming capsomers and subsequently the assembly into VLPs under given conditions.

The ecDHFR was integrated into the HI-loop on the surface of VP1, which is located on the outside when VP1 assembles into capsids. Insertion of a protein in this loop should place it upright from VP1 thus minimizing steric hindrance and blocking the sialic acid binding site of VP1. Circular permutation studies on several proteins have shown that the disconnection of loop structures in proteins does not have severe effects on the overall structure and stability of proteins in contrast to circular permutation within secondary structure elements (Buchwalder et al., 1992; Ay et al., 1998b; Hennecke et al., 1999). Especially interesting for the case discussed here proves to be the β -glucanase, which also possesses a jelly-roll topology, similar to the structure of VP1. β -glucanase was circularly permuted with new termini in a loop connecting two strands of one β -sheet (Ay et al., 1998b). Based on these data, the HI-loop of VP1 was selected for insertion of DHFR. To increase the probability that within the fusion VP1 and DHFR could act as independent entities, the fusion was performed using two Ser-Gly linkers (eight and nine residues, respectively). These linkers should warrant a flexibility of the DHFR on the surface of the VP1 pentamer. In this first design, the fusion impairs the structural integrity of DHFR. One indicator for a change of DHFR in this fusion

protein was the activity of the DHFR part. In the absence of cofactors, thermal denaturation occurred below 10 °C. Despite this drastic destabilization, DHFR was shown to be fully active, with K_M values for the substrates changing only by a factor of 8 and 50, respectively. The appearance of enzymatic activity in principle shows that the DHFR part in the fusion protein is quite functional. It remains open what reason can be found for the increased K_M value. To answer this question, detailed enzymatic analyses have to be performed. At present, it is not possible to decide if the altered kinetic parameters are a result of a limited access of substrate and cofactor, due to the steric vicinity of five DHFR molecules, or the need of DHFR for large subdomain movements during the catalytic cycle (Sawaya & Kraut, 1997). Due to the connection of N- and C-termini of DHFR with VP1, the flexibility of DHFR might be reduced dramatically. The reduction of subdomain movement could contribute to the raise of the K_M value. These aspects would suggest that the reduction in enzymatic activity might be at least partially a specific problem of the very complex model system DHFR.

Furthermore, the significant reduced thermal stability seems to be an indication for structural changes and thereby triggering problems within the fusion protein. However, the extreme positive effect of NADPH suggests that structural changes alone are not the reason for the reduced stability and activity. Despite these shortcomings, the designed fusion protein is capable of placing a functional enzyme on the surface of VP1.

More important than the model enzyme DHFR, VP1 is not significantly affected by the insertion. The temperature transition is comparable to that of wtVP1. Despite the destabilization of DHFR, VP1 at least seems to act as an independent folding unit, structurally not affected. Beside the structural characteristics, we investigated how far functional parameters of VP1 were changed in the fusion protein. It could be demonstrated that within the fusion VP1 is in a pentameric state. No equilibrium with monomers nor aggregation that would indicate dissociation with concomitant off-pathway reactions could be detected. As expected from the location of DHFR in the fusion protein, DHFR blocks the sialic acid binding site of VP1. Thus, in contrast to wtVP1, VP1-DHFR does not show any affinity to the surface of erythrocytes.

The next characteristic of VP1-DHFR to be investigated was the assembly into VLPs, which have diameters of about 50 nm if constituted of wtVP1. The assembly of wtVP1 into VLPs is a reaction that only depends on the presence of Ca^{2+} and oxidizing conditions (Salunke et al., 1986, 1989). The VLPs can be obtained simply by dialysis against an assembly buffer. Similar to wtVP1, the fusion protein VP1-DHFR is capable of the formation of VLPs. The predominant population consists of particles with a size distribution from 40 to 20 nm. Additionally, there are several particles with a diameter of 50 nm, which is found as the predominant assembly product of wtVP1. It is known from wtVP1, however, that this protein has the ability to form capsids of different size under different conditions, depending on pH and ionic strength (Salunke et al., 1989). The other particles represent VLPs formed of 18 and 24 pentamers of VP1, respectively. In the case of VP1-DHFR, the formation of the 50 nm VLPs is probably hindered by the presence of the five DHFR molecules on top of the pentamer, so that the equilibrium in VLP formation is shifted toward the smaller products.

In summary, we report the possibility to modify the functional aspects of polyoma VP1 by combining VP1 with another protein. A new functional domain could be placed on the surface of VP1

without significant changes in the original functions of VP1. This is the basis for the development of VLPs, which can be modified according to different purposes such as cell-type specific targeting or escape of the immune system. With the insertion of functional domains, new characteristics of the VLP could be achieved. We could show that the assembly of such a modified virus coat protein into VLPs is possible even though a new protein sequence was inserted in a surface loop of VP1. In the first design of experiments this assembly resulted in inhomogeneous particles. To achieve a homogeneous population of VLPs with a diameter of 50 nm, as described for wtVP1, the mixed assembly of wt-protein with the insertion mutant could be a possibility. This should generate larger particles that are better suitable of packaging multiple therapeutics currently used for different therapy strategies. Therefore, the fusion protein described is a promising basis for further attempts to construct fusions with functional modules essential for a gene therapeutic approach.

Materials and methods

Plasmid construction

The coding sequence for the fusion protein VP1-DHFR was constructed by polymerase chain reaction (PCR) of three overlapping fragments A, B, C. Fragment A encoding residues 1–293 of VP1 and a (Ser-Gly)₄ linker sequence at the C-terminal end was amplified from pALVTAC (Leavitt et al., 1985) with primers vpnde (5'-TATACATATGGCCCCAAAAGAAAAGC-3') and vp1li (5'-GCCGCTACCGCTACCGCTGCCGCTGTTTCTTGTAACTCTCAGCCC-3'). Fragment B encoding the linker sequence (GlySer)₄Gly at the N-terminal end of this fragment and residues 294–385 of VP1 was amplified from the same plasmid with primers livp1 (5'-GGCAGCGGTAGCGGCAGCGGTAGCTATGATGCCATCACTGGAGAG-3') and vpnot (5'-TATAGTTTAGCGGCCGCTTAATTCCAGGAAATACAGTCT-3'). Fragment C consisted of the ecDHFR-gene amplified from plasmid DHFR6, which encoded the genomic ecDHFR (U. Fiedler, pers. comm.) with primer lidhfr (5'-AGCGGCAGCGGTAGCGGTAGCGGCAGCATGATCAGTCTGATTGCGGCGTTAG-3') and primer dhfrli (5'-GCTACCGTGCCGCTACCGTGCCCCGCCGCTCCAGAATCTCAAGCA-3'), which also encoded the N- and C-terminal linker sequences, thus overlapping with the fragment A and B.

Amplification products were separated on 1% (w/v) agarose gel (ICN) and purified with the GFX-PCR-purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Fragments B and C were combined for an overlapping amplification with the primer lidhfr and vp1not. The PCR product was again isolated from an agarose gel and used in a PCR with fragment A and primer vpnde and primer vpnot to give the entire VP1-DHFR fusion gene. The fragment was then cloned in pET21a (+) (Novagen, Madison, Wisconsin) using the restriction enzymes *Nde*I and *Not*I.

To make use of a one step affinity purification system (IMPACT-system, NEB), VP1-DHFR sequence was again amplified in a PCR with primers vp1Nimp (5'-TATACATATGGCCCCAAAAGAAAAGC-3') and VP1Cimp (5'-ATATCCCGGGAGGAAAATACAGTCTTTGTTTTTCC-3'). This introduces a *Xma*I site at the 3'-end of the vp1-dhfr gene, required for cloning into the vector with the intein gene. Additionally, the last two C-terminal residues were changed from Gly384–Asn385 to Pro384–Gly385, as recommended in the manufacturer's manual. This fragment was cloned

2568

S. Gleiter et al.

in a pET21a(+) vector (Novagen), which already contained the intein gene. The resulting plasmid was termed pVP1DHFR.

Protein expression

The plasmid pVP1DHFR was transformed into the expression strain BL21(DE3) (Stratagene, La Jolla, California). To optimize expression conditions of the fusion protein cultivations were carried out at different temperatures in 1 L shake flasks with 250 mL LB-media.

At $OD_{500} = 0.5$, protein expression was induced with IPTG (Biomol) at a final concentration of 1 mM. After induction, cells were incubated overnight and then harvested by centrifugation. The pellet was resuspended in buffer 100 (20 mM HEPES, 1 mM EDTA, 5% (w/v) glycerol, 100 mM NaCl, 10 μ M NADPH, pH 8.0). Cell lysis was achieved using a bead mill. Soluble and insoluble fractions were analyzed by SDS-PAGE.

For large scale preparation of VP1-DHFR *E. coli* strain BL21(DE3) was cultivated on mineral salt medium at 30 °C at a scale of 5 L in a Biostat-Fermenter (Fa. Braun) using the fed-batch technique (Teich et al., 1998). The recombinant expression of VP1-DHFR was induced by 0.4 mM IPTG after 3 h of feeding start. At the time of induction, the temperature was set to 18 °C and was maintained until the end of fermentation at 15 h. Cells were harvested by centrifugation (8,000 g, 15 min) and stored at -70 °C. For purification cell lysis was achieved by high pressure dispersion (Gaulin Micron Lab 40). The crude extract was centrifugated for 1 h at 20,000 rpm. Soluble VP1-DHFR was purified from the supernatant.

Protein purification

The soluble fraction of the cell lysate was incubated with 5 mL chitin beads (NEB) equilibrated in buffer 100 in a batch procedure. Afterward, beads were washed with 15 mL buffer 100 and incubated for 15 min. The beads were then washed with 15 mL of buffer, consisting of equal amounts buffer 100 and buffer 2000 (20 mM HEPES, 1 mM EDTA, 5% (w/v) glycerol, 2,000 mM NaCl, 10 μ M NADPH, pH 8.0) and incubated the same time. This was followed by a washing step with 40 mL of buffer 2000 and 15 mL of buffer 100. Elution of the bound fusion protein was obtained by inducing the self-splicing mechanism of the intein with 15 mL of buffer 100 supplemented with 50 mM DTT. For complete cleavage, the beads were incubated in 5 mL of this buffer overnight at 4 °C. Finally, the fusion protein was eluted with 10 mL of buffer.

For further purification of VP1-DHFR, a fractionated ammonium sulfate precipitation between 25–45% was performed. The ammonium sulfate precipitate was resuspended in buffer 200 (50 mM Tris/HCl, 5% (w/v) glycerol, 2 mM EDTA, 2 mM DTT, 200 mM NaCl, 10 μ M NADPH, pH 7.2) and dialyzed against the same buffer overnight.

To separate aggregated VP1-DHFR from the native pentamer gel filtration was performed using a Superdex 200 prep grade column (120 mL Amersham Pharmacia Biotech) equilibrated in buffer 200.

Analytical gel filtration

The analytical gel filtration was performed on a TSK5000 column (TosoHaas). The column was equilibrated in 10 mM HEPES,

200 mM NaCl, 0.5 mM CaCl₂, 5% (w/v) glycerol, pH 7.2. The flow rate was 0.7 mL/min. Specific elution volumes of pentameric protein and VLPs were determined using wtVP1.

Temperature denaturation

Temperature transitions of VP1 and VP1-DHFR were determined using a Hitachi F-4500 fluorescence spectrometer in stirrable cuvettes with a light path of 10 mm. The excitation wavelength was set to 295 nm and the emission was measured at 340 nm. A programmable water bath was used to ensure a heating rate of about 0.3 °C/min. Temperature transitions were measured in the range from 2–80 °C.

Enzymatic assay of DHFR

Enzymatic activity of DHFR was measured photometrically at 340 nm and 20 °C (Zywno-van et al., 1997). The reaction mixture consisted of 10 mM β -mercaptoethanol, 0.1 mM NADPH, 0.1 mM dihydrofolate and 50 μ L of VP1-DHFR solution in a standard buffer of 50 mM potassium phosphate, 1 mM EDTA, pH 7.0. The total assay volume was 1 mL.

The reaction was started by addition of dihydrofolate after incubation of all required components at 20 °C. In case of determining the K_M value for NADPH, the reaction mixture consisted of dihydrofolate instead of NADPH and the reaction was started with NADPH.

Protein analysis

Proteins were separated on 12% SDS-gels. The gels were stained with Coomassie Blue (Fairbanks et al., 1971).

Analytical ultracentrifugation

Pentameric VP1 with a concentration of 0.45 mg/mL in buffer VP1-200 (without glycerol) was analyzed in a Beckman Optima XL-A centrifuge with the AN60Ti rotor. Sedimentation velocity experiments were performed at 30,000 rpm and 10 °C. Sedimentation equilibrium centrifugation was performed at 10,000 rpm, 10 °C for 40 h. The sedimentation velocity of VLPs was measured in assembly buffer at 10 °C, 10,000 rpm. The data were analyzed using the software provided by Beckman Instruments (Fullerton, California).

Assembly of VP1-DHFR

Assembly of VP1-DHFR to VLPs was induced by dialyzing VP1-DHFR against assembly buffer (0.75 M ammonium sulfate, 20 mM Tris, 5% (w/v) glycerol, 1 mM CaCl₂, 10 μ M NADPH, pH 7.3) for 2 days. After dialysis the samples were centrifugated at 13,000 rpm for 30 min at 4 °C. Aliquots were used for electron microscopy, analytical gel filtration, and ultracentrifugation.

Electron microscopy

Formvar and carbon coated grids were used for negative staining as described by Dyksta (1992). Transmission electron microscopy was performed with a LEO EM 920 Omega microscope. Photographs were taken at magnifications of 100,000-fold.

Fusion protein of VP1

2569

Haemagglutination assay

Haemagglutination was determined in 0.9% (w/v) NaCl solution using U-well plates (Nunc, Roskilde, Denmark) and sheep erythrocytes (Dade-Behring, Behring, Germany). Protein concentrations in the assay ranged from 0.05 mg/mL to 0.5×10^{-6} mg/mL for both wtVP1 and VP1-DHFR, respectively. Dilutions were made in a 0.9% NaCl solution. Forty-five microliters of each dilution was supplemented with 45 μ L of a 20-fold diluted erythrocyte stock solution. Haemagglutination was assayed after 3 h incubation at 7 °C.

Acknowledgments

We thank Uli Schmidt for providing us with the intein expression vector, and Ulrike Fiedler for plasmid DHFR6 encoding ecDHFR, Dieter Neuman who made the electron microscope available for us, and Peter Neubauer who instructed the fermentation of VP1-DHFR. Gerald Boehm and Rainer Rudolph are acknowledged for fruitful discussion and Sarah Olson, Martin Kleinschmidt, and Lars Waldmann for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

References

- Ay J, Gotz F, Borriess R, Heinemann U. 1998a. Structure and function of the bacillus hybrid enzyme GluXyn-1: Native-like jellyroll fold preserved after insertion of autonomous globular domain. *Proc Natl Acad Sci USA* 95:6613–6618.
- Ay J, Hahn M, Decanniere K, Piotukh K, Borriess R, Heinemann U. 1998b. Crystal structures and properties of de novo circularly permuted 1,3-1,4-beta-glucanases. *Proteins* 30:155–167.
- Bothmann H, Pluckthun A. 1998. Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nat Biotechnol* 16:376–380.
- Braun H, Boller K, Lower J, Bertling WM, Zimmer A. 1999. Oligonucleotide and plasmid DNA packaging into polyoma VP1 virus-like particles expressed in *Escherichia coli*. *Biotechnol Appl Biochem* 29(Part 1):31–43.
- Buchvalder A, Szadkowski H, Kirschner K. 1992. A fully active variant of dihydrofolate reductase with a circularly permuted sequence. *Biochemistry* 31:1621–1630.
- Clackson T, Hoogenboom HR, Griffiths AD, Winter G. 1991. Making antibody fragments using phage display libraries. *Nature* 352:624–628.
- Crawford LV, Crawford EM. 1963. A comparative study of polyoma and papilloma viruses. *Virology* 21:258–263.
- Dyksta MJ. 1992. *Biological electron microscopy*. New York: Plenum Press.
- Eckhart W. 1990. Polyomavirinae and their replication. In: Fields BN, Knipe DM, eds. *Fundamental virology*. New York: Raven Press. pp 727–742.
- Fairbanks G, Steck T, Wallach D. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606–2617.
- Forstova J, Krauzewicz N, Sandig V, Elliott J, Palkova Z, Strauss M, Griffin BE. 1995. Polyoma virus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells. *Hum Gene Ther* 6:297–306.
- Forstova J, Krauzewicz N, Wallace S, Street AJ, Dilworth SM, Beard S, Griffin BE. 1993. Cooperation of structural proteins during late events in the life cycle of polyomavirus. *J Virol* 67:1405–1413.
- Freund R, Garcea RL, Sahli R, Benjamin TL. 1991. A single-amino-acid substitution in polyomavirus VP1 correlates with plaque size and hemagglutination behavior. *J Virol* 65:350–355.
- Garcea RL, Salunke DM, Caspar DL. 1987. Site-directed mutation affecting polyomavirus capsid self-assembly in vitro. *Nature* 329:86–87.
- Hennecke J, Sebbel P, Glockshuber R. 1999. Random circular permutation of DsbA reveals segments that are essential for protein folding and stability. *J Mol Biol* 286:1197–1215.
- Konishi H, Ochiya T, Chester KA, Begent RH, Muto T, Sugimura T, Terada M. 1998. Targeting strategy for gene delivery to carcinoembryonic antigen-producing cancer cells by retrovirus displaying a single-chain variable fragment antibody. *Hum Gene Ther* 9:235–248.
- Kosukegawa A, Arisaka F, Takayama M, Yajima H, Kaidow A, Handa H. 1996. Purification and characterization of virus-like particles and pentamers produced by the expression of SV40 capsid proteins in insect cells. *Biochim Biophys Acta* 1290:37–45.
- Leavitt AD, Roberts TM, Garcea RL. 1985. Polyoma virus major capsid protein, VP1. Purification after high level expression in *Escherichia coli*. *J Biol Chem* 260:12803–12809.
- Leontiev VV, Uversky VN, Gudkov AT. 1993. Comparative stability of dihydrofolate reductase mutants in vitro and in vivo. *Protein Eng* 6:81–84.
- Martin F, Kupsch J, Takeuchi Y, Russell S, Cosset FL, Collins M. 1998. Retroviral vector targeting to melanoma cells by single-chain antibody incorporation in envelope. *Hum Gene Ther* 9:737–746.
- Montross L, Watkins S, Moreland RB, Mamon H, Caspar DL, Garcea RL. 1991. Nuclear assembly of polyomavirus capsids in insect cells expressing the major capsid protein VP1. *J Virol* 65:4991–4998.
- Moreland RB, Garcea RL. 1991. Characterization of a nuclear localization sequence in the polyomavirus capsid protein VP1. *Virology* 185:513–518.
- Moreland RB, Montross L, Garcea RL. 1991. Characterization of the DNA-binding properties of the polyomavirus capsid protein VP1. *J Virol* 65:1168–1176.
- Penner MH, Frieden C. 1987. Kinetic analysis of the mechanism of *Escherichia coli* dihydrofolate reductase. *J Biol Chem* 262:15908–15914.
- Salunke DM, Caspar DL, Garcea RL. 1986. Self-assembly of purified polyomavirus capsid protein VP1. *Cell* 46:895–904.
- Salunke DM, Caspar DL, Garcea RL. 1989. Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophys J* 56:887–900.
- Savaya MR, Kraut J. 1997. Loop and subdomain movements in the mechanism of *Escherichia coli* dihydrofolate reductase: Crystallographic evidence. *Biochemistry* 36:586–603.
- Slilaty SN, Berns KI, Aposhian HV. 1982. Polyoma-like particle: Characterization of the DNA encapsidated in vitro by polyoma empty capsids. *J Biol Chem* 257:6571–6575.
- Soeda E, Krauzewicz N, Cox C, Stokrova J, Forstova J, Griffin BE. 1998. Enhancement by polylysine of transient, but not stable, expression of genes carried into cells by polyoma VP1 pseudocapsids. *Gene Ther* 5:1410–1419.
- Stehle T, Harrison SC. 1996. Crystal structures of murine polyomavirus in complex with straight-chain and branched-chain sialyloligosaccharide receptor fragments. *Structure* 4:183–194.
- Stehle T, Harrison SC. 1997. High-resolution structure of a polyomavirus VP1-oligosaccharide complex: Implications for assembly and receptor binding. *Embo J* 16:5139–5148.
- Stehle T, Yan Y, Benjamin TL, Harrison SC. 1994. Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* 369:160–163.
- Stubenrauch K, Bachmann A, Rudolph R, Lilie H. 1999. Purification of a viral coat protein by an engineered polyionic sequence. *J Chromatogr. Forthcoming*.
- Tang S, van RR, Silvera D, Andino R. 1997. Toward a poliovirus-based simian immunodeficiency virus vaccine: Correlation between genetic stability and immunogenicity. *J Virol* 71:7841–7850.
- Teich A, Lin HY, Andersson L, Meyer S, Neubauer P. 1998. Amplification of ColE1 related plasmids in recombinant cultures of *Escherichia coli* after IPTG induction. *J Biotechnol* 64:197–210.
- Zyvno-van GS, Dooley TP, Suling WJ, Barrow WW. 1997. Identification and cloning of the *Mycobacterium avium* folA gene, required for dihydrofolate reductase activity. *FEMS Microbiol Lett* 156:69–78.

Coupling of antibodies via protein Z on modified polyoma virus-like particles

STEFAN GLEITER AND HAUKE LILIE

Martin-Luther-Universität Halle, Institut für Biotechnologie, D-06120 Halle, Germany

(RECEIVED July 27, 2000; FINAL REVISION November 14, 2000; ACCEPTED November 21, 2000)

Abstract

Therapeutic application of virus-based delivery systems often implies a change of the tropism of these vectors. This can be achieved by insertion of polypeptides (e.g., antibody fragments) in viral coat proteins. Such fusion proteins have only been used in viral vectors so far and, as part of a virus, they have not been available for a detailed biophysical characterization. We analyzed a fusion protein called VP1-Z, which is based on the polyoma virus coat protein VP1 and protein Z. Protein Z is an engineered antibody-binding domain derived from protein A from *Staphylococcus aureus*. The fusion VP1-Z was constructed by insertion of protein Z in the HI-loop of VP1. As wild-type VP1, VP1-Z formed pentameric capsomers and assembled to VLPs in vitro. The stability of these particles was very similar compared to that of VLPs of wild-type VP1. Protein Z was fully structured in the fusion protein and was still capable of binding antibodies on the surface of VLPs of VP1-Z. Using this fusion protein, we could change the tropism of polyoma VLPs toward cells presenting on their surface the antigen of the coupled antibody.

Keywords: Polyoma; VP1; protein Z; antibody; stability; targeting; fluorescence microscopy

Murine polyoma virus is a nonenveloped DNA virus that belongs to the family Papovaviridae. The viral coat consists of three coat proteins VP1, VP2, and VP3 (Eckhart 1990). However, the formation of VLPs does not depend on the presence of all three polyoma coat proteins. It is possible to form VLPs from isolated recombinant VP1 in vitro (Salunke et al. 1986, 1989; Garcea et al. 1987). Usually these VLPs, which are ~50 nm in diameter, consist of 360 VP1 molecules arranged in 72 pentamers. However, depending on the conditions used for assembly, smaller particles that contain only 24 or 18 pentamers can be produced (Salunke et al. 1989).

VLPs of VP1 can be used to package DNA and they have successfully been applied in cell transfection experiments

(Forstova et al. 1995; Soeda et al. 1998; Braun et al. 1999; Krauzewicz et al. 2000) thus showing their value as vector system for gene or cancer therapy. In the case of cell transfection, the adhesion of VP1 to the cell surface is mediated by its natural sialic acid-binding activity (Freund et al. 1991; Stehle and Harrison 1996, 1997). As for the presence of sialic acid residues on the surface of almost all mammalian cells, this specific binding leads to cell-type-unspecific targeting of VLPs, based on VP1.

To achieve cell-type specificity of this potentially useful vector system, two modifications have to be established: Suppression of the natural tropism of polyoma VP1 and generation of a new cell-type-specific targeting function. Several different approaches have been pursued to endow viral vector systems with targeting functions. One attempt is the insertion of single-chain Fv fragments, which are the smallest antigen-binding antibody fragments, consisting of only the variable domains VL and VH in viral coat proteins (Chu et al. 1994; Jiang et al. 1998; Konishi et al. 1998; Martin et al. 1998). However, this strategy is limited by the modification of the virus coat protein and the development and characterization of single-chain Fv fragments. Furthermore, the fusion of all parts to a stable fusion protein that

Reprint requests to: Hauke Lilie, Institut für Biotechnologie, Martin-Luther-Universität Halle, Kurt-Mothes Straße 3, D-06120 Halle, Germany; e-mail: lilie@biochemtech.uni-halle.de; fax: 490345-5527011.

Abbreviations: DTT, dithiothreitol; ecDHFR, dihydrofolate reductase from *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl- β -D-thiogalactopyranoside; PDB, Protein Database Brookhaven; VLP, virus-like particle; VP1-Z, fusion protein with protein Z inserted in VP1; wt, wild type.

Article and publication are at www.proteinscience.org/cgi/doi/10.1110/ps.31101.

has the desired properties is essential. Another strategy is the insertion of docking modules on the surface of the viral coat protein. This would allow one to work with a once modified and characterized viral coat protein in several different applications. Such an approach was developed by Ohno and coworkers (Ohno and Meruelo 1997; Ohno et al. 1997). They used an immunoglobulin-binding domain from *Staphylococcus aureus*, the protein Z, which was inserted in the viral coat protein of Sindbis virus and retroviruses. Binding of a cell-specific antibody to the modified virus led to a change in the tropism of Sindbis virus toward the target cells of the respective antibody.

We transferred this elegant approach to VLPs of polyoma VP1. VP1 is a pentameric protein with monomers of 42.5 kD molecular mass. The VP1 structure shows mainly β -sheets, which are arranged in a jelly-roll topology (Stehle et al. 1994; Stehle and Harrison 1996, 1997). The VP1 monomer possesses three protruding loops, which are faced outward in the assembled state of the VLPs. Recently, we showed that the 18-kD ecDHFR could be functionally inserted in the HI-loop of VP1 (Gleiter et al. 1999). Therefore, we decided to insert the sequence of the immunoglobulin-binding domain, the protein Z, into the HI-loop of VP1 (Fig. 1). With this docking module it should be possible to create a recombinant carrier for proteins or DNA, which could be targeted via a bound antibody to a specific cell surface. Here, we characterize the fusion protein VP1-Z biochemically, with emphasis on the structural and functional parameters of the fusion protein.

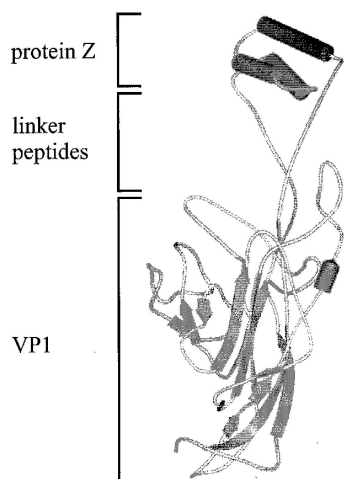


Fig. 1. Scheme of the fusion protein VP1-Z. The model is composed of the structures of VP1 (PDB entry 1vps) and the protein Z (PDB entry 2spz). The serine-glycine linkers were introduced with the program SYBYLL. No extensive attempts were made to energy minimize the structure of the fusion protein.

Results

Expression and purification

Recombinant expression of the VP1-Z gene in *Escherichia coli* led to formation of predominantly soluble VP1-Z in the host cell, only ~10–20 % of the recombinant protein formed insoluble aggregates (not shown). VP1-Z was purified as described in the Materials and Methods section. The homogeneity of the purified protein is documented in Figure 2. Only one minor contamination was copurified. As described previously for wtVP1 (Leavitt et al. 1985), western blot analyses proved that this is a degradation product of VP1-Z, which is still integrated in the oligomeric structure of VP1-Z (data not shown).

Structural characterization of VP1-Z

To analyze whether protein Z is structured within the fusion protein, CD spectroscopy was used. As an α -helical protein, protein Z showed a strong signal in the far UV region (Fig. 3A). The α -helical content of isolated protein Z was determined to be 57% by quantitative analysis of the CD spectrum, using the program CDNN (Bohm et al. 1992), which is in accordance with 54% α -helical content of the three helices that constitute the protein Z structure (Tashiro et al. 1997). To determine the helix content of the Z domain in VP1-Z, CD spectra of wtVP1, VP1-Z, and protein Z were compared.

The predominantly β -sheet containing VP1 possessed a rather small amplitude in the far UV region (Fig. 3A). The CD spectrum of VP1-Z proved to be identical to the sum of spectra from wtVP1 and protein Z (Fig. 3B). This clearly indicated that the three helices of protein Z were maintained in the fusion protein. Thus both proteins, VP1 and protein Z, possessed a secondary structure that was not significantly altered within the fusion protein.

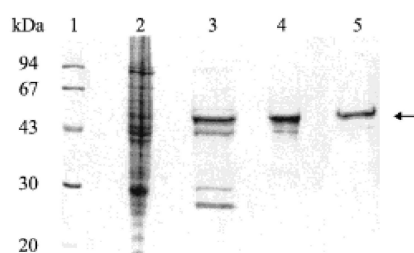


Fig. 2. Purification of VP1-Z. *Escherichia coli* strain BL21(DE3) that contained the plasmid pVP1Z was treated as described. Along the purification scheme, aliquots were taken and analyzed by 12% SDS-PAGE. VP1-Z is marked by an arrow. (1) Molecular weight standard; (2) soluble cell lysate; (3) VP1-Z after S-Sepharose; (4) VP1-Z pentamers after gel filtration; (5) VLPs of VP1-Z after gel filtration.

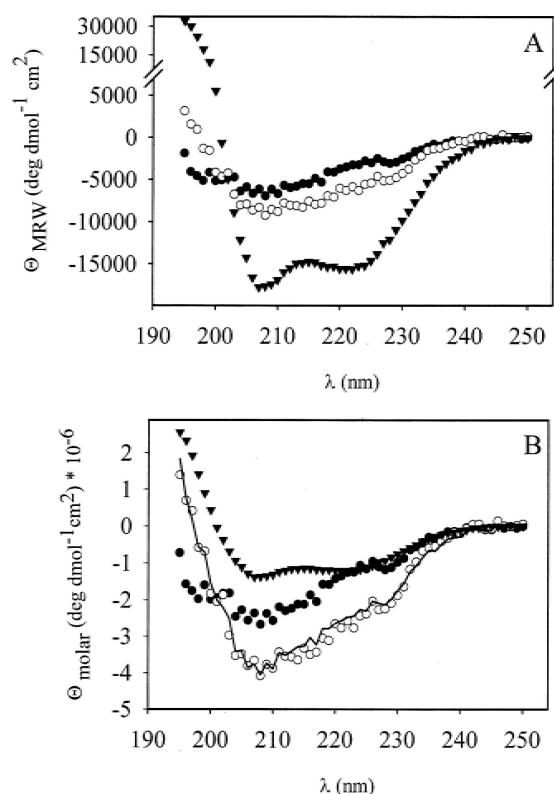


Fig. 3. Secondary structure of VP1-Z. CD spectra from wtVP1, VP1-Z, and protein Z. VP1 variants were measured in 50 mM Tris at pH 7.4, 200 mM NaCl, 5% (v/v) glycerol, 0.5 mM EDTA, and 1 mM DTT at concentrations of 0.423 mg/mL (wtVP1) and 0.38 mg/mL (VP1-Z), respectively. Protein Z at a protein concentration of 0.15 mg/mL was analyzed in 10 mM Na-phosphate, pH 6.8. (A) Far UV CD spectra of pentameric wtVP1 (filled circle), VP1-Z (open circle) and protein Z (triangles) measured from 250 nm to 195 nm. The CD signals were normalized to Θ_{MRW} . (B) Comparison of the calculated CD spectrum of VP1-Z (solid line), that is the sum of wtVP1 (filled circle) and protein Z (triangles), with the measured spectrum of VP1-Z (open circle). The CD data are normalized to Θ_{molar} .

The oligomerization state of VP1-Z depends on the correct fold of the VP1 part in the fusion protein. WtVP1 and several variants were found previously to form pentamers in solution (Leavitt et al. 1985; Salunke et al. 1986; Schmidt et al. 1999; Gleiter et al. 1999; Schmidt et al. 2000; Stubenrauch et al. 2000). This association state could also be verified for VP1-Z. Using analytical ultracentrifugation the sedimentation velocity of VP1-Z pentamers was determined with $s_{(w,20)} = 7.8$ S (Fig. 4), which is similar to that of pentameric wtVP1 ($s_{app} = 7.5$ S; Salunke et al. 1986). From sedimentation equilibrium measurements the molecular mass was calculated to be 237 kD (data not shown), corresponding to a pentamer of VP1-Z with a theoretical molecular mass of $M_t = 247$ kD.

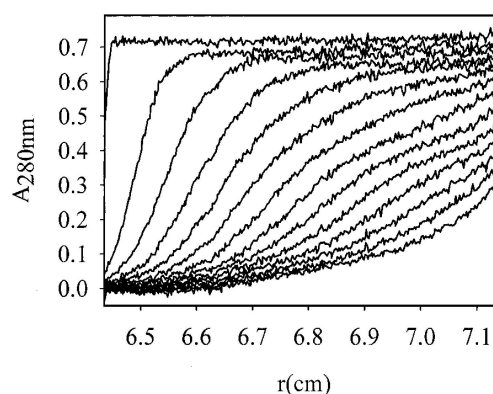


Fig. 4. Analytical ultracentrifugation of VP1-Z pentamers. The sedimentation velocity of VP1-Z (30,000 rpm, 20°C) was determined at a protein concentration of 0.45 mg/mL. Scans were taken every 10 min (displayed are every 20 min). Quantitative analysis yielded a value of $s_{(w,20)} = 7.8$ S.

Stability of VP1-Z

The insertion of peptides or proteins between position Asn²⁹³ and Tyr²⁹⁴ of VP1 results in decreased stability of either VP1 (Stubenrauch et al. 2000) or the inserted protein (Gleiter et al. 1999). To analyze the stability of VP1-Z, thermal denaturation measurements of the fusion protein were performed either measuring fluorescence or CD. As shown in Figure 5, the isolated protein Z did not possess significant changes in secondary structure up to a temperature of ~63°C. Unfolding of pentameric wtVP1 started at ~48°C. The following aggregation on denaturation led to an increase in the CD signal at higher temperatures. Pentameric

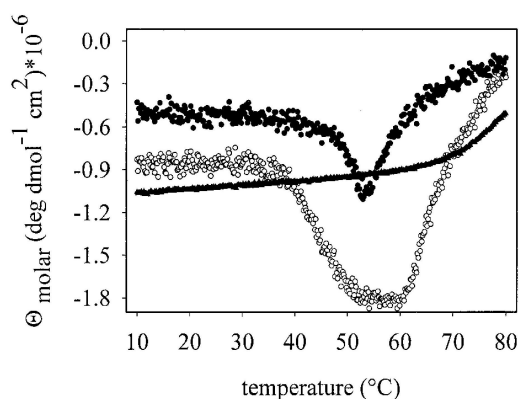


Fig. 5. Thermal denaturation of VP1-Z, wtVP1, and protein Z. Structural changes of VP1-Z (open circle), protein Z (triangles), and wtVP1 (filled circle) at protein concentrations of 200 μ g/mL, respectively, in 20 mM Tris at pH 7.4, 5% glycerol, 200 mM NaCl, 0.5 mM EDTA were analyzed by CD at 220 nm and a heating rate of 1° C/min.

VP1-Z unfolded at $\sim 38^\circ\text{C}$, a significantly lower temperature than for denaturation of wtVP1. In contrast to wtVP1, the CD signal of VP1-Z reached a plateau during thermal denaturation. Denaturation of the Z-domain of VP1-Z was observed only at $\sim 62^\circ\text{C}$, which is similar to that of the isolated protein Z. Simultaneously, the fusion protein started to aggregate. Comparison of these data led to the conclusion that the VP1-part in the fusion protein is destabilized to some extent. Because all processes were irreversible, quantitative data could not be obtained. The Z domain in the fusion protein seemed to be as stable as the isolated protein Z.

Functional analysis of VP1-Z

Considering the potential use of VP1-Z as the basic structure of a cell-type-specific delivery system, three functional aspects of VP1-Z are of major importance: The capability to assemble into VLPs, the inhibition of the natural sialic acid-binding activity, which leads to a cell-type-unspecific targeting of wtVP1 VLPs, and the binding of, for example, tumor-specific antibodies to protein Z on the surface of VLPs of VP1-Z.

The formation of VLPs was induced by dialysis of VP1-Z pentamers against assembly buffer. Sedimentation velocity analysis of assembled samples resulted in an S -value of $s_{\text{app}} = 64 \text{ S}$ (Fig. 6), which clearly indicates that an assembly process took place. The sizes of the VLPs obtained were comparable to those observed for other mutants of VP1 under similar conditions (Gleiter et al. 1999; Stubenrauch et al. 2000). The VLPs could also be visualized by electron microscopy after negative staining of VLP preparations (Fig. 6). Both analytical methods showed that the VLPs of VP1-Z pentamers were rather homogeneous in size, with a diameter of about 45 nm. Under identical conditions wtVP1 assembled predominantly to VLPs, comparable in size to those of VP1-Z ($s_{\text{app}} = 63 \text{ S}$; diameter of $\sim 50 \text{ nm}$; Fig. 6). As shown by a less pronounced cooperativity in sedimentation, part of wtVP1 also assembled to larger particles ($s_{\text{app}} = 96 \text{ S}$). This inhomogeneity might be caused by the respective buffer conditions, optimized for assembly of VP1-Z.

The stability of VLPs of VP1-Z and wtVP1 was very similar. The transition of thermal denaturation of VLPs of VP1-Z, measured by fluorescence, had a midpoint of $T_M = 51^\circ\text{C}$, whereas VLPs of wtVP1 showed a $T_M = 54^\circ\text{C}$ (Fig. 7). Compared to the stability of nonassembled proteins, the VP1 variants are significantly stabilized on assembly, most likely because of additional interactions among the VP1 pentamers in the VLPs.

Another ability of wtVP1 is its binding to sialic acid residues, which are located on the surface of most mammalian cells. The sialic acid-binding site of VP1 is located directly beneath the HI-loop (Stehle and Harrison 1997). As

described previously, it is possible to diminish the binding of VP1 to its cellular target by the insertion of a polypeptide sequence in the HI-loop (Gleiter et al. 1999; Stubenrauch et al. 2000). In a hemagglutination assay, VLPs of wtVP1 induced agglutination of sheep erythrocytes at protein concentrations of $\geq 0.05 \mu\text{g/mL}$. In contrast, the presence of VP1-Z did not lead to agglutination even at protein concentrations of $\leq 50 \mu\text{g/mL}$ (Fig. 8), which proves that the insertion of protein Z in the HI-loop of VP1 blocks binding of the natural ligand sialic acid to the protein. This is an essential prerequisite for establishing cell-type-specific targeting of modified polyoma VLPs. It should be mentioned, however, that the lack of hemagglutination does not prove the inability of VP1-Z to bind to any other cell. It is known that wtVP1 can bind sialyloligosaccharides in their branched and nonbranched forms, which seems to determine the pathogenicity of polyoma viruses in mice (Bauer et al. 1999). Whether the insertion of protein Z directly above the sialic acid binding site of VP1 not only reduces its affinity to the respective sialyloligosaccharides but also changes the specificity toward sugar residues, which are not present on erythrocytes, cannot be estimated from the present data.

To analyze the function of the inserted polypeptide, the binding of a humanized anti-carcino embryogenic antigen (CEA) antibody to VP1-Z was measured. This antibody bound isolated protein Z with a dissociation constant of $K_D = 10 \text{ nM}$ as determined by surface plasmon resonance measurements (data not shown). To show binding of the antibody on the surface of VP1-Z, the antibody was labeled with 5-nm gold particles. This gold-labeled antibody was used to visualize the specific binding on the surface of VP1-Z VLPs by electron microscopy. As shown in Figure 6E, the electron-dense, 5-nm gold particles coupled to the antibody were attached to the VLPs. These ordered structures of the gold-labeled antibody could not be observed in samples containing VLPs of wtVP1 (data not shown). Thus the anti-CEA antibody specifically associated with the Z domain within the fusion protein VP1-Z. Furthermore, this result directly showed that, as predicted, the Z-domain, and thus the bound antibody, are presented on the surface of the VLPs. Titration of the antibody to VLPs of VP1-Z that were analyzed by HPLC gel filtration revealed that almost every Z domain on the surface of VP1 bound an antibody molecule, which led to a stoichiometry of about 0.8 anti-CEA antibody molecules per VP1-Z monomer. Furthermore, the appearance of the titration curve without any detectable curvature but, instead, with the sharp bend at a stoichiometry of 0.8 indicates that protein Z possessed a high affinity to the antibody, even within the fusion protein (Fig. 9). In contrast, no interaction between antibodies and wtVP1 could be observed, showing the specificity of the coupling of antibodies to VP1-Z (data not shown).

These antibody-binding characteristics were not limited

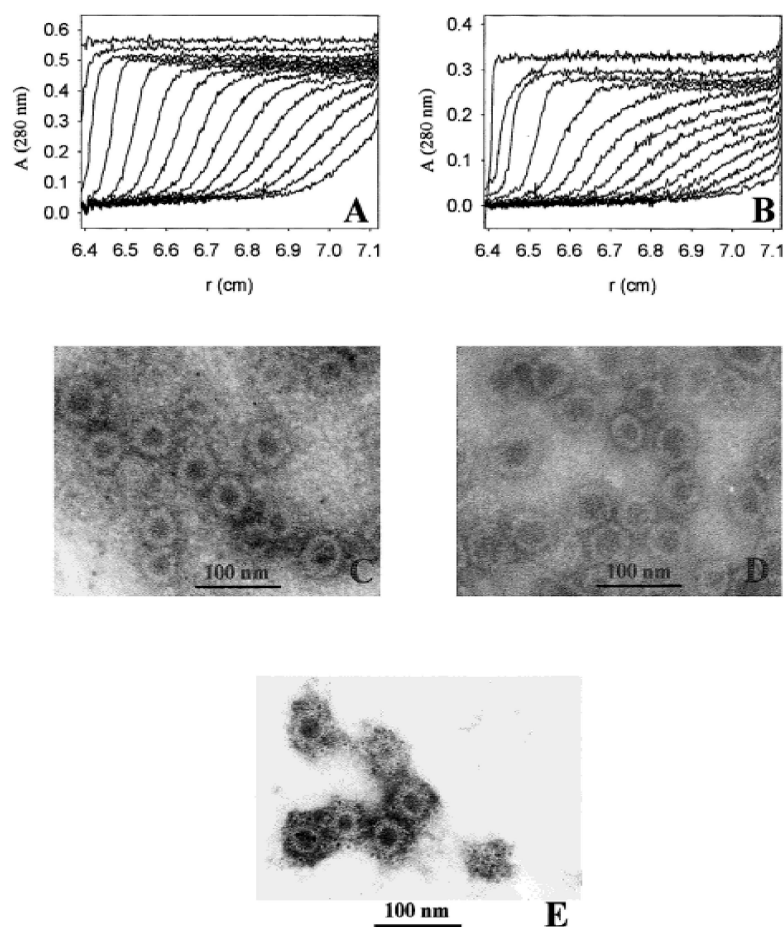


Fig. 6. Formation of VLPs of VP1-Z. VP1-Z and wtVP1 at a protein concentration of ~ 1 mg/mL was assembled to VLPs by dialysis against 20 mM Tris at pH 7.4, 200 mM NaCl, 5% glycerol, 0.5 M ammonium sulfate. The VLPs were analyzed by analytical ultracentrifugation. For electron microscopy the ammonium sulfate, which was present in the assembly buffer, was removed by dialysis. (A) Sedimentation velocity analysis of VP1-Z VLPs. An apparent S -value of 64 S was calculated. (B) Sedimentation velocity analysis of VLPs of wtVP1. The majority of the VLPs sedimented with $s_{app} = 63$ S. A small fraction assembled to larger particles ($s_{app} = 96$ S). VLPs of VP1-Z (C) and wtVP1 (D) were adsorbed to formvar-coated copper grids. The negative staining was performed using uranyl acetate. The electron micrograph was taken at a nominal magnification of 85,000 $\times$. (E) VLPs of VP1-Z adsorbed on Ni grids were coupled with gold-labeled antibody TU2. Afterward the complexes were stained with uranyl acetate. The electron micrograph was taken at a nominal magnification of 85,000 $\times$.

to the anti-CEA antibody. Another humanized antibody, Herceptin, could also be coupled to VLPs of VP1-Z with a similar stoichiometry (data not shown).

Cell-specific targeting of antibody presenting VLPs

The presentation of a cell-type-specific antibody on the surface of VP1-Z VLPs allowed us to analyze whether or not the respective antibody leads to a change in the tropism of the modified VLPs. The antibody Herceptin is known to

bind selectively to the glycoprotein p185^{Her2}, a member of the EGF receptor family, which is present on several different human tumor cells (De Potter 1994; Molland et al. 1996). Using this antibody, a cell-type-specific targeting of immunoliposomes could be shown (Kirpotin et al. 1997). We compared the targeting of VLPs of VP1-Z, unmodified and decorated with Herceptin, to SK-BR-3 cells that expressed the mutated EGF receptor. To this end, rhodamine-labeled plasmid DNA was packaged into the respective VLPs and incubated with the cells for 90 min. As analyzed by laser scanning microscopy, almost no cellular-associated

Fusion of protein Z to polyoma VP1

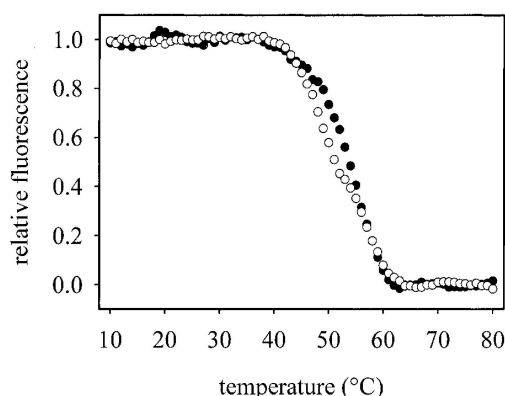


Fig. 7. Stability of VLPs of VP1-Z and wtVP1. VP1-Z and wtVP1 at a protein concentration of 20 $\mu\text{g/mL}$ in assembly buffer without ammonium sulfate, respectively, were incubated at a heating rate of 0.3°C/min from 5°C to 80°C. The change in fluorescence during heating was monitored using 295 nm for excitation and 340 nm for emission. The transition midpoint of denaturation of VP1-Z VLPs (open circle) and wtVP1 VLPs (filled circle) was determined with 51°C and 54°C, respectively.

rhodamine fluorescence could be observed using VLPs in the absence of antibody (data not shown). A still very weakly detectable fluorescence signal was also obtained if DNA alone or in combination with the antibody was used. In contrast, if Herceptin was bound to the VLPs, nearly all of the cells were fluorescence labeled and the quantity of fluorescence per cell showed that this targeting was highly efficient (Fig. 10A). This specific targeting function could be confirmed using MCF-7 cells as another antigen presenting cell line (data not shown). Furthermore, the fluorescence signal could not only be detected on the surface of the cells but also inside the cells (Fig. 10B–G), which indicates that an uptake of the fluorescence-labeled particles occurred. Using the cell line HT-29, which does not present the antigen, no VLP complexes were associated with the cells. The same negative result was observed when the cell line SK-BR-3 was addressed by VLPs coupled with an antibody that was not directed against these cells (data not shown). Thus VLPs of VP1-Z that are decorated with a cell-type-specific antibody can be used specifically to deliver these particles to cells that express the antigen on the surface and to transport them across the cytoplasmic membrane.

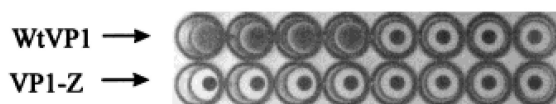


Fig. 8. Suppression of cell-type-unspecific cell adhesion of VP1-Z. Hemagglutination of sheep erythrocytes in the presence of VLPs of VP1-Z and wtVP1 was analyzed. Protein concentrations ranged from 50 $\mu\text{g/mL}$ to 5 $\mu\text{g/mL}$ (10-fold dilution from left to right). Only wtVP1 agglutinated the erythrocytes at a protein concentration of ≥ 50 $\mu\text{g/mL}$.

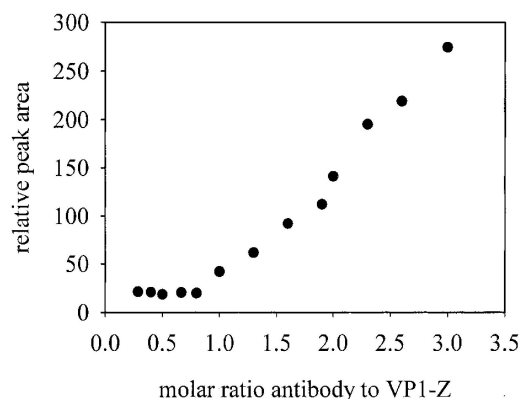


Fig. 9. Titration of VP1-Z VLPs with anti-CEA antibody. The concentration of VP1-Z capsids was held constant at 1.5 μM referred to as the monomer of VP1-Z. The anti-CEA antibody was titrated to VP1-Z from 0 μM to 7.5 μM . The samples were analyzed after overnight incubation at 20°C on HPLC gel filtration. The relative area of the antibody that was not bound to VP1-Z capsids was measured and plotted against the molar ratio of VP1-Z monomer to anti-CEA antibody.

Discussion

Recently we showed that an 18-kDa protein can be functionally inserted in polyoma VP1 and presented on the surface of the respective VLPs. In this case, ecDHFR was used as a model system (Gleiter et al. 1999). Although some alterations regarding stability, enzymatic activity, and assembly of the VP1-DHFR fusion were observed compared with wtVP1 and ecDHFR, respectively, the results prompted us to go one step further in modifying the surface of VLPs of polyoma VP1 that should allow cell-type-specific targeting of those modified particles. To enable a change in tropism, the antibody-binding-domain protein Z was placed on the surface of VP1, thus making it possible to couple antibodies to VLPs of VP1-Z. The interaction of protein Z with antibodies is rather specific, with dissociation constants in the range of 15 nM (Starovasnik et al. 1997).

The inserted Z domain is rather small with a molecular mass of ~6.8 kD. As the only secondary structure elements it consists of three helices, two of which are involved in antibody binding (Deisenhofer 1981). Importantly, the relative position of these helices does not change on binding of an antibody (Jendeborg et al. 1996), thus facilitating modeling of the fusion protein. We inserted protein Z via short serine-glycine linkers to enable an optimal flexibility on the surface of VP1. The structural integrity, not only of VP1 but also of protein Z, was completely maintained within the fusion protein, which led to a soluble, functional fusion protein. This was expected because protein Z is a derivative of the B-domain of protein A. Protein A consists of alto-

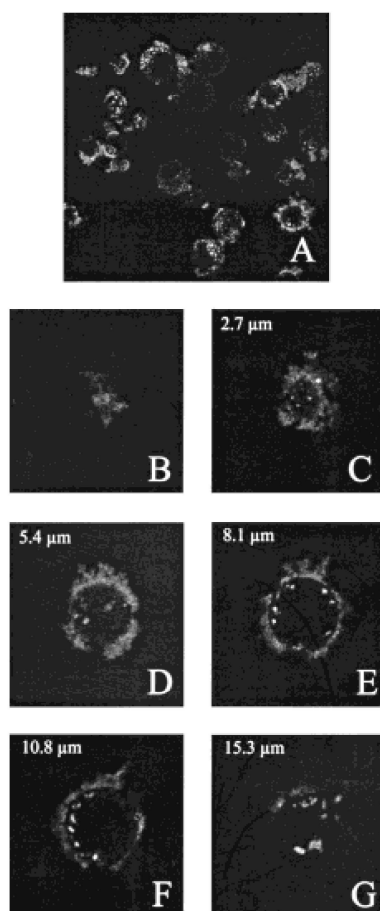


Fig. 10. Cell type-specific targeting of antibody-presenting VLPs of VP1-Z. SK-BR-3 cells were grown to a confluency of ~70%. Subsequently, the cells were incubated with VLPs of VP1-Z, which were decorated with the antibody Herceptin and packaged with fluorescence-labeled DNA. After overnight incubation nonbound VLP were removed by excessive washing and the cells were fixed using paraformaldehyde. (A) Overview of fluorescence-labeled cells. (B–G) Z series of a single cell. Fluorescence staining could be observed on the cell surface and inside the cells (D,E,F).

gether five immunoglobulin-binding domains, which means that the B domain is flanked by other protein domains in protein A (Moks et al. 1986). Furthermore, there are several known artificial fusion proteins of protein Z (Chu et al. 1994; Martin et al. 1998), for example, the insertion of Z domain in Sindbis virus coat protein (Ohno and Meruelo 1997) or the use of a tandem Z domain as purification tag (Larsson et al. 1996) or as fusion partner to enhance solubility and refolding (Samuelsson et al. 1994; Samuelsson and Uhlen 1996). In all of these examples a functional fusion protein could be obtained.

The insertion of the model protein ecDHFR in VP1 at the same localization as that used for protein Z led to a dramatic destabilization of the DHFR part in the fusion protein (Gleiter et al. 1999). In contrast, protein Z does not seem to be significantly destabilized in the fusion protein compared with the stability of the isolated domain as revealed by CD measurements. Therefore, in contrast to DHFR, protein Z is still considerably more stable than VP1 within the fusion protein. VLPs of VP1-Z do not denature at temperatures <43°C, which indicates that the stability of these particles does not limit their potential as a gene therapeutic vector system at body temperature.

Whereas the apparent stability of VLPs of VP1-Z and wtVP1 is very similar, with a midpoint of thermal denaturation of $T_M = 51^\circ\text{C}$ and $T_M = 54^\circ\text{C}$, respectively, the stability of the pentameric state of both proteins is quite different. Denaturation of wtVP1 occurs at 48°C . In contrast, the VP1-part of VP1-Z starts to denature at 38°C (Fig. 5A). The observed stabilization, especially of VP1-Z in VLPs compared with VP1 pentamers, may result from two different types of additional interactions that occur on assembly. The C-terminal sequence of one molecule becomes a β -strand of the β -sheet of a VP1-Z molecule of a neighboring pentamer. Furthermore, the reduced VP1-Z is oxidized under assembly conditions. This oxidation process leads at least to one disulfide bridge between monomers within the pentamer (Schmidt et al. 2000).

The functional parameters of the fusion protein can be divided into three parts: The function of the VP1 part to assemble into VLPs, binding of antibody on the surface of VLPs, and suppression of the sialic acid binding on mammalian cells.

The assembly of VP1-Z into VLPs could be shown by different techniques. In all cases the data imply that the VLPs that are formed are a homogeneous population of particles with a diameter of ~45 nm, which is very similar to particles of wtVP1 that are assembled under identical conditions. For wtVP1, particles of different sizes between 20 nm and 50 nm in diameter, depending on the assembly conditions, were described (Salunke et al. 1989). Once assembled, the different VLPs possess a similar stability against irreversible thermal denaturation.

The structural and functional integrity of the Z domain inserted in VP1 was proven by CD spectroscopy and antibody-binding activity. Quantitative analysis of the CD spectra clearly showed that protein Z, in solution and as fusion protein with VP1, consists of its natural α -helical content as described in the NMR structure (Tashiro et al. 1997; Gouda et al. 1998). In another study the crystal structure of protein Z was solved with only two of the three helices present (Deisenhofer 1981). However, this difference to the NMR structure of protein Z could be shown to be caused by crystal contacts of the protein in the crystal (Tashiro et al. 1997; Gouda et al. 1998).

Fusion of protein Z to polyoma VP1

Incubation of VLPs of VP1-Z with humanized monoclonal antibodies leads to a stoichiometry of binding of maximal 0.8, which indicates that almost every protein Z of the VLPs can bind one antibody molecule. Because of such a high loading rate, one cannot expect that every antigen-binding site of every bound antibody is accessible for the respective antigen. However, the titration clearly showed that the amount of antibody that is coupled can easily be controlled by loading substoichiometric amounts of antibody to VP1-Z. Although not analyzed so far, a low coupling rate would probably also ensure binding of different antibodies to VP1-Z simultaneously.

Placing cell-type-specific antibodies on the surface of VLPs, together with suppression of cell-type-unspecific sialic acid binding by the modification of VP1, should lead to a tropism of these particles based on the antibody–antigen interaction of the respective antibody. Such an antibody-specific cellular targeting could be shown using the tumor-specific antibody Herceptin. This antibody, directed against a mutant of a receptor of the EGF receptor family, directed the respective particles toward the antigen presenting cells SK-BR-3 or MCF-7. This targeting led also to an incorporation of these particles. Without the antibody coupled to the VLPs, no targeting could be observed at all. These results indicate that the modified polyoma VP1 particles that are described in this study are an important achievement in developing a cell-type-specific delivery system.

Materials and methods

Plasmid construction

The gene encoding the fusion protein VP1-Z was obtained by polymerase chain reaction (PCR) with three overlapping fragments: A, B, and C. Fragment A, which encodes residues 1–293 of VP1 and a C-terminal (Ser-Gly)₃ linker sequence, was amplified from plasmid pVP1-DHFR (Gleiter et al. 1999) with primers NT7PpET (5'-CGATCCCGCGAAATTAATACGACTCAC-3') and vp1linkrev (5'-ACCGCTACCGCTGCCGCTGTTCTTG-TAACTCTCCACC-3'). Fragment B encoded residues 294–385 of VP1, with an additional N-terminal (Ser-Gly)₂-Ser linker sequence, and was amplified from the same plasmid with primers linkvp1fwd (5'-TCTGGTCTGGTAGCTATGATGTCCATCAC TGGAGAGG-3') and revvp1not (5'-CCTAGGTCCAGCGCC GCTCATTAATTCCAGGAAATACAGTCTTTGTTTTTC-3'). Using this reverse primer, the last two amino acids of the VP1 sequence were changed to Gly³⁸⁴-Asn³⁸⁵, which is the natural C terminus of VP1. In addition, a NotI site was introduced. Fragment C consisted of the sequence encoding the Z domain amplified from plasmid pZR8 that comprised a C-terminal extended Z domain. Only the originally described sequence of protein Z (Larsson et al. 1996) was amplified from this plasmid with forward primer linkZfwd (5'-AGCGGCAGCGGTAGCGGTGTAGACAACAAA TTCAACAAAGAAC-3') and the reverse primer zlinkrev (5'-GC TACCAGAACCAGATTTCGGCGCCTGAGCATCATTTAG-3'), thereby introducing the N- and C-terminal (Ser-Gly)-linker sequences, complementary to the C terminus of fragment A and to the N terminus of fragment B. Amplification products were separated on 1% agarose gels (ICN) and purified with the GFX puri-

fication kit (Amersham Pharmacia). Fragments B and C were combined for an overlapping amplification using primers linkZfwd and revvp1not.

The PCR product was again isolated from agarose gel and purified. This PCR product was used for the last overlapping PCR with fragment A and primers NT7PpET and revvp1not. The fragment was cloned into pET 21a(+) (Novagen) using the restriction enzymes *Xba*I and *Nor*I. The resulting plasmid, which contained the complete sequence of the fusion protein VP1-Z, was termed pVP1Z.

Protein expression

The expression strain BL21(DE3) was transformed with the plasmid pVP1Z. To optimize the expression conditions for the fusion protein cultivations were performed at different temperatures in 1 l shake flasks with 250 mL LB medium. At OD₅₀₀ = 0.5 protein expression was induced by addition of IPTG to a final concentration of 1 mM. After induction, cells were incubated at temperatures between 15°C and 37°C overnight and then harvested by centrifugation. The pellet was resuspended in buffer A0 (50 mM Tris at pH 7.4, 200 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA, 2 mM DTT). Cell lysis was achieved using a bead mill. Soluble and insoluble fractions were analyzed by SDS-PAGE.

For large-scale preparation of VP1-Z, *E. coli* BL21(DE3) transformed with pVP1Z was cultivated on mineral salt medium at 30°C at a scale of 5 L in a Biostat-Fermenter (B. Braun) using the fed-batch technique (Teich et al. 1998). The recombinant expression of VP1-Z was induced by adding 0.4 mM IPTG 3 hrs after feeding started. At the time of induction the temperature was set to 15°C and was maintained until the end of fermentation at 15 h. Cells were harvested by centrifugation (8000 g, 15 min) and stored at –70°C.

Protein purification

For preparation of VP1-Z 20 g cells (wet weight) were resuspended in 50-mL buffer A0, supplemented with two tablets of complete protease inhibitor cocktail, EDTA free (Roche Diagnostics). After high-pressure dispersion, the cell lysate was diluted with an equal volume of H₂O. To digest DNA bound to the N terminus of VP1-Z, the cell lysate was incubated with Benzonase (Merck) in the presence of 2 mM MgCl₂ for 30 min at 4°C. Afterward EDTA was added to a final concentration of 4 mM and the lysate was centrifugated in a Sorvall SS-34 rotor at 20,000 rpm for 40 min.

The supernatant that contained the soluble fraction of VP1-Z was diluted with 50 mL buffer 1 (20 mM sodium acetate, pH 5.5, 5% (v/v) glycerol, 2 mM EDTA, 2 mM DTT) and the pH adjusted to 5.5. Because the pH shift led to slight aggregation the solution was again centrifugated (20,000 rpm, 40 min, SS-34 rotor) before the protein solution was applied to a S-Sepharose column (Amersham Pharmacia), equilibrated with buffer 1. The bound VP1-Z protein was eluted in a linear gradient from 0 to 2 M NaCl over 14 column volumes.

VP1-Z in the flow through was subjected to a second DNA digest after readjusting the pH and the Mg²⁺ concentration. Afterward the sample was applied again to the S-Sepharose column with the pH adjusted to 5.5. The VP1-Z containing fractions were pooled and concentrated by incubation with PEG 35,000 (Fluka) to a final volume of 6 mL for each run.

After dialysis against buffer A0 overnight VP1-Z was applied on a Superdex 200 prep grade gel filtration column (Amersham Pharmacia) equilibrated in buffer A0. Pentameric VP1-Z was concentrated and then dialyzed against VP1-Z assembly buffer (0.5 M

Gleiter and Lillie

ammonium sulfate, 20 mM Tris at pH 7.4, 5% (v/v) glycerol, 1 mM CaCl_2) overnight at 20°C. The assembled VP1-Z again was applied on the Superdex 200 column, this time equilibrated in assembly buffer without ammonium sulfate (200 mM NaCl, 20 mM Tris at pH 7.4, 5% (v/v) glycerol, 1 mM CaCl_2), thus separating VLPs and nonassembled pentamers. The overall yield of purified VP1-Z was in the range of 2 mg protein per 20 g of cells. Purification steps were analyzed on 12% SDS-PAGE, stained with Coomassie blue (Fairbanks et al. 1971).

The single IgG-binding domain of protein A (protein Z), which contained a fusion peptide of eight arginine residues and one cysteine at the C terminus of the protein, was also expressed in BL21(DE3). Cells expressing protein Z for about 14 hr were harvested and resuspended in buffer Z1 (10 mM phosphate, 10 mM EDTA, pH 6.8). After high-pressure disruption of the cells, the crude extract was centrifuged at 25,000 rpm for 1 hr. The clear supernatant was applied on a Resource S column (Amersham Pharmacia), which was equilibrated with buffer Z1. The recombinant protein was eluted in a linear gradient from 0–1 M NaCl. For further purification, the fractions containing protein Z were pooled and applied on a Superdex 75 column (Amersham Pharmacia), which was equilibrated in buffer Z1. Again, fractions containing protein Z were pooled and dialyzed against buffer Z1. For plasmon resonance measurements, the protein was dialyzed against buffer Z1 without EDTA.

Spectroscopic techniques

CD spectra were collected with an AVIV CD spectrophotometer. Protein concentrations for pentameric wtVP1 and VP1-Z were 0.423 mg/mL and 0.38 mg/mL, respectively. An average of 12 spectra were measured in the range of 250–195 nm in a 0.2-mm cuvette at 10°C.

The concentration of protein Z was 0.15 mg/mL in 10 mM phosphate buffer pH 6.8. For protein Z, an average of six spectra were measured in a 1-mm cuvette.

Thermal denaturation of all three proteins was measured in a Jasco CD spectrophotometer. The protein concentration for all proteins was 200 µg/mL. Measurements were performed in a 1-mm cuvette. The protein solution was heated using a programmable water bath with a heating rate of 1°C/min.

In addition, temperature transitions of the different proteins were determined using a Hitachi F-4500 fluorescence spectrometer in a stirrable cuvette with a light path of 10 mm. The excitation wavelength was set to 295 nm and the emission was measured at 340 nm. A programmable water bath was used to ensure a heating rate of about 0.3°C/min. Temperature transitions were measured in a range between 5°C and 80°C with protein concentrations of 20 µg/mL.

Analytical ultracentrifugation

VP1-Z at a concentration of ~0.25 mg/mL in buffer A0 (pentamers) or assembly buffer (VLPs) was analyzed in a Beckman Optima XL-A centrifuge using an AN60Ti rotor and double sector cells. Sedimentation velocity experiments were performed at 30,000 rpm and 20°C for the pentameric state and at 10,000 rpm, 20°C in the case of VLPs, respectively. Sedimentation equilibrium centrifugation was performed at 10,000 rpm, 20°C. The data were analyzed using the software provided by Beckman Instruments.

Assembly of VP1-Z

Assembly of VP1-Z to VLPs was induced by dialyzing VP1-Z against assembly buffer (0.5 M ammonium sulfate, 20 mM Tris at

pH 7.4, 5% (v/v) glycerol, 1 mM CaCl_2) overnight. After the end of assembly the ammonium sulfate was removed by dialysis against assembly buffer without ammonium sulfate (200 mM NaCl, 20 mM Tris at pH 7.4, 5% (v/v) glycerol, 1 mM CaCl_2). After the second dialysis the samples were centrifuged at 13,000 rpm for 30 min at 4°C. Aliquots were used for electron microscopy and ultracentrifugation.

Association of VP1-Z with anti-CEA antibody

Because protein Z has highest affinity to the Fc part of human immunoglobulins of the type IgG, we used the monoclonal antibody MAK TU2. This antibody consists of a human Fc and Fab fragments from mouse, directed against the antigen CEA (Alfred Engel, Roche Diagnostics).

For association of VP1-Z capsids with anti-CEA antibody VP1-Z was used at a concentration of 1.5 µM (corresponding to the monomer). Variable concentrations of anti-CEA antibody (0 µM–7.5 µM) were incubated with VLPs of VP1-Z in assembly buffer without ammonium sulfate (200 mM NaCl, 20 mM Tris at pH 7.4, 5% (v/v) glycerol, 1 mM CaCl_2). After incubation at 20°C overnight, the samples were applied to a YMC Diol-300 gel filtration column, which was equilibrated in the same buffer. The relative peak area of the remaining nonassociated antibody was analyzed.

Gold labeling of anti-CEA antibody

1-mL of gold solution (Sigma, 5-nm colloidal gold) adjusted to pH 7.3 with 0.2 M K_2CO_3 was incubated with 0.1 mL of anti-CEA solution ($c = 0.6$ mg/mL in 10 mM phosphate pH 6.8) for 30 min at room temperature. Afterward bovine serum albumin was added to a final concentration of 1%. Aggregates were sedimented by centrifugation for 1 hr at 30,000 rpm in a Beckman tabletop ultracentrifuge. The supernatant was discarded and the pellet was diluted with PBS to a final concentration of 0.1 mg/mL antibody–gold conjugate.

Electron microscopy

Formvar- and carbon-coated copper grids were used for negative staining of VLPs as described by Dyksta (1992) at protein concentrations of ~0.2 mg/mL. Transmission electron microscopy was performed with a Zeiss EM 900. Photographs were taken at a nominal magnification of 85,000-fold.

Complexes of VLPs of VP1-Z and gold-labeled anti-CEA antibody were analyzed on formvar-coated Ni grids. VLPs with a concentration of ~0.5 mg/mL were adsorbed to the grid for 30 min. Afterward the grid was blocked with 2% acetylated BSA and 0.2% Tween for 5 min. Finally, the grid was incubated for 10 min with gold-labeled antibody and then stained with uranyl acetate. Between each step excessive protein solution was carefully removed from the grid.

Hemagglutination assay

Hemagglutination of sheep erythrocytes (Dade-Behring) was determined in 0.9% (w/v) NaCl using U-well plates (NUNC). Protein concentrations in the assay ranged from 50 µg/mL to 0.5×10^{-9} µg/mL for both wtVP1 and VP1-Z, respectively. Fivefold dilutions of the protein solution were made in 0.9% NaCl. 40 µL of each dilution was supplemented with 40 µL of a 20-fold diluted

Fusion of protein Z to polyoma VP1

erythrocyte stock solution, resulting in a 10-fold dilution per well. Hemagglutination was assayed after a 3-hr incubation at 7°C.

Cell targeting

The cells SK-BR-3 (ATCC: HTB-30) and MCF-7 (ATCC: HTB-22) were grown at 37°C in a humidified atmosphere, containing 5% CO₂, using DMEM and RPMI 1640 medium, respectively. The medium was supplemented with 1% Glutamax (Gibco) and 10% heat-inactivated fetal calf serum (FCS). For targeting experiments, the cells were seeded in chamber slides, each chamber containing ~70,000 cells. At a confluency of 70%, the targeting experiments were performed.

To analyze cellular targeting of the VLPs, 16 µg of VP1-Z or wtVP1 VLPs were incubated with 2 µg of rhodamine-labeled DNA of the plasmid pELI 92 (Yerushalmi et al. 2000; labeling procedure according to the manufacturer's guidelines [Mirus]). For specific targeting, 16 µg of MAK<Her2>rH-IgG (Herceptin) were added to the VLPs. The final sample volume was 150 µL of assembly buffer. Before adding the VLPs to the cells, the cells were supplemented with fresh, FCS-free medium. After at least 90-min incubation the cells were washed with PBS. For long-term storage the labeled cells were fixed with 4% (w/v) paraformaldehyde. Subsequently, mounting medium (Sigma) was added and the chamber slides were sealed.

Fluorescence microscopy was performed with a Zeiss LSM 410, equipped with a He/Ne-Laser. Samples were excited at 543 nm and the emission filter was set to 570 nm. For scanning a single cell, Z series of 20 individual sectional planes were monitored with a pitch of 0.9 µm. Each plane was scanned four times to improve the signal-to-noise ratio.

Acknowledgments

We are grateful to S. Ståhl and M. Larsson for the kind gift of the plasmid encoding protein Z and to A. Engel and K.H. Sellinger (Roche Diagnostics GmbH) for the anti-CEA antibody TU-2 and Herceptin. G. Hause (Biozentrum, Universität Halle) is acknowledged for performing electron microscopy of the VLPs as is B. Hause for help with the LSM. We thank T. Jahn for technical assistance, F. Broda for help in modeling, and D. Wildemann for relief with the CD. R. Rudolph is acknowledged for fruitful discussions as is P. Bayer for critical reading of the manuscript. This work was supported by a grant of the Deutsche Forschungsgemeinschaft to H.L.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Bauer, P.H., Cui, C., Liu, R., Stehle, T., Harrison, S.C., DeCaprio, J.A., and Benjamin, T.L. 1999. Discrimination between sialic acid-containing receptors and pseudoreceptors regulates polyomavirus spread in the mouse. *J. Virol.* **73**: 5826–5832.
- Bohm, G., Muhr, R., and Jaenicke, R. 1992. Quantitative analysis of protein far UV circular dichroism spectra by neural networks. *Protein Eng.* **5**: 191–195.
- Braun, H., Boller, K., Lower, J., Bertling, W.M., and Zimmer, A. 1999. Oligonucleotide and plasmid DNA packaging into polyoma VP1 virus-like particles expressed in *Escherichia coli*. *Biotech. & App. Biochem.* **29 Part 1**: 31–43.
- Chu, T.H., Martinez, I., Sheay, W.C., and Dornburg, R. 1994. Cell targeting with retroviral vector particles containing antibody-envelope fusion proteins. *Gene Ther.* **1**: 292–299.
- De Potter, C.R. 1994. The neu-oncogene: More than a prognostic indicator? *Hum. Pathol.* **25**: 1264–1268.
- Deisenhofer, J. 1981. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry* **20**: 2361–2370.
- Dyksta, M.J. 1992. *Biological Electron Microscopy*. Plenum Press, New York.
- Eckhart, W. 1990. Polyomavirinae and their replication. In *Fundamental virology*, (eds. B.N. Fields and D.M. Knipe), pp. 727–742. Raven Press, New York.
- Fairbanks, G., Steck, T., and Wallach, D. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**: 2606–2617.
- Forstova, J., Krauzewicz, N., Sandig, V., Elliott, J., Palkova, Z., Strauss, M., and Griffin, B.E. 1995. Polyoma virus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells. *Hum. Gene Ther.* **6**: 297–306.
- Freund, R., Garcea, R.L., Sahli, R., and Benjamin, T.L. 1991. A single-amino-acid substitution in polyomavirus VP1 correlates with plaque size and hemagglutination behavior. *J. Virol.* **65**: 350–355.
- Garcea, R.L., Salunke, D.M., and Caspar, D.L. 1987. Site-directed mutation affecting polyomavirus capsid self-assembly in vitro. *Nature* **329**: 86–87.
- Gleiter, S., Stubenrauch, K., and Lilie, H. 1999. Changing the surface of a virus shell fusion of an enzyme to polyoma VP1. *Protein Sci.* **8**: 2562–2569.
- Gouda, H., Shiraishi, M., Takahashi, H., Kato, K., Torigoe, H., Arata, Y., and Shimada, I. 1998. NMR study of the interaction between the B domain of staphylococcal protein A and the Fc portion of immunoglobulin G. *Biochemistry* **37**: 129–136.
- Jendeborg, L., Tashiro, M., Tejero, R., Lyons, B.A., Uhlen, M., Montelione, G.T., and Nilsson, B. 1996. The mechanism of binding staphylococcal protein A to immunoglobulin G does not involve helix unwinding. *Biochemistry* **35**: 22–31.
- Jiang, A., Chu, T.H., Nocken, F., Cichutek, K., and Dornburg, R. 1998. Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies. *J. Virol.* **72**: 10148–10156.
- Kirpotin, D., Park, J.W., Hong, K., Zalipsky, S., Li, W.L., Carter, P., Benz, C.C., and Papahadjopoulos, D. 1997. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry* **36**: 66–75.
- Konishi, H., Ochiya, T., Chester, K.A., Begent, R.H., Muto, T., Sugimura, T., Terada, M., and Begent, R.H. 1998. Targeting strategy for gene delivery to carcinoembryonic antigen-producing cancer cells by retrovirus displaying a single-chain variable fragment antibody. *Hum. Gene Ther.* **9**: 235–248.
- Krauzewicz, N., Cox, C., Soeda, E., Clark, B., Rayner, S., and Griffin, B.E. 2000. Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids. *Gene Ther.* **7**: 1094–1102.
- Larsson, M., Brundell, E., Nordfors, L., Hoog, C., Uhlen, M., and Stahl, S. 1996. A general bacterial expression system for functional analysis of cDNA-encoded proteins. *Protein Expr. Purif.* **7**: 447–457.
- Leavitt, A.D., Roberts, T.M., and Garcea, R.L. 1985. Polyoma virus major capsid protein, VP1. Purification after high level expression in *Escherichia coli*. *J. Biol. Chem.* **260**: 12803–12809.
- Martin, F., Kupsch, J., Takeuchi, Y., Russell, S., Cosset, F.L., and Collins, M. 1998. Retroviral vector targeting to melanoma cells by single-chain antibody incorporation in envelope. *Hum. Gene Ther.* **9**: 737–746.
- Moks, T., Abrahamsen, L., Nilsson, B., Hellman, U., Sjoquist, J., and Uhlen, M. 1986. Staphylococcal protein A consists of five IgG-binding domains. *Eur. J. Biochem.* **156**: 637–643.
- Molland, J.G., Barraclough, B.H., Gebbski, V., Milliken, J., and Bilous, M. 1996. Prognostic significance of c-erbB-2 oncogene in axillary node-negative breast cancer. *Aust. N.Z. J. Surg.* **66**: 64–70.
- Ohno, K. and Meruelo, D. 1997. Retrovirus vectors displaying the IgG-binding domain of protein A. *Biochem. Mol. Med.* **62**: 123–127.
- Ohno, K., Sawai, K., Iijima, Y., Levin, B., and Meruelo, D. 1997. Cell-specific targeting of *Sindbis* virus vectors displaying IgG-binding domains of protein A. *Nat. Biotechnol.* **15**: 763–767.
- Salunke, D.M., Caspar, D.L., and Garcea, R.L. 1986. Self-assembly of purified polyomavirus capsid protein VP1. *Cell* **46**: 895–904.
- Salunke, D.M., Caspar, D.L., and Garcea, R.L. 1989. Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophys. J.* **56**: 887–900.
- Samuelsson, E., Moks, T., Nilsson, B., and Uhlen, M. 1994. Enhanced in vitro refolding of insulin-like growth factor I using a solubilizing fusion partner. *Biochemistry* **33**: 4207–4211.

Gleiter and Lillie

- Samuelsson, E. and Uhlen, M. 1996. Chaperone-like effect during in vitro refolding of insulin-like growth factor I using a solubilizing fusion partner. *Ann. N.Y. Acad. Sci.* **782**: 486–494.
- Schmidt, U., Kenkies, J., Rudolph, R., and Bohm, G. 1999. Site-specific fluorescence labelling of recombinant polyomavirus-like particles. *Biol. Chem.* **380**: 397–401.
- Schmidt, U., Rudolph, R., and Bohm, G. 2000. Mechanism of assembly of recombinant murine polyomavirus-like particles [In Process Citation]. *J. Virol.* **74**: 1658–1662.
- Soeda, E., Krauzewicz, N., Cox, C., Stokrova, J., Forstova, J., and Griffin, B.E. 1998. Enhancement by polylysine of transient, but not stable, expression of genes carried into cells by polyoma VP1 pseudocapsids. *Gene Ther.* **5**: 1410–1419.
- Starovasnik, M.A., Braisted, A.C., and Wells, J.A. 1997. Structural mimicry of a native protein by a minimized binding domain. *Proc. Natl. Acad. Sci.* **94**: 10080–10085.
- Stehle, T. and Harrison, S.C. 1996. Crystal structures of murine polyomavirus in complex with straight-chain and branched-chain sialyloligosaccharide receptor fragments. *Structure* **4**: 183–194.
- Stehle, T. and Harrison, S.C. 1997. High-resolution structure of a polyomavirus VP1-oligosaccharide complex: implications for assembly and receptor binding. *EMBO J.* **16**: 5139–5148.
- Stehle, T., Yan, Y., Benjamin, T.L., and Harrison, S.C. 1994. Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* **369**: 160–163.
- Stubenrauch, K., Bachmann, A., Rudolph, R., and Lillie, H. 2000. Purification of a viral coat protein by an engineered polyionic sequence. *J. Chromatogr. B. Biomed. Sci. Appl.* **737**: 77–84.
- Tashiro, M., Tejero, R., Zimmerman, D.E., Celda, B., Nilsson, B., and Montellione, G.T. 1997. High-resolution solution NMR structure of the Z domain of staphylococcal protein A. *J. Mol. Biol.* **272**: 573–590.
- Teich, A., Lin, H.Y., Andersson, L., Meyer, S., and Neubauer, P. 1998. Amplification of ColE1 related plasmids in recombinant cultures of *Escherichia coli* after IPTG induction. *J. Biotechnol.* **64**: 197–210.
- Yerushalmi, N., Brinkmann, U., Brinkmann, E., Pai, L., and Pastan, I. 2000. Attenuating the growth of tumors by intratumoral administration of DNA encoding Pseudomonas exotoxin via cationic liposomes. *Cancer Gene Ther.* **7**: 91–96.

Cell-type specific targeting and gene expression using a variant of polyoma VP1 virus like particles

Stefan Gleiter and Hauke Lilie

Martin-Luther-Universität Halle, Institut für Biotechnologie, Kurt-Mothes
Straße 3, D-06120 Halle, Germany

Running title: Functional gene transfer with VP1-Z

Reprint requests to :

Hauke Lilie
Institut für Biotechnologie
Martin-Luther-Universität Halle
Kurt-Mothes Straße 3
D-06120 Halle, Germany

Phone: +49 345 55 24900

Fax: +49 345 55 27013

e-mail: lilie@biochemtech.uni-halle.de

Keywords

Polyomavirus, VP1, VP1-Z, gene transfer, targeting, ErbB2-receptor, cell-type specificity

Abstract

The variant VP1-Z of the polyomavirus coat protein VP1 has been described recently as an engineered fusion protein of VP1 and the antibody binding domain protein Z. This construct is able to specifically bind and functionally present antibodies on the surface of virus like particles of VP1-Z. Here we demonstrate that with the binding of Herceptin, an antibody directed against the receptor tyrosine kinase ErbB2, a cell type specific targeting was established. ErbB2 positive cell lines were transduced with different plasmids encoding eGFP or β -galactosidase. With both reporter systems functional gene expression in transduced cells could be observed. The transduction was strictly dependent on the use of a ternary complex formed of VLPs of VP1-Z, Herceptin, and the reporter plasmid DNA. The use of single components or ErbB2-negative cell lines did not result in functional gene transfer. The transduction was completely dependent on the use of chloroquine, a lysosomotropic reagent. This indicates that the complex is internalized by ErbB2-mediated endocytosis.

Introduction

The mechanisms of viruses to enter host cells and transduce their DNA are multifaceted. Cell binding, cell penetration and transport of nucleic acids into host cells are mediated by structural and functional domains of viruses, and many of these components are the basis for gene delivery systems. The murine polyomavirus belongs to the non-enveloped DNA viruses with their DNA encapsulated in a virus shell consisting of the major coat protein VP1, and the minor coat proteins VP2 and VP3 [6]. Recombinantly produced pentameric VP1 can be assembled *in vitro* into virus like particles (VLPs) in the presence of Ca^{2+} ions [18;28;29]. These VLPs can be loaded with DNA by osmotic shock [8]. DNA is bound with a high affinity to the N-terminal part of VP1 and therefore protected from degradation [21]. Polyoma VLPs have been used as gene transfer systems both *in vitro* and *in vivo* [8;16;17;31;34;41]. Also the use of VLPs as peptide and protein transfer system has been demonstrated [31]. One of the natural cellular ligands for these particles is sialic acid [1;12;35-37] that is present on many eucaryotic cells. Binding to these sugar residues leads to a cell-type unspecific targeting. Structure determination of VP1 revealed that the binding site of sialic acid is located on the surface of the viral coat, beneath the HI-loop [35;36]. Based on this structural knowledge different approaches for the development of an artificial cell-type specific VP1 vector have been followed. One approach is the insertion of a polyionic glutamate stretch in the HI-loop of VP1. This abolishes the natural tropism and also serves as part of a docking module. A Fv-fragment with a poly-arginine tag could be coupled via polyionic interactions on the surface of the modified VP-1 [39;40]. This leads to the generation of a new tropism of the particles if a cell-type specific Fv-fragment is used.

A second approach is the insertion of the docking module protein Z in the HI-loop that binds complete antibodies [10]. Obviating the necessity to develop new Fv-Fragments, this variant,

VP1-Z was previously shown to assemble into VLPs and binds cell-type specific to cells presenting the appropriate antigen that is recognized by the antibody coupled on VP1-Z.

Here we show that the cell-type specific targeting against the ErbB2-receptor leads to the transduction of ErbB2 positive cell lines MCF 7 and SK-BR3. The ErbB2-receptor belongs to the family of the epidermal growth factor receptors. ErbB2-receptor, a tyrosine kinase, serves as a co-receptor for several epidermal growth factor-like ligands that bind and activate other epidermal growth factor receptors. The overexpression of the ErbB2-receptor is observed in several human adenocarcinomas and leads to a constitutive ErbB2-receptor expression. Cell-type specific targeting of tumors overexpressing the ErbB2-receptor has been achieved with the fully humanized anti-ErbB2 antibody Herceptin [33]. Using this antibody we demonstrate that the uptake of the VLPs of VP1-Z depends solely on the antibody/antigen interaction and no unspecific transduction could be detected.

Material and methods

Cell lines:

The human adenocarcinoma cell line SK-BR3 (ATCC: HTB-30), and HT-29 (ATCC: HTB-38), a colorectal adenocarcinoma cell line, were maintained in DEMEM (Invitrogen), supplemented with 10% FCS (Invitrogen) and 0.5% Gentamicin (Invitrogen).

HeLa CD4⁺ Clone 1022 (NIH AIDS Research & Reference Reagent Program), and MCF 7 (ATCC: HTB-22), a human adenocarcinoma cell line, were maintained in RPMI 1640 (Invitrogen), supplemented with 10% FCS, 1% Glutamax and G418 (1 mg/ml final concentration) for HeLa CD4⁺ or 0.5% Gentamicin for MCF 7. CHO-AA8-Luc tet-off from Clontech was maintained in MEM (Invitrogen), supplemented with 10% FCS and 100 µg/ml G418 and 100 µg/ml Hygromycin B. NIH/3T3 mouse fibroblast cells (ATTC: CRL-1658) were maintained in RPMI 1640 (Invitrogen), supplemented with 10% CS, 1% Glutamax and 0.5% Gentamicin. All cells were cultivated in a humidified atmosphere at 37°C and 5% CO₂.

Proteins:

VP1-Z, a variant of the polyoma coat protein VP1, was prepared as described previously [10]. The concentration of VP1-Z was determined by UV-VIS spectroscopy using an extinction coefficient of $\epsilon_{280\text{ nm}; 1\text{ cm}} = 1.3$ for a protein solution of 1 mg/ml. Humanized anti-ErbB2 antibody (MAK<Her2>rH-IgG, Herceptin) was kindly provided by Roche Diagnostics (Penzberg, Germany).

Transduction protocol:

For transduction with the plasmid pEGFP-C1 (Clontech), cells were seeded in 6-well plates containing sterile coverslips. For the β -galactosidase reporter assay cells were seeded in 12-well plates. At a confluency of about 60-70% cells were washed three times with cold medium in the absence of any supplements. After addition of medium and transduction agent, cells were incubated for 1h at 37°C /5% CO₂. Afterwards chloroquine (final concentration of 50 µM) as well as FCS or CS, respectively, were added. Cells were then incubated, depending

on how long chloroquine in the medium was tolerated: MCF 7: incubation for 72 hours, HeLa CD4⁺, SK-BR3, NIH 3T3, and CHO-AA8-Luc tet-off for 12-14 hours.

Subsequently cells were washed carefully 3 times with PBS and fresh medium without antibiotics was added. All cells were incubated at least 72 hours post transduction before expression of β -galactosidase or GFP was analyzed.

Transduction reagents:

For transduction of cells, VLPs of VP1-Z, the humanized anti-ErbB2 antibody Herceptin and a GFP encoding plasmid (pEGFP-C1, Clontech) or a β -galactosidase encoding plasmid pELI92 [42] were used. First VP1-Z was incubated with antibody before DNA was added. Afterwards the mixture was replenished with buffer (20 mM Tris pH 7.4, 200 mM NaCl, 5% (v/v) glycerol, 1 mM CaCl₂) to a final volume of 100 μ l. The mixture was then incubated at 20°C for 20 minutes to allow formation of the ternary complex. Afterwards the transduction reagent was added to the cell culture medium to a final volume of 2 ml and incubated with the cells. The amount of protein and the ratio of antibody and DNA to the VLPs of VP1-Z is stated in the result section and figure legends.

Reporter plasmid assays:

GFP expression was visualized using a Zeiss fluorescence microscope LSM 100M.

Cells were grown in 6-well plates on coverslips. After the transduction experiment was finished, the cells were washed extensively with cold PBS and fixed with paraformaldehyde (3% (w/v)). Subsequently the coverslips were mounted with Mowiol 4-88 (Calbiochem) containing DABCO (0.2% (w/v)) and the cells were analyzed by fluorescence microscopy .

The β -galactosidase activity was measured according to the manufacturers protocol (Promega β -galactosidase enzyme assay system with reporter lysis buffer, Promega corporation, Mannheim, Germany). Absorption of ortho-nitrophenol was measured at 405 nm using a polarstar microplate reader. All measured values were corrected for light scattering using the

signal at 544 nm. All transduction experiments with the β -galactosidase encoding plasmid were replicated at least three times.

DNA binding studies:

2.8 nM of plasmid pELI 92 were incubated with different concentrations (0.093 to 11.2 nM) of VLPs of VP1-Z. Each sample was incubated at 20°C for 20 minutes. DNA binding was analyzed by sedimentation velocity experiments in an analytical ultracentrifuge using an AN50Ti rotor and double sector cells. The experiments were performed at 10,000 rpm, 20°C. Scans were taken every 10 min at 260 nm. The data were analyzed using the software provided by Beckman instruments.

Plasmid preparation:

Plasmids were propagated in *E.coli* XL1-blue (Stratagene, CA). Plasmid preparation was performed according to the manufactures protocol (Qiagen, Mini-Spin Prep or Qiagen Qia-Filter Mega Prep). The concentration of the plasmid was determined using UV-VIS spectroscopy. To verify the identity of the plasmids, analytical restriction digests were performed.

Results

Binding of plasmid DNA to VP1-Z

The insertion of the antibody binding domain Z in the HI-loop of VP1 had no influence on several biophysical parameters of VP1 compared to those of the wildtype protein [10]. However, binding and transportation of DNA using VLPs of VP1-Z as an important factor of gene delivery systems has not been analyzed so far. To demonstrate that the insertion of protein Z did not influence the DNA binding properties of VP1 [11;20;21], the interaction of VLPs of VP1-Z with supercoiled plasmid DNA of 7.2 kb was analyzed by analytical ultracentrifugation. Under the chosen experimental conditions, the supercoiled plasmid DNA at a concentration of 2.8 nM showed only a very slow sedimentation (Fig. 1A). Addition of a 4-fold molar excess of VLPs of VP1-Z over plasmid DNA resulted in a quantitative co-sedimentation of DNA and VLPs of VP1-Z, indicating an efficient binding of DNA to VLPs of VP1-Z (Fig. 1C). Even at stoichiometric concentration of VLPs of VP1-Z to DNA an interaction of both partners could be observed (data not shown). In addition the DNA binding ability of VLPs of VP1-Z could be verified using a gel shift assay. A similar behavior could also be shown for the VLPs of wtVP1 (data not shown). The ability of the unspecific DNA binding site of VP1 to interact with DNA seems therefore not to be changed in the variant VP1-Z.

Transduction of cells expressing the ErbB2 antigen

After showing the ability of VP1-Z to bind DNA, we analyzed the use of VLPs of VP1-Z as a cell-type specific gene delivery system. To test this, we prepared a ternary complex consisting of VLPs of VP1-Z, reporter gene DNA and the tumor specific anti-ErbB2 antibody Herceptin. As demonstrated earlier, Herceptin could be coupled on the surface of VLPs of VP1-Z with very high efficiency. Here a coupling rate of 72 antibodies Herceptin per VLP was used. Herceptin binds to the overexpressed ErbB2-receptor on the surface of several human cancer

cell lines including human breast cancer cell lines and ovarian carcinomas [5]. As a reporter system of transduction the expression of green fluorescent protein was documented. Since the antibody bound to the ErbB2-receptor is internalized by receptor-mediated endocytosis [7;9;14], transduction experiments were performed in the presence of the lysosomotropic chloroquine to ensure at least a partial release of the vector into the cytosol of the cells. During the whole time scale of the experiment cells showed a normal shape and no noticeable abnormalities (Fig. 2A), indicating that the cells survived the transduction process and the incubation with chloroquine for 72 hours. 48 hours after transduction expressed GFP started to become visible and reached a maximum at about 72 hours. In Fig. 2B the fluorescence of the same cells shown in Fig. 2A is documented. A significant fraction of cells expressed GFP. Obviously, the fluorescence of GFP is located inside the cells. This was also confirmed using CLS (data not shown).

The transduction of MCF 7 cells totally depend on the presence of chloroquine. Without this lysosomotropic agent no GFP expression could be detected (data not shown), indicating that the vector is directed from the endosomes to the lysosomes and is degraded.

The transduction of MCF 7 was strictly based on the Herceptin-mediated targeting of the VLPs of VP1-Z. Without the coupled Herceptin MCF 7 could not be transduced with VLPs of VP1-Z, indicating that the cell-type unspecific but efficient targeting of wtVP1 by its natural sialic acid binding activity was completely suppressed in our mutant VP1-Z. Similarly, incubation of MCF 7 with Herceptin and DNA but in the absence of VLPs of VP1-Z did not result in GFP expression (data not shown). Thus no unspecific internalization of the reporter plasmid could be observed under any conditions.

Similar results were obtained if β -galactosidase encoding plasmid was used for transduction.

For MCF 7, only transduction with the complete ternary complex of VLPs of VP1-Z, plasmid DNA encoding β -galactosidase and Herceptin, led to expression of β -galactosidase. If one or two components of this vector system were omitted the enzymatic activity measured after

transduction was identical to the endogenous β -galactosidase activity of untreated cells within the range of error (Fig 3A). The same results were obtained for another erbB2-positive cell line SK-BR3. Since SK-BR3 cells were much more sensitive to the cytotoxic effect of chloroquine, the incubation time was kept significantly shorter than for MCF 7. Furthermore, a cytotoxic and anti-proliferative effect of Herceptin on these cells has been reported [5;25;33]. Therefore the overall transduction rate was noticeable lower than for MCF 7, but differences between the transduction and control experiment were still significant. The cell-type specific effect of targeting and gene delivery could be shown only with the ternary complex of VLPs of VP1-Z, DNA and antibody (Fig. 3B).

Efficiency of the transduction

After obtaining positive results for the cell-type specific gene transfer, we attempted to optimize the vector system. All three components of the system were titrated and the resulting vector assayed regarding its transduction efficiency on MCF 7 cells. The ratio of VLPs of VP1-Z, presenting approximately 70 antibody molecules, to plasmid DNA was changed in a range from no plasmid DNA to a ten fold molar excess of plasmid over VLP. As shown in Figure 4A, at a molar ratio of VLP to plasmid of 1:1 to 1:5 a maximum of transduction was obtained. The decrease in transduction at very high concentrations of plasmid DNA might be due to extensive binding of DNA on the surface of VLPs of VP1-Z, thus shielding the coupled antibody and diminishing cell targeting. Also the coupling rate of antibody was varied from the absence of any Herceptin to a three-fold molar excess of antibody over the VP1-Z monomer, which resulted in an almost complete saturation of VLPs of VP1-Z with Herceptin. The β -galactosidase activity obtained after gene delivery was plotted against the ratio of VP1-Z monomers to antibody (Fig. 4B). The transduction efficiency reached a plateau at a ratio of one antibody per pentamer of VP1-Z, resulting in 36 coupled antibodies per VLP.

We further analyzed, if increasing amounts of transduction-active ternary complex in optimal composition, lead to an advance in transduction and expression of β -galactosidase. In Figure 4C the result of this titration is shown. At low concentrations of transduction complex, an increase of transduction could be observed with increasing the amount of complex. At 3 μ g VP1-Z (and a ratio of 1 plasmid per VLP and 72 antibodies per VLP of VP1-Z) a plateau of expressed activity was reached. This demonstrates that with relatively low amounts of the ternary complex, the best transduction and gene expression could be obtained.

With this optimized vector system and a titre of $5 \cdot 10^{13}$ particles (corresponding to 3 μ g) per $1 \cdot 10^6$ cells of MCF7 a transduction efficiency of 0.45% as analyzed by FACS analysis could be obtained. This transduction efficiency is comparable to that of wtVP1, which was shown to be in the range of 0.1-0.5% using the same titres [16;17].

Cell-type specificity of gene delivery

The transduction of the cell lines MCF 7 and SK-BR3 demonstrated that functional gene delivery strictly depends on the interaction of the antibody Herceptin with cellular ErbB2-receptor. However, these results did not rule out that with cell lines not overexpressing the ErbB2-receptor on the cell surface the vector system might be internalized via other mechanisms. Therefore we screened cell lines that are not described to be positive for the overexpression of ErbB2-receptor. NIH/3T3 is reported to express high amounts of sialic acids on its surface. Since sialic acid is the natural target of polyomavirus, we used this cell line to analyze whether VLPs of VP1-Z may still possess a residual affinity to sialic acids in the absence of ErbB2. Furthermore, we chose a human cervix adenocarcinoma cell line, a hamster ovarian cell line, and a human colorectal adenocarcinoma as other negative cell lines. Whereas transduction of MCF 7 led to a significant increase in β -galactosidase expression compared to the control (Fig. 5), for none of the control cell lines the transduction resulted in

a signal significantly higher than the background signal, strongly indicating that the gene transfer indeed is strictly dependent on the antibody-mediated cellular targeting.

Discussion

Many different transduction systems for gene or cancer therapy have been evolved in the last decade. The main focus has been the development and improvement of viral systems. Adenoviruses, AAV and retroviral systems are the main topics of recent research. The major advantage of the viral system is the utilization of the viral DNA packaging, cell entry and uncoating mechanism. But these natural mechanisms bear also a major disadvantage. The natural tropism of the virus directs the vector system to its natural target cells. Usually this includes rather different tissues than specific cell types. Many attempts have been made to circumvent this natural tropism and to create a new, cell-type specific targeting mechanism. For example, scFv fragments were fused to the coat proteins of retroviruses [2;3;13;19]. Ohno et al. and Morizono et al. inserted the tandem repeat of protein Z in the coat protein of sindbis virus and retrovirus [3;22-24] and coupled cell-type specific antibodies to this modified viruses. Even in the case of non-viral vectors such as liposomes attempts have been reported to create a cell-type specific targeting of so called immunoliposomes by coupling of cell type specific antibodies [14;26].

Virus-like particles of the major coat protein VP1 of murine polyoma virus represent a combination of both, the non-viral and the viral systems. Derived from a murine virus, the recombinant and safe production in *E.coli* yields high amounts of protein [18]. The ability of the pentameric form of VP1 to self-assemble *in vitro* allows the formation of VLPs that can be used to transduce cells [8;34;38]. Because the natural ligand of polyomavirus, sialic acids, is present on almost all mammalian cells, this transduction proved to be cell-type unspecific.

The structure of VP1 was solved to high resolution [36], allowing rational attempts to modify the VP1 protein. One attempt to change the natural tropism of the wtVLPs was based on the insertion of a polyionic glutamate stretch in the HI-loop. This polyionic peptide blocked the sialic acid binding of VP1 and additionally served as an anchor to couple a tumor specific antibody Fv-fragment. Using this vector system a cell-type specific targeting and gene

transfer could be demonstrated [40]. Because this approach always depends on the modification of a Fv-fragment and a coupling reaction of VP1 and Fv-fragment, we decided to insert a coupling module for intact antibodies in the HI-loop of VP1. With the insertion of the IgG binding domain from protein A, protein Z, we recently described a fusion protein that is able to form VLPs and is capable of binding antibodies on its surface. Besides this, the binding to sialic acids was diminished [10].

Here we show that this vector based on VP1-Z, in principal can be used as a cell-type specific gene delivery system. Whereas in this study only large plasmids were used merely as an analytical detection system of cellular transduction reasonable therapeutic approaches can make use of smaller DNA fragments in order to enhance the packaging in the VLP and enhance protection of the transported DNA. Also the use of small DNA or RNA molecules like DNAzymes or ribozymes opens the possibility to bind or package more than one copy of biologically active nucleic acid per particle, raising the effective concentration of therapeutic molecules per VLP that transduces a cell.

For the cell-type specific targeting of VLPs of VP1-Z carrying an appropriate marker gene, we chose the monoclonal antibody Herceptin which is directed against the tumor marker ErbB2, a receptor tyrosine kinase. This receptor is overexpressed in several cancer cell lines, in particular in breast cancer [32]. ErbB2 as target for the therapeutic antibody Herceptin has been used very successfully in the therapy of cancer. The effect of this therapeutic antibody is not yet fully understood, but several modes of action are discussed, e.g. the activation of the ADCC pathway, a cell cytotoxic effect, and a decrease in cell growth [4;5;15;33]. It was also described that the specific binding of this antibody and its effects depend on the amount of antigen overexpressed. For this reason we used a cell line expressing high amounts of ErbB2 (SK-BR3)[5;14] and MCF 7, a cell line expressing moderate levels of ErbB2 [5]. As read out system for transduction GFP or β -galactosidases expression was used.

The cell-type specificity of gene transfer could be demonstrated, because expression of either GFP or β -galactosidase could only be observed in cells overexpressing the ErbB2-receptor. Cells that do not overexpress ErbB2-receptor could not be transduced. The cell-type specific transduction depends on the antibody coupled to VLPs of VP1-Z and is not provoked by any cell binding ability from the VP1 part of the fusion protein. Only if the ternary complex of VP1-Z, Herceptin and DNA was used functional gene transfer could occur. Best results were obtained with a coupling rate of at least 36 antibodies per VLP, resulting in a transduction rate of 0.45%.

However the transduction of antigen presenting cells is dependent on the use of chloroquine so far. The natural route of cellular uptake of wt polyomavirus occurs presumably via caveolae [27], for which polyoma is adapted. In our system the internalization is mediated by receptor mediated endocytosis of ErbB2-receptor, the target of the tumor-specific antibody used for cell recognition [14]. Thus particles end up in endosomes. It has not yet been described that VP1 or the complete murine polyomavirus utilizes mechanisms for the release from endosomes into the cytosol or requires the low pH in the lysosomes to escape. Therefore, the presence of the lysomotropic agent chloroquine was essential to prove functional gene transfer via the antibody coupled on VLPs of VP1-Z. This clearly indicates that, after establishing cell-type specificity of gene transfer the next step in developing polyoma VLPs as vector system is the further modification of these VLPs in order to ensure the release of the vector from endosomes biochemically.

The necessity of binding of only 36 antibodies to the VLPs for cell specific targeting provides a wide range of possibilities for further applications. One could imagine that the binding of different cell-type specific antibodies at the same time on the surface of VLPs may result in a highly selective targeting of cells, an effect reported for an immunotoxin containing two different antibody fragments [30]. Also the use of VP1-Z in a modular context is possible. Because only 36 antibodies are required for efficient gene transfer, it is conceivable to

assemble VLPs from VP1-Z and other VP1 derivatives, e.g. VP1E8C [40]. This would allow the additional presentation of peptides and proteins other than antibodies with functions necessary for the intracellular trafficking of the vector system, thus further improving the transduction efficiency of polyoma VLPs.

In summary, the insertion of an IgG binding domain in the HI-loop of VP1 leads to a cell-type specific transduction system, depending on the binding of the appropriate antibody. Because of a rapid development of new humanized antibodies and the increasing knowledge about cell-type specific antigens, this system can easily be adapted to different applications. Not only the cell-type specific gene transfer but also its modular design that allows a change in tropism just by replacing the antibody used, offers a wide range of applications. The *in vitro* assembly of polyoma VLPs should allow the combination of different VP1 variants in one particle. In this modular design VP1-Z could serve as docking module for cell-type specific antibodies whereas other VP1 variants, e.g. VP1E8C [40], might be used for coupling of peptides and proteins with other functions such as disruption of endosomes and reduction of immunogenicity of polyoma VP1.

Acknowledgements

We thank U. Brinkmann and R. Rudolph for fruitful discussion and J. Winter for critically reading the manuscript. We are grateful to K.H. Sellinger from Roche Diagnostics for the kind gift of Herceptin. D. Mayer and A. Navarrete Santos are acknowledged for their help with FACS analysis. This work was supported by a grant of the DFG to H. Lilie and a scholarship of the Land Sachsen-Anhalt to S. Gleiter.

Abbreviations

FCS, fetal calf serum; CS, calf serum; eGFP, enhanced green fluorescent protein; VLP, virus like particle; PFA, paraformaldehyde; wtVP1, wildtype VP1; mab, monoclonal antibody

Reference List

1. Bauer, P. H., C. Cui, R. Liu, T. Stehle, S. C. Harrison, J. A. DeCaprio, and T. L. Benjamin. (1999). Discrimination between sialic acid-containing receptors and pseudoreceptors regulates polyomavirus spread in the mouse. *J Virol.* **73**, 5826-5832
2. Chu, T. H. and R. Dornburg. (1997). Toward highly efficient cell-type-specific gene transfer with retroviral vectors displaying single-chain antibodies. *J Virol.* **71**, 720-725
3. Chu, T. H., I. Martinez, W. C. Sheay, and R. Dornburg. (1994). Cell targeting with retroviral vector particles containing antibody- envelope fusion proteins. *Gene Ther.* **1**, 292-299
4. Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. (2000). Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med* **6**, 443-446
5. Cuello, M., S. A. Ettenberg, A. S. Clark, M. M. Keane, R. H. Posner, M. M. Nau, P. A. Dennis, and S. Lipkowitz. (2001). Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand- mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. *Cancer Res.* **61**, 4892-4900
6. Eckhart, W. 1990. Polyomavirinae and their replication, p. 727-742. *In* B. N. Fields and D. M. Knipe (eds.), *Fundamental virology*. Raven Press
7. Fendly, B. M., M. Winget, R. M. Hudziak, M. T. Lipari, M. A. Napier, and A. Ullrich. (1990). Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. *Cancer Res.* **50**, 1550-1558
8. Forstova, J., N. Krauzewicz, V. Sandig, J. Elliott, Z. Palkova, M. Strauss, and B. E. Griffin. (1995). Polyoma virus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells. *Hum. Gene Ther.* **6**, 297-306
9. Foster, B. J. and J. A. Kern. (1997). HER2-targeted gene transfer. *Hum. Gene Ther.* **8**, 719-727
10. Gleiter, S. and H. Lilie. (2001). Coupling of antibodies via protein Z on modified polyoma virus-like particles. *Protein Sci* **10**, 434-444
11. Henke, S., A. Rohmann, W. M. Bertling, T. Dingermann, and A. Zimmer. (2000). Enhanced in vitro oligonucleotide and plasmid DNA transport by VP1 virus-like particles. *Pharm. Res.* **17**, 1062-1070
12. Herrmann, M., C. W. der Lieth, P. Stehling, W. Reutter, and M. Pawlita. (1997). Consequences of a subtle sialic acid modification on the murine polyomavirus receptor. *J Virol.* **71**, 5922-5931
13. Jiang, A., T. H. Chu, F. Nocken, K. Cichutek, and R. Dornburg. (1998). Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies. *J Virol.* **72**, 10148-10156

14. Kirpotin, D., J. W. Park, K. Hong, S. Zalipsky, W. L. Li, P. Carter, C. C. Benz, and D. Papahadjopoulos. (1997). Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry* **36**, 66-75
15. Klapper, L. N., H. Waterman, M. Sela, and Y. Yarden. (2000). Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res.* **60**, 3384-3388
16. Krauzewicz, N., C. Cox, E. Soeda, B. Clark, S. Rayner, and B. E. Griffin. (2000). Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids. *Gene Ther.* **7**, 1094-1102
17. Krauzewicz, N., J. Stokrova, C. Jenkins, M. Elliott, C. F. Higgins, and B. E. Griffin. (2000). Virus-like gene transfer into cells mediated by polyoma virus pseudocapsids. *Gene Ther.* **7**, 2122-2131
18. Leavitt, A. D., T. M. Roberts, and R. L. Garcea. (1985). Polyoma virus major capsid protein, VP1. Purification after high level expression in *Escherichia coli*. *J. Biol. Chem.* **260**, 12803-12809
19. Martin, F., J. Kupsch, Y. Takeuchi, S. Russell, F. L. Cosset, and M. Collins. (1998). Retroviral vector targeting to melanoma cells by single-chain antibody incorporation in envelope. *Hum. Gene Ther.* **9**, 737-746
20. Moreland, R. B. and R. L. Garcea. (1991). Characterization of a nuclear localization sequence in the polyomavirus capsid protein VP1. *Virology* **185**, 513-518
21. Moreland, R. B., L. Montross, and R. L. Garcea. (1991). Characterization of the DNA-binding properties of the polyomavirus capsid protein VP1. *J. Virol.* **65**, 1168-1176
22. Morizono, K., G. Bristol, Y. Xie, S. K. Kung, and I. S. Chen. (2001). Antibody-Directed Targeting of Retroviral Vectors via Cell Surface Antigens. *J Virol.* **75**, 8016-8020
23. Ohno, K. and D. Meruelo. (1997). Retrovirus vectors displaying the IgG-binding domain of protein A. *Biochem. Mol. Med* **62**, 123-127
24. Ohno, K., K. Sawai, Y. Iijima, B. Levin, and D. Meruelo. (1997). Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A. *Nat. Biotechnol.* **15**, 763-767
25. Park, B. W., H. T. Zhang, C. Wu, A. Berezov, X. Zhang, R. Dua, Q. Wang, G. Kao, D. M. O'Rourke, M. I. Greene, and R. Murali. (2000). Rationally designed anti-HER2/neu peptide mimetic disables P185HER2/neu tyrosine kinases in vitro and in vivo. *Nat. Biotechnol* **18**, 194-198
26. Park, J. W., K. Hong, P. Carter, H. Asgari, L. Y. Guo, G. A. Keller, C. Wirth, R. Shalaby, C. Kotts, W. I. Wood, and . (1995). Development of anti-p185HER2 immunoliposomes for cancer therapy. *Proc Natl Acad Sci U S A* **92**, 1327-1331
27. Richterova, Z., D. Liebl, M. Horak, Z. Palkova, J. Stokrova, P. Hozak, J. Korb, and J. Forstova. (2001). Caveolae Are Involved in the Trafficking of Mouse Polyomavirus Virions and Artificial VP1 Pseudocapsids toward Cell Nuclei. *J Virol.* **75**, 10880-10891

28. Salunke, D. M., D. L. Caspar, and R. L. Garcea. (1986). Self-assembly of purified polyomavirus capsid protein VP1. *Cell* **46**, 895-904
29. Salunke, D. M., D. L. Caspar, and R. L. Garcea. (1989). Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophys J* **56**, 887-900
30. Schmidt, M., N. E. Hynes, B. Groner, and W. Wels. (1996). A bivalent single-chain antibody-toxin specific for ErbB-2 and the EGF receptor. *Int. J Cancer* **65**, 538-546
31. Schmidt, U., C. Gunther, R. Rudolph, and G. Bohm. (2001). Protein and peptide delivery via engineered polyomavirus-like particles. *FASEB J* **15**, 1646-1648
32. Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A. Ullrich, and . (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707-712
33. Sliwkowski, M. X., J. A. Lofgren, G. D. Lewis, T. E. Hotaling, B. M. Fendly, and J. A. Fox. (1999). Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). *Semin. Oncol.* **26**, 60-70
34. Soeda, E., N. Krauzewicz, C. Cox, J. Stokrova, J. Forstova, and B. E. Griffin. (1998). Enhancement by polylysine of transient, but not stable, expression of genes carried into cells by polyoma VP1 pseudocapsids. *Gene Ther.* **5**, 1410-1419
35. Stehle, T. and S. C. Harrison. (1996). Crystal structures of murine polyomavirus in complex with straight- chain and branched-chain sialyloligosaccharide receptor fragments. *Structure.* **4**, 183-194
36. Stehle, T. and S. C. Harrison. (1997). High-resolution structure of a polyomavirus VP1-oligosaccharide complex: implications for assembly and receptor binding. *Embo J* **16**, 5139-5148
37. Stehle, T., Y. Yan, T. L. Benjamin, and S. C. Harrison. (1994). Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* **369**, 160-163
38. Stokrova, J., Z. Palkova, L. Fischer, Z. Richterova, J. Korb, B. E. Griffin, and J. Forstova. (1999). Interactions of heterologous DNA with polyomavirus major structural protein, VP1. *FEBS Lett.* **445**, 119-125
39. Stubenrauch, K., A. Bachmann, R. Rudolph, and H. Lilie. (2000). Purification of a viral coat protein by an engineered polyionic sequence. *J Chromatogr. B Biomed. Sci Appl.* **737**, 77-84
40. Stubenrauch, K., S. Gleiter, U. Brinkmann, R. Rudolph, and H. Lilie. (2001). Conjugation of an antibody Fv fragment to a virus coat protein: cell- specific targeting of recombinant polyoma-virus-like particles. *Biochem J* **356**, 867-873
41. Yang, Y. W. and L. H. Chen. (2000). Gene delivery via polyomavirus major capsid protein VP(1), isolated from recombinant Escherichia coli. *Biotechnol Appl. Biochem* **32 (Pt 1)**, 73-79

42. Yerushalmi, N., U. Brinkmann, E. Brinkmann, L. Pai, and I. Pastan. (2000). Attenuating the growth of tumors by intratumoral administration of DNA encoding *Pseudomonas* exotoxin via cationic liposomes. *Cancer Gene Ther.* **7**, 91-96

Figure Legends

Figure 1: Binding of plasmid DNA to VLPs of VP1-Z.

DNA binding was analyzed using analytical ultracentrifugation at 10,000 rpm. The samples were incubated at 20°C for 20 minutes before starting the measurements. Scans were taken every 10 minutes (displayed every 20 min.). The concentration of the plasmid pELI92 and VLPs of VP1-Z was 2.8 nM and 11.2 nM, respectively.

A: Sedimentation of plasmid pELI92.

B: Sedimentation of VLPs of VP1-Z without DNA.

C: Sedimentation of the complex, formed with a 4-fold molar excess of VLPs of VP1-Z over plasmid DNA.

Figure 2: Fluorescence microscopy of MCF 7 cells expressing eGFP

MCF 7 cells, grown on coverslips, were transduced with 5 µg VLPs of VP1-Z, and a molar ratio of 1 molecule Herceptin per VP1-Z pentamer and 1 plasmid encoding eGFP per VLP of VP1-Z. Coverslips were prepared 72 hours after transduction. Cells were fixed with PFA and mounted in Mowiol/DABCO.

A: Transmission view of cells at 200-fold magnification. The detail of the slide shows MCF 7 cells after transduction.

B: Fluorescence view of the same detail and same magnification as in figure A.

Figure 3: β -galactosidase expression after transduction of ErbB2-receptor overexpressing cells.

A: MCF 7 cells were transduced with the plasmid pELI92 encoding the β -galactosidase gene. For transduction, 10 µg VLPs of VP1-Z loaded with approx. 90 molecules of the monoclonal antibody (mab) Herceptin and 1 plasmid molecule were used. Enzymatic activity was measured 72 hours after transduction and subsequent cell lysis. For control, the single

components of the ternary complex as well as different combinations were used for transduction. The concentrations used were the same as in the transduction with the ternary complex. Furthermore, cells were treated only with the buffer, used to replenish the transduction samples. Also cells were only treated with chloroquine (control cells), without any other reagent. Measured activities were obtained from at least three independent experiments.

B: SK-BR3 cells transduced with the β -galactosidase encoding plasmid. The amount of VLPs of VP1-Z was 5 μ g and a coupling rate of 72 monoclonal antibodies (mab) Herceptin per VLP of VP1-Z and 1 plasmid per VLP of VP1-Z was utilized. The same control experiments as described for A were performed.

Figure 4: Optimization of the transduction protocol.

Upon the preparation of the vector system, the concentration of VLPs of VP1-Z was kept constant and the concentrations of Herceptin and plasmid DNA were titrated. Afterwards the optimal ratios were used to titrate the amount of ternary complex used for transduction. All transduction experiments were performed using the MCF 7 cell line and were replicated at least three times.

A: Titration of plasmid DNA over VLPs of VP1-Z

For titration of plasmid DNA, 10 μ g of VLPs of VP1-Z and a coupling rate of 90 antibodies per VLP of VP1-Z was used. The ratio of VLPs of VP1-Z to plasmid DNA is plotted against the measured β -galactosidase activity.

B: Titration of Herceptin over on VLPs of VP1-Z.

The amount of Herceptin on the surface of VLPs of VP1-Z was titrated using 5 μ g of VLPs of VP1-Z and 1 plasmid per VLP of VP1-Z. The molar ratio of Herceptin to VP1-Z monomer is plotted against the measured β -galactosidase activity of transduced cells.

C: Optimization of the amount of ternary complex used.

To determine whether the transduction efficiency is dependent on the amount of the ternary complex, the optimal ratios of VLP of VP1-Z to Herceptin (1 antibody per pentamer) and DNA (1 plasmid per VLP of VP1-Z) were used and the overall amount of transduction agent was titrated. As control, the background activity in cells treated only with chloroquine was measured.

Figure 5: Cell-type specificity of transduction

Different cell lines were transduced with the ternary complex to analyze the specificity and efficiency of gene transfer. CHO-AA8-Luc tet-off, NIH/3T3 and HeLaCD4⁺ are not described to overexpress the ErbB2-receptor and are therefore the negative control for the cell-type specific gene transfer. MCF7 is the cell line used in the prior experiments. Transduction was carried out under optimal conditions with 5 µg VLPs of VP1-Z, a coupling rate of 72 antibodies Herceptin per VLP of VP1-Z, and 1 plasmid molecule per VLP in the presence of chloroquine. The β -galactosidase expression was analyzed in comparison to cells untreated or cells treated with the plasmid only.

Figure 1

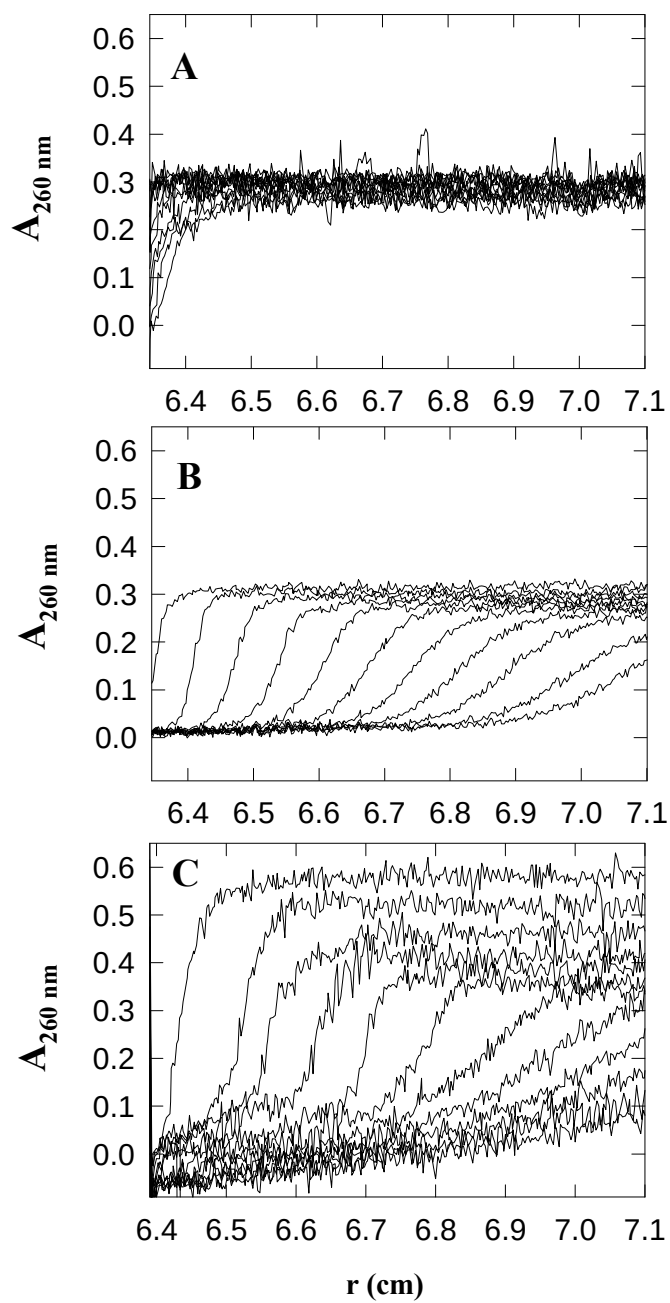


Figure 2

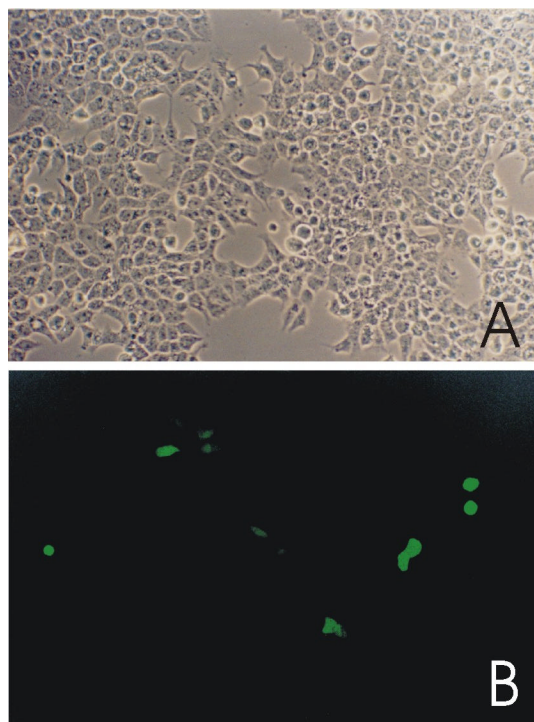


Figure 3A

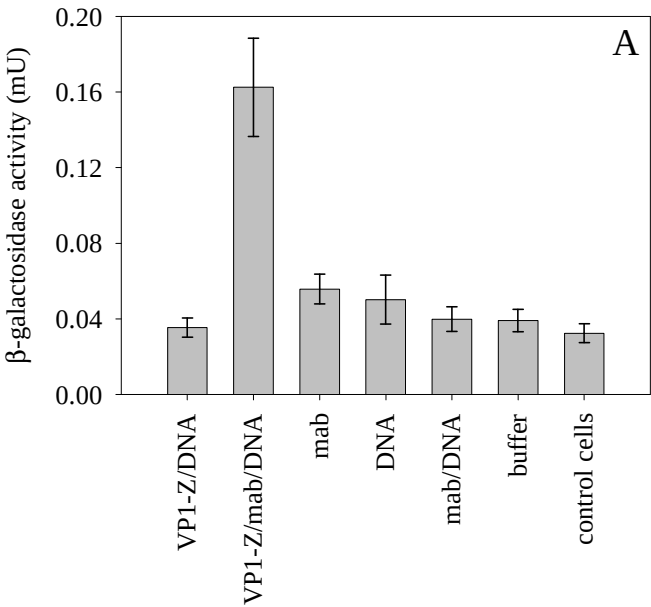


Figure 3B

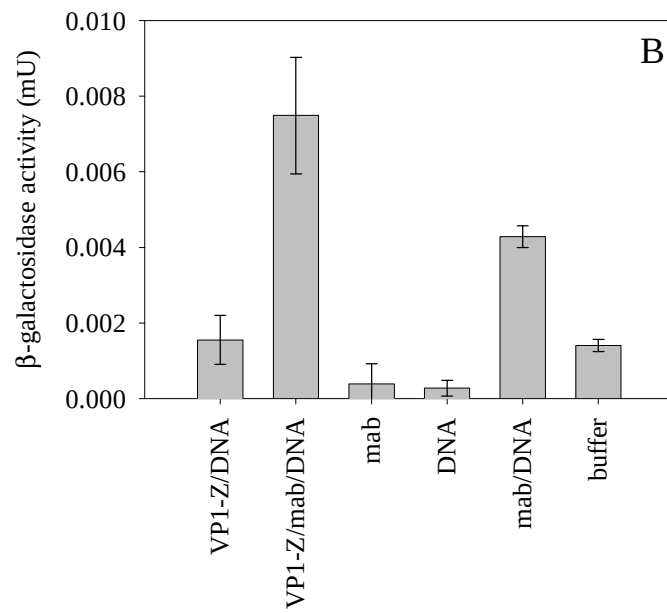


Figure 4A

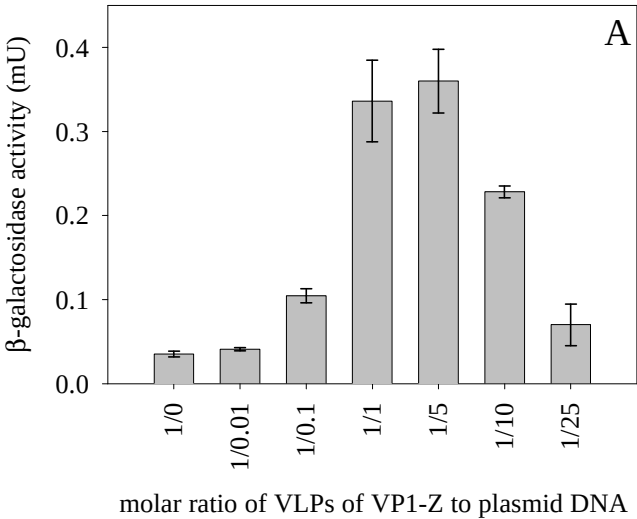


Figure 4B

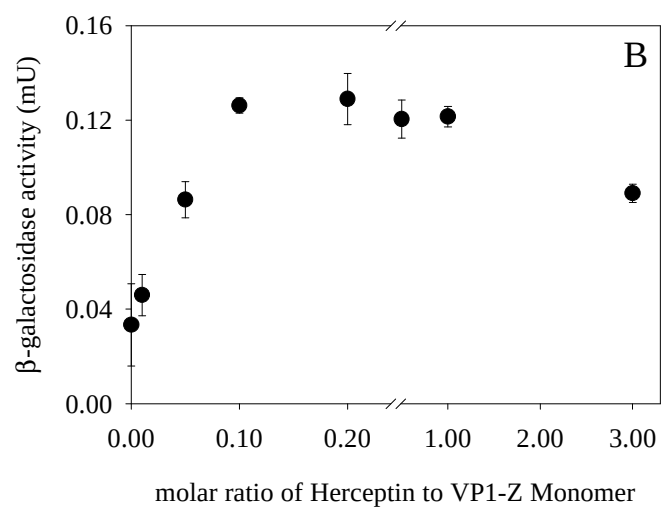


Figure 4C

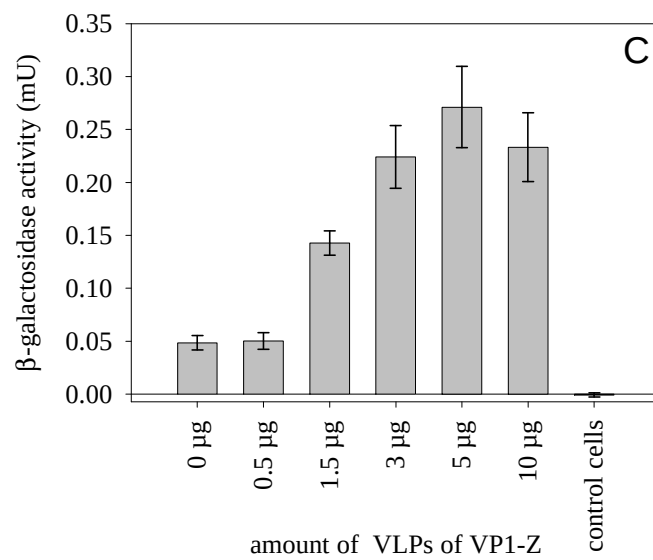
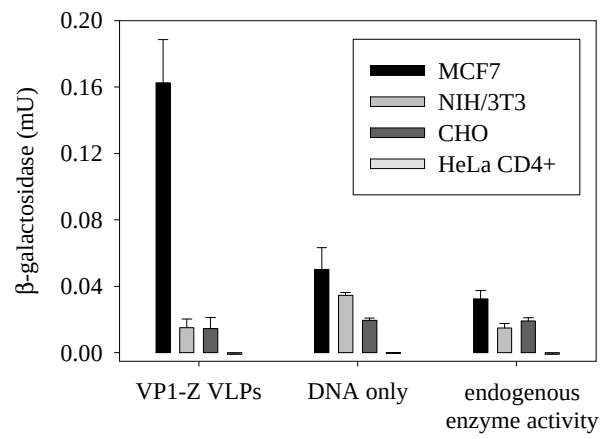


Figure 5



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

Kay STUBENRAUCH*, Stefan GLEITER*, Ulrich BRINKMANN†, Rainer RUDOLPH* and Hauke LILIE*¹

*Institut für Biotechnologie, Universität Halle, Kurt Mothes Strasse 3, D-06120 Halle, Germany, and †Epidauros Biotechnology, Am Neuland 1, D-82347 Bernried, Germany

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.

¹ To whom correspondence should be addressed (e-mail lilie@biochemtech.uni-halle.de).

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 (42 000 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 promoter, 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 10 000-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

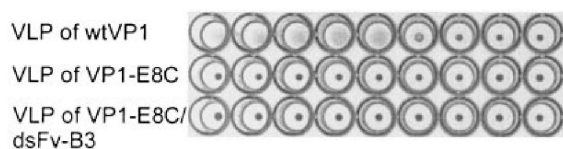


Figure 3 Haemagglutination assay for VP1 VLPs

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 Glu₃Cys 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

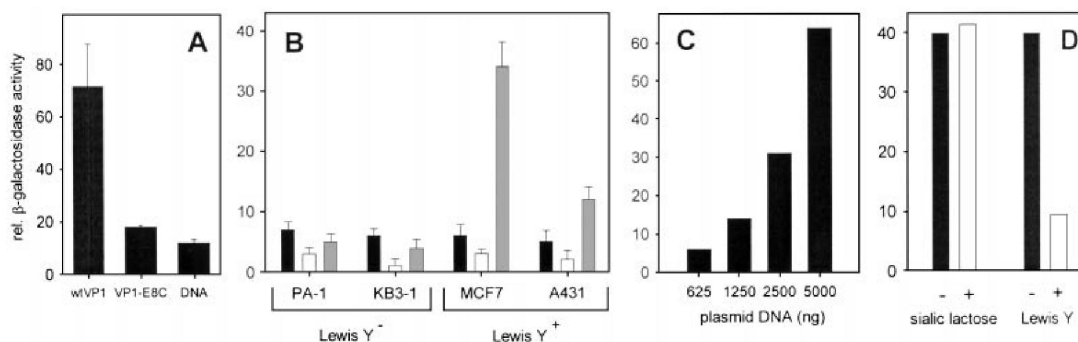


Figure 4 Cell-type-specific targeting of particles of VP1-E8C/dsFv-B3-R8C

(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 20 000-fold molar excess of 3'-sialyl-lactose and a 40 000-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.

We thank Elisabeth Brinkmann and Peter Neubauer for advice in performing cell culture experiments and fermentation respectively, and Ira Pastan for the gift of expression clones of dsFv-B3 and for helpful discussions. This work was supported by a grant of the Deutsche Forschungsgemeinschaft to H.L.

REFERENCES

- Robbins, P. D., Tahara, H. and Ghivizzani, S. C. (1998) Viral vectors for gene therapy. *Trends Biotechnol.* **16**, 35–40.
- Russell, S. J., Hawkins, R. E. and Winter, G. (1993) Retroviral vectors displaying functional antibody fragments. *Nucleic Acids Res.* **21**, 1081–1085.
- Jiang, A., Chu, T. H., Nocken, F., Cichutek, K. and Dornburg, R. (1998) Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies. *J. Virol.* **72**, 10148–10156.
- Konishi, H., Ochiya, T., Chester, K. A., Begent, R. H., Muto, T., Sugimura, T., Terada, M. and Begent, R. H. (1998) Targeting strategy for gene delivery to carcinoembryonic antigen-producing cancer cells by retrovirus displaying a single-chain variable fragment antibody. *Hum. Gene Ther.* **9**, 235–248 (erratum, p. 1257).
- Engelstadter, M., Bobkova, M., Baier, M., Stitz, J., Holtkamp, N., Chu, T. H., Kurth, R., Dornburg, R., Buchholz, C. J. and Cichutek, K. (2000) Targeting human T cells by retroviral vectors displaying antibody domains selected from a phage display library. *Hum. Gene Ther.* **11**, 293–303.
- Ohno, K., Sawai, K., Iijima, Y., Levin, B. and Meruelo, D. (1997) Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A. *Nat. Biotechnol.* **15**, 763–767.
- Ohno, K. and Meruelo, D. (1997) Retrovirus vectors displaying the IgG-binding domain of protein A. *Biochem. Mol. Med.* **62**, 123–127.
- Johnson, J. E., Schnell, M. J., Buonocore, L. and Rose, J. K. (1997) Specific targeting to CD4+ cells of recombinant vesicular stomatitis viruses encoding human immunodeficiency virus envelope proteins. *J. Virol.* **71**, 5060–5068.
- Schnierle, B. S., Stitz, J., Bosch, V., Nocken, F., Mergel-Millitzer, H., Engelstadter, M., Kurth, R., Groner, B. and Cichutek, K. (1997) Pseudotyping of murine leukemia virus with the envelope glycoproteins of HIV generates a retroviral vector with specificity of infection for CD4-expressing cells. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8640–8645.
- Nguyen, T. H., Pages, J. C., Farge, D., Briand, P. and Weber, A. (1998) Amphotropic retroviral vectors displaying hepatocyte growth factor-envelope fusion proteins improve transduction efficiency of primary hepatocytes. *Hum. Gene Ther.* **9**, 2469–2479.
- Erbacher, P., Remy, J. S. and Behr, J. P. (1999) Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. *Gene Ther.* **6**, 138–145.
- Forstova, J., Krauzewicz, N., Sandig, V., Elliott, J., Palkova, Z., Strauss, M. and Griffin, B. E. (1995) Polyoma virus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells. *Hum. Gene Ther.* **6**, 297–306.
- Soeda, E., Krauzewicz, N., Cox, C., Stokrova, J., Forstova, J. and Griffin, B. E. (1998) Enhancement by polylysine of transient, but not stable, expression of genes carried into cells by polyoma VP1 pseudocapsids. *Gene Ther.* **5**, 1410–1419.
- Krauzewicz, N., Cox, C., Soeda, E., Clark, B., Rayner, S. and Griffin, B. E. (2000) Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids. *Gene Ther.* **7**, 1094–1102.
- Salunke, D. M., Caspar, D. L. and Garcea, R. L. (1986) Self-assembly of purified polyomavirus capsid protein VP1. *Cell* **46**, 895–904.
- Leavitt, A. D., Roberts, T. M. and Garcea, R. L. (1985) Polyoma virus major capsid protein, VP1. Purification after high level expression in *Escherichia coli*. *J. Biol. Chem.* **260**, 12803–12809.
- Freund, R., Garcea, R. L., Sahli, R. and Benjamin, T. L. (1991) A single-amino-acid substitution in polyomavirus VP1 correlates with plaque size and hemagglutination behavior. *J. Virol.* **65**, 350–355.
- Stehle, T., Yan, Y., Benjamin, T. L. and Harrison, S. C. (1994) Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature (London)* **369**, 160–163.
- Stehle, T. and Harrison, S. C. (1996) Crystal structures of murine polyomavirus in complex with straight-chain and branched-chain sialyloligosaccharide receptor fragments. *Structure* **4**, 183–194.
- Salunke, D. M., Caspar, D. L. and Garcea, R. L. (1989) Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophys. J.* **56**, 887–900.
- Schmidt, U., Kenkies, J., Rudolph, R. and Bohm, G. (1999) Site-specific fluorescence labelling of recombinant polyomavirus-like particles. *Biol. Chem.* **380**, 397–401.
- Schmidt, U., Rudolph, R. and Bohm, G. (2000) Mechanism of assembly of recombinant murine polyomavirus-like particles. *J. Virol.* **74**, 1658–1662.
- Gleiter, S., Stubenrauch, K. and Lilie, H. (1999) Changing the surface of a virus shell fusion of an enzyme to polyoma VP1. *Protein Sci.* **8**, 2562–2569.
- Stubenrauch, K., Bachmann, A., Rudolph, R. and Lilie, H. (2000) Purification of a viral coat protein by an engineered polyionic sequence. *J. Chromatogr. B Biomed. Sci. Appl.* **737**, 77–84.

- 25 Brinkmann, U., Pai, L. H., FitzGerald, D. J., Willingham, M. and Pastan, I. (1991) B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8616–8620
- 26 Brinkmann, U., Reiter, Y., Jung, S. H., Lee, B. and Pastan, I. (1993) A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7538–7542
- 27 Reiter, Y., Brinkmann, U., Lee, B. and Pastan, I. (1996) Engineering antibody Fv fragments for cancer detection and therapy: disulfide-stabilized Fv fragments. *Nat. Biotechnol.* **14**, 1239–1245
- 28 Reiter, Y., Brinkmann, U., Jung, S. H., Pastan, I. and Lee, B. (1995) Disulfide stabilization of antibody Fv: computer predictions and experimental evaluation. *Protein Eng.* **8**, 1323–1331
- 29 Rudolph, R., Boehm, G., Lilie, H. and Jaenicke, R. (1997) Folding proteins. In *Protein function* (Creighton, T. E., ed.), pp. 57–99, IRL Press, Oxford
- 30 Barr, S. M., Keck, K. and Aposhian, H. V. (1979) Cell-free assembly of a polyoma-like particle from empty capsid and DNA. *Virology* **96**, 656–659
- 31 Yerushalmi, N., Brinkmann, U., Brinkmann, E., Pai, L. and Pastan, I. (2000) Attenuating the growth of tumors by intratumoral administration of DNA encoding *Pseudomonas* exotoxin via cationic liposomes. *Cancer Gene Ther.* **7**, 91–96
- 32 Moreland, R. B., Montross, L. and Garcea, R. L. (1991) Characterization of the DNA-binding properties of the polyomavirus capsid protein VP1. *J. Virol.* **65**, 1168–1176
- 33 Sillat, S. N., Berns, K. I. and Aposhian, H. V. (1982) Polyoma-like particle: characterization of the DNA encapsidated *in vitro* by polyoma empty capsids. *J. Biol. Chem.* **257**, 6571–6575
- 34 Sonia, N. V., Zoppe, M. and Verna, I. M. (1995) Generation of targeted retroviral vectors by using single-chain variable fragment: an approach to *in vivo* gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7570–7574
- 35 Martin, F., Kupsch, J., Takeuchi, Y., Russell, S., Cosset, F. L. and Collins, M. (1998) Retroviral vector targeting to melanoma cells by single-chain antibody incorporation in envelope. *Hum. Gene Ther.* **9**, 737–746
- 36 Pack, P. and Pluckthun, A. (1992) Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*. *Biochemistry* **31**, 1579–1584
- 37 Pluckthun, A. and Pack, P. (1997) New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology* **3**, 83–105
- 38 O'Shea, E. K., Lumb, K. J. and Kim, P. S. (1993) Peptide 'velcro': design of a heterodimeric coiled coil. *Curr. Biol.* **3**, 658–667
- 39 Chang, H.-C., Bao, Z.-Z., Yao, Y., Tse, A. G. D., Goyarts, E. C., Madsen, M., Kawasaki, E., Brauer, P. P., Sacchetti, J. C., Nathenson, S. G. and Reinherz, E. L. (1994) A general method for facilitating heterodimeric pairing between two proteins: application to expression of a and b T-cell receptor extracellular segments. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11408–11412
- 40 Pai, L. H., Batra, J. K., FitzGerald, D. J., Willingham, M. C. and Pastan, I. (1992) Antitumor effects of B3-PE and B3-LysPE40 in a nude mouse model of human breast cancer and the evaluation of B3-PE toxicity in monkeys. *Cancer Res.* **52**, 3189–3193
- 41 Reiter, Y., Pai, L. H., Brinkmann, U., Wang, Q. C. and Pastan, I. (1994) Antitumor activity and pharmacokinetics in mice of a recombinant immunotoxin containing a disulphide-stabilized Fv fragment. *Cancer Res.* **54**, 2714–2718

Received 20 November 2000/27 February 2001; accepted 12 April 2001

Assessment of cell type specific gene transfer of polyoma virus like particles presenting a tumor specific antibody Fv fragment

Tobias May, Stefan Gleiter, Rainer Rudolph, and Hauke Lilie[#]

Institut für Biotechnologie, Universität Halle, Kurt Mothes Str. 3,
D-06120 Halle, Germany

Running title: Cell specific targeting of polyoma VLPs

Corresponding author: Hauke Lilie
Institut für Biotechnologie
Universität Halle
Kurt Mothes Strasse 3
D-06120 Halle, Germany

Tel: +49 345 55 24900

Fax: +49 345 55 27013

Email: Lilie@biochemtech.uni-halle.de

Abstract

Application of delivery systems in cancer therapy is restricted as a result of the lack of cell specificity of the respective vectors. Recently, we described a vector system based on virus like particles of modified polyoma-VP1. These particles were able to specifically bind a tumor-specific antibody fragment, thus directing the vector system towards tumor cells. Here we analyzed the functional gene transfer using the VP1 variant VP1-E8C, coupled with the antibody fragment of the tumor-specific antibody B3. The specific targeting of the antigen presenting cells was highly efficient as determined by fluorescence microscopy. However, only a low percentage of these cells showed a functional gene transfer. This discrepancy could be accounted for by a rather low capacity of the virus like particles to transport DNA and the mechanism of their internalization by the target cells, which led to a lysosomal degradation of the particles. These limitations could be partially surmounted in cell culture experiments, principles suitable for approaching an application of this vector system in vivo are discussed.

Key words: virus like particle, polyoma, polyionic peptides, targeting, gene transfer, endosome, Fv fragment

Introduction

Targeting to tumor specific antigens is a powerful approach to obtain efficient therapeutics in cancer gene therapy. The basic vector systems that have been used so far, such as adenovirus, AAV, and retroviruses (Robbins et al., 1998) do not show intrinsic cell type specificity. However, several attempts have been put forward to generate cell specific vector systems. One approach is based on the fusion of tumor specific antibody fragments to the surface of viral vectors which were in most cases retroviruses (Engelstadter et al., 2000; Jiang et al., 1998; Konishi et al., 1998). Furthermore, cell type specific targeting of vector systems dependent on proteins and peptides others than antibodies has been developed (Nguyen et al., 1998; Schnierle et al., 1997; Johnson et al., 1997).

In contrast to directly displaying cell specific ligands on the surface of viral vector systems, another strategy is the insertion of docking modules in the viral vectors. This would ensure that a once modified and characterized basic vector can be used in several different applications. As one docking module an immunoglobulin binding domain from *S. aureus*, protein ZZ, was inserted in the viral coat protein of Sindbis virus and retroviruses. Binding of a cell specific antibody to the modified viruses led to a change in the tropism towards the target cells of the respective antibody (Ohno and Meruelo, 1997; Ohno et al., 1997; Morizono et al., 2001). This approach was further transferred to virus like particles (VLPs) of the polyoma VP1 coat protein (Gleiter and Lilie, 2001).

Recently, we established another docking principle for coupling antibody fragments to VLPs and, thus, created a cell type specific vector system, based on VLPs of a variant of polyoma VP1 (Stubenrauch et al., 2001). This major coat protein of the murine polyoma virus was previously shown to assemble to VLPs in vitro (Salunke et al., 1986, 1989). The VLPs can package DNA and deliver it into cells both in cell culture and in vivo (Forstova et al., 1995, Soeda et al., 1998, Krauzewicz et al., 2000). Using wtVP1 the cell targeting is mediated by the binding activity of VP1 to sialic acid derivatives residing on cell surfaces of mammalian

cells (Freund et al., 1991). The crystal structure of VP1 revealed that the sialic acid binding site is located in a cleft beneath the HI-loop of the beta-sheeted protein (Stehle et al., 1994; Stehle and Harrison, 1996). The modification of this HI-loop by inserting another protein or a peptide sequence blocked the natural sialic acid binding completely (Gleiter et al., 1999, Gleiter and Lilie, 2001; Stubenrauch et al., 2001). If the inserted peptide consists of eight glutamic acid residues and one cysteine it enables the coupling of a tumor specific antibody Fv fragment, fused to a Arg₈Cys-peptide, to the VLPs. This antibody-decorated VLPs could be used as cell type specific gene delivery system (Stubenrauch et al., 2001).

Here, we used this antibody-decorated VLPs to analyze different steps of the transfection reaction. The analysis led to the identification of reactions limiting the efficacy of transfection. Based on this knowledge we were able to substantially improve the transfection efficiency of this vector system in cell culture.

Methods

Production of polyoma VLPs in vitro

Recently, we engineered a mutant of polyoma VP1 with a peptide, containing eight glutamic acid residues and one cysteine, inserted in the HI-loop (VP1-E8C) (Stubenrauch et al., 2000). After expression of VP1-E8C in its native pentameric form in *E. coli* the purification scheme consists of a fractionated ammonium sulfate precipitation followed by anion exchange and size exclusion chromatography (Stubenrauch et al., 2000). For assembly of VLPs, VP1-E8C was dialyzed for two days at 20°C against 0.02 M Tris, pH 7.4, 200 mM NaCl, 1 mM CaCl₂, 0.75 M ammonium sulfate, upon which VLPs form spontaneously (Salunke et al., 1986, 1989). Subsequently, VLPs were purified by size exclusion chromatography on a Superdex 200 prep grade column (Pharmacia, vol. 120 ml, flow rate 1ml/min) equilibrated with 0.02 M Tris, pH 7.4, 200 mM NaCl, 1 mM CaCl₂.

Preparation of recombinant dsFv-B3-R8C antibody fragment

For cell type specific targeting of the VLPs an Fv fragment of the tumor specific antibody B3 was chosen. This Fv fragment is stabilized by an engineered interchain disulfide bond (Brinkmann et al., 1993). The VH-domain, modified at the C-terminus by introduction of a sequence encoding the peptide Arg₈CysPro, and VL-domain were expressed separately in *E. coli* and isolated as inclusion bodies (ib's) (Rudolph et al., 1997). Ib's were refolded to dsFv-B3-R8C and purified as described (Stubenrauch et al., 2001).

Decoration of the 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, pH 7.4, 200 mM NaCl, 5% glycerol, 1 mM CaCl₂, 1.6 mM GSSG and 0.4 mM GSH at protein concentrations of 40 µg/ml dsFv-B3-R8C and 0.3 mg/ml VP1-E8C VLPs. After 5 h coupling at 10°C the reaction mixtures were applied to a size exclusion column as described for

unconjugated VLPs. The coupling reaction led to a decoration of the VLPs with 30-40 antibody fragments statistically (Stubenrauch et al., 2001).

DNA –association/packaging in VLPs

The packaging of DNA into VLPs was performed by incubating ca. 2 nM plasmid DNA pELI92, encoding the bacterial β -galactosidase (Yerushalmi et al., 2000), or pEGFP, a GFP expression plasmid (Clontech) with 10 nM VLPs at room temperature for at least 1 h. Tight DNA binding/packaging was determined either by analytical ultracentrifugation or a gel shift assay. Analytical ultracentrifugation experiments were performed in an analytical ultracentrifuge Optima XL-A, using an An50Ti rotor and double sector cells. The samples were analyzed at 20°C and a rotor speed of 10 000 rpm. Every 10 min the sedimentation of DNA and VLPs were monitored at a wavelength of 260 nm, respectively.

The gel shift assay was performed using 1% agarose gels in 20 mM Tris/acetate buffer, pH 7.4. 800 ng DNA of a 1 kb-molecular weight marker (Gibco BRL) was incubated with 10 μ g VLPs for 1 h at room temperature under different buffer conditions and subsequently loaded on the gel.

Cell culture experiments

MCF7 (breast carcinoma), which express the oligosaccharide Lewis Y, the antigen recognized by dsFv-B3-R8C on their surface, were grown as monolayers on cover slips in 6-well plates in RPMI 1640 (ICN) supplemented with 10% FBS, 0.5 % Gentamicin, and 1 % Glutamax. The Lewis Y-negative cell line HT-29 was cultured in DMEM/high glucose (Gibco BRL) supplemented with 10% FBS, 0.5 % Gentamicin, and 1 % Glutamax. All cultures were maintained in a humidified atmosphere of 5 % CO₂ in air at 37°C.

For analysis of transfected cells, the cells were preserved. To this end the cells were washed two times with 2 ml PBS and then fixed with 1.5 ml 3 % paraformaldehyde for 30 min.

Subsequently, the cells were again washed with PBS and the excess paraformaldehyde inactivated by adding of 2 ml 80 mM glycine, pH 8 (dissolved in PBS) for 10 min. Afterwards the glycine was removed by two additional washing steps with PBS and the cell preparation was sealed in Mowiol 4-88 containing 0.2 % DABCO.

Cell type specific targeting

Cell type specific targeting of VLPs of VP1-E8C either coupled with dsFv-B3-R8C or not was analyzed using fluorescent labeled VLPs. To this end the particles were labeled with the fluorescence dye Texas Red according to the manufacturer's protocol (Molecular Probes). The concentrations of VLPs and Texas Red were adjusted to ensure a coupling rate of one Texas Red molecule per VP1 pentamer. After labeling non-bound dye was removed by extensive dialysis against 20 mM Tris, pH 7.4, 200 mM NaCl, 5 % glycerol, 1 mM CaCl₂ at 4°C.

The fluorescent labeled VLPs (0.5 – 10 µg/ml) were added to the cells and incubated in ice cold medium for 1 h. Then the temperature was shifted to 37°C and the samples incubated for another hour. Afterwards the cells were preserved and cell targeting of the VLPs analyzed by fluorescence microscopy using a Zeiss fluorescence microscope Axiovert 100M or a Zeiss laser scanning microscope Axiovert 100M, equipped with a He/Ne laser.

Cellular uptake of VLPs

To analyze the mechanism of internalisation of dsFv-B3-R8C-coupled VLPs, phagocytosis was blocked by addition of the drug chlorpromazine. Cells were preincubated with chlorpromazine at a concentration of 50 µM for 1 h at 37°C. Subsequently, Texas Red labeled VLPs of VP1-E8C/dsFv-B3-R8C were added to a final amount of 1.4 µg. After incubating the cells 60 min at 37°C the cells were preserved and analyzed by fluorescence microscopy.

In another approach co-localization of VLPs with transferrin was monitored. To this end MCF

7 cells, maintained in ice cold medium, were incubated with 1.4 µg Texas Red labelled VLPs of VP1-E8C/dsFv-B3-R8C and 40 µg Oregon Green labelled transferrin (labelling in borate buffer, pH 8, 200 mM NaCl according to the manufacturers protocol (Molecular Probes)). After 10 min incubation at 37°C the cells were washed with PBS and preserved. Co-localization was analysed by laser scanning fluorescence microscopy. The excitation wavelength was set to 543 nm to visualize the VLPs (emission 560 nm) and 488 nm for transferrin (emission 505 nm), respectively. VLPs are shown in red, transferrin in green. Co-localization of both proteins resulted in yellow staining.

Gene transfer

MCF 7 and HT 29 cells were grown in 6-well plates to a confluency of 40 –50 %. Then the medium was removed and the cells were overlaid with 2 ml PBS, pH 5. After addition of the transfection reagent the cells were incubated for 30 min at 37°C. Subsequently, the solution was removed and 2 ml fresh medium was added to the cells. After an additional incubation of 1 h at 37°C the medium was changed and chloroquine was added to a final concentration of 50 µM. MCF 7 was incubated in the chloroquine containing medium for 2 days. In case of HT 29, the cells were supplemented with fresh medium after 12 h. GFP expression was analysed by fluorescence microscopy 60-70 h post transfection.

Results

Coupling of antibody fragments to VLPs of modified polyoma VP1-E8C led to a cell type specific gene transfer in target cells (Stubenrauch et al., 2001). However, the efficacy of functional gene transfer was rather low, which was surprising, since this vector system contained a specific cell binding activity. To analyze the reasons for the limited transfection rate and to functionally optimize the VP1-E8C-based vector system, we experimentally addressed different steps that might confine an efficient gene transfer: (i) the capacity of DNA transportation, (ii) the efficiency of antibody-mediated cell targeting, and (iii) subcellular localization of the internalized particles.

Interaction of VP1-E8C with DNA

VLPs of wtVP1 have been shown previously to associate in vitro with plasmid DNA (Forstova et al., 1995; Soeda et al., 1998; Krauzewicz et al., 2000) and linear DNA fragments (Henke et al., 2000). This capability of wtVP1 to interact and package DNA could be compromised in case of the mutant VP1-E8C due to the high density of negative charges on its VLPs and a resulting electrostatic repulsion of VLPs and DNA. To quantitatively analyze the interaction of VP1-E8C and plasmid DNA we used analytical ultracentrifugation. As shown in Fig. 1 the sedimentation velocity of purified VLPs was much higher than that of isolated plasmid DNA. Under conditions suitable for wtVP1 to associate with DNA, the sedimentation of VLPs of VP1-E8C and DNA led to a complete separation of both components (Fig. 1C); the analysis of the respective amplitudes clearly indicated that a stable complex was not formed. This lack of complex formation was observed even at a fivefold molar excess of VLPs over DNA. The only detectable effect was a slight increase in the sedimentation velocity of the DNA in the presence of VLPs compared to that of isolated DNA (Fig. 1A,C). This might indicate a very weak interaction of DNA and VLPs without a stable complex being formed.

The observed lack of VP1-E8C in binding DNA is presumably due to electrostatic repulsion of both components and interfere with a functional gene transfer via these VLPs in cell culture. Therefore, we changed the conditions for VLP/DNA interaction by lowering the pH. In case of wtVP1 it has been shown that packaging of short DNA fragments is about two times more efficient at pH 5 than at pH 7 (Henke et al., 2000). Furthermore, at pH 5 a fraction of the glutamic acid residues of VP1-E8C should be titrated, thus leading to a decreased electrostatic repulsion. The DNA binding capacity of VP1-E8C under these conditions, analysed by analytical ultracentrifugation, is shown in Fig. 1E. As could be quantified by the amplitude of the differently sedimenting species about 40 % of the plasmid DNA was bound to VP1-E8C at pH 5. The binding of DNA led to the formation of particles inhomogeneous in size, resulting in a less cooperative sedimentation behavior.

The complex formation of VP1-E8C and DNA was confirmed using a gel shift assay. In this case, DNA fragments of different length were used (Fig. 2). The incubation of DNA with VLPs of VP1-E8C at pH 5 led to a significant reduction of DNA, stained in the gel. Instead some DNA remained in the slot indicating binding to the VLPs with a diameter of 50 nm. At pH 7 this effect was far less pronounced. Besides the importance of the pH for DNA binding from these data it could be concluded that VLPs of VP1-E8C did not exhibit any preferences in binding DNA of a certain length.

These results suggest that one reason for the low efficacy of gene transfer using the VP1-E8C-based vector system is its very limited interaction with recombinant DNA. However, the pH shift upon complex formation of VP1-E8C and DNA should partially overcome this limitation.

Cellular targeting of VLPs of VP1-E8C via the antibody fragment dsFv-B3-R8C

The antibody fragment dsFv-B3-R8C, directed against the tumor specific antigen Lewis Y, has been used for the construction of immunotoxins, previously (Brinkmann et al., 1991;

1993). However, the tumor specific dsFv-B3-R8C only possesses a low affinity to its antigen with a dissociation constant of the complex in the millimolar range. In order to test whether this low affinity results in an inefficient cell targeting of dsFv-B3-R8C-coupled VLPs, fluorescent labelled VP1-E8C was utilized. If the particles, not presenting dsFv-B3-R8C on their surface, were incubated with the Lewis Y positive cell line MCF 7, virtually no cell-associated fluorescence could be observed (data not shown). In contrast, with dsFv-B3-R8C coupled to the VLPs almost every cell was highly fluorescent, indicating a high efficiency in targeting the particles to the cells via the antibody/antigen interaction (Fig. 3). Despite the low affinity of dsFv-B3-R8C to its antigen this high targeting efficiency might depend on coupling of not only one antibody fragment to a VLP but about 30 fragments (Stubenrauch et al., 2001) which increases the affinity substantially. If the antigen Lewis Y was not present on the cell surface as in case of the control cell lines HT 29, SKBR-3 and HeLa, targeting could not be observed, neither for dsFv-B3-R8C coupled nor uncoupled VLPs (data not shown). Thus, specificity and efficacy of targeting of the dsFv-B3-R8C decorated VLPs were not limiting for gene transfer.

The cellular targeting of VLPs of VP1-E8C via dsFv-B3-R8C to MCF 7 did not only lead to adhesion of the particles to the outer surface of the cells, but also an internalisation of VLPs was observed. As shown in Fig. 3C intracellularly localized fluorescent spots could be detected that resemble vesicular structures (see below). The kinetic of internalisation was finished in a time range of about 60 min, as estimated by the decrease of the cell membrane-associated fluorescence (data not shown). The intracellularly located fluorescence remained stably vesicle-associated over several hours.

dsFv-B3-R8C mediated internalisation of VLPs via the phagocytotic pathway

The intracellularly localized fluorescent spots already suggested the cellular uptake of the VLPs in vesicles. Whether these vesicles belong to the receptor-mediated phagocytotic

pathway was analyzed by colocalization experiments with transferrin. If MCF 7 cells were incubated with fluorescent labelled transferrin and dsFv-B3-R8C-coupled VLPs the internalisation and subcellular localization of both components could be observed. Using different fluorescence labels for transferrin and VLPs, respectively, a colocalization of both proteins could be proven (Fig. 4). After 10 min incubation of the cells at 37°C in the presence of both proteins, most of the proteins were colocalized on the cell surface. However, a significant fraction of the proteins did also colocalize intracellularly.

Since the transferrin receptor is part of the coated pits on the cell surface and transferrin is only localized in endocytotic vesicles during its metabolic pathway, this result strongly indicates that also VLPs of VP1-E8C coupled with dsFv-B3-R8C were internalised via receptor-mediated phagocytosis and clathrin-coated vesicles.

The suggestion that dsFv-B3-R8C-mediated internalisation of VLPs occurred via receptor-mediated phagocytosis was confirmed by suppressing phagocytosis with the drug chlorpromazine. This drug specifically inhibits the formation of clathrin-coated vesicles, thus blocking the initial step of the receptor-mediated phagocytosis (Wang et al., 1993). In the presence of 50 µM chlorpromazine, the uptake of VLPs was substantially inhibited (data not shown). The targeting, however, as estimated from the cell-surface associated fluorescence, seemed to be as efficient as in the absence of the drug. These results strongly suggest that dsFv-B3-R8C decorated VLPs were internalised into cells via the clathrin-dependent endocytotic pathway. Furthermore, the long persistence of the intracellularly fluorescent stained vesicles suggests that after internalisation the predominant fraction of VLPs did not enter the cytosol but remained in the endosomes and were subsequently degraded in the lysosomes. Thus, the failure in intracellular trafficking of the VLPs due to the lack of endosomal release probably represents another limiting step in the functional gene transfer based on this vector system.

Functional gene transfer mediated by dsFv-B3-R8C coupled VLPs

With the very limited DNA binding of VLPs and their persistence in endosomes after cellular uptake, two steps were identified that might cause the low efficiency in functional gene transfer using these VLPs. In order to test this hypothesis transfection experiments using MCF 7 cells were performed. For these experiments a GFP encoding plasmid was loaded on the VLPs at pH 5, allowing ca. 40% of DNA being bound to the VLPs (cf. Fig. 1). Furthermore, the cells were transfected in the presence of 50 μ M chloroquine. This drug functionally destroys endosomes, thus leading to a partial release of endosomal encapsulated material.

Using this strategy to at least partially overcome the identified limitations of the overall reaction, transfection of MCF 7 cells could be demonstrated unequivocally (Fig. 5). Quantification by FACS analysis yielded a transfection rate of 0.12 $\pm$ 0.02 %. This observed transfection efficacy is similar to that of VLPs of wtVP1 which was described to be in the range of 0.1 – 0.5 % (Krauzewicz et al., 2000a,b). In contrast, if the transfection of MCF 7 cells was performed either in the absence of chloroquine or with DNA bound to VLPs at pH 7 instead of pH 5, no GFP expression could be observed at all (data not shown). Similarly, VLPs that did not present dsFv-B3-R8C on the surface could not transfect cells even under optimum conditions. This indicates that the observed GFP expression is the result of a specific transfection reaction. Thus, the characterized limitations of the VLPs in binding DNA and their persistence in endosomes after cellular uptake are responsible for the low efficiency in functional gene transfer. Bypassing these limitations led to a significant increase in transfection. However, the overall transfection rate still proved to be low indicating that additional limiting steps remained unresolved.

Discussion

Various attempts have been made in the last few years to develop cell-type specific delivery systems for application in gene therapy. These delivery systems are mainly based on different viruses, such as Adenovirus, AAV, and retroviruses. Whereas Adenovirus and AAV are predominantly used in cell-type unspecific therapeutic approaches, several attempts have been put forward to generate cell-type specific retroviruses. To this end, cell specific scFv fragments were fused to the coat protein of retroviruses (Engelstadter et al., 2000; Jiang et al., 1998; Konishi et al., 1998). In another approach the antibody binding domain protein ZZ was inserted into a viral coat protein, thus allowing binding of cell-type specific antibodies to this modified virus (Ohno et al., 1997, Ohno and Meruelo, 1997, Morizono et al., 2001).

Recently, we established a cell-type specific gene transfer using VLPs of a modified polyoma VP1 coat protein (Stubenrauch et al., 2001). VP1 can be expressed recombinantly in *E. coli* and assembled to VLPs in vitro (Leavitt et al., 1985, Salunke et al., 1986). These VLPs possess a cell-type unspecific gene transfer activity (Forstova et al., 1995, Soeda et al., 1998, Krauzewicz et al., 2000) due to their affinity to sialic acid derivatives (Freund et al., 1991, Stehle et al., 1994; Stehle and Harrison, 1996), present on almost all eucaryotic cells. In order to switch the tropism of polyoma VLPs from a cell-type unspecific targeting to a cell-type specific targeting, (i) the natural tropism has to be diminished and (ii) a new targeting function has to be established. We constructed two different variants of VP1, in which the solvent exposed HI-loop was mutated. Alterations of this loop, which is located directly above the sialic acid binding site of VP1, abolished binding of VP1 to this sugar and thus the cell-type unspecific targeting. In a first approach, we inserted the antibody binding domain protein Z in the HI-loop of VP1. Similar to the presentation of protein ZZ on the surface of modified Sindbis virus and retrovirus (Ohno et al., 1997, Meruelo and Ohno, 1997, Morizono et al., 2001), this variant, VP1-Z, allowed coupling of tumor-specific antibodies on the surface of the VLPs, thus enabling a cell-type specific targeting and gene transfer (Gleiter et al.,

2001). The other variant of VP1, called VP1-E8C, made use of the polyionic peptide Glu₈Cys, inserted in the HI-loop (Stubenrauch et al., 2000, 2001). This peptide served as docking module for coupling an antibody fragment containing the complementary charged peptide Arg₈Cys. These polyionic fusion peptides can function as highly specific dimerization motifs for construction of artificial bifunctional proteins (Richter et al., 2001). VLPs of VP1-E8C, coupled with the tumor-specific antibody fragment dsFv-B3-R8C, could also be used for a cell-type specific gene transfer (Stubenrauch et al., 2001). The efficiency of transfection, however, was very low. In this study we analyzed three different aspects of the overall transfection process in order to identify limiting reactions. As could be shown by fluorescence labeling the targeting of VLPs coupled with dsFv-B3-R8C to antigen presenting cells was very efficient. Furthermore, the particles were internalized by the clathrin-dependent endocytotic pathway. This is in accordance with data on dsFv-B3-R8C-containing immunotoxins. These chimeric proteins consisting of dsFv-B3-R8C and a fragment of the pseudomonas exotoxin, PE38, are also targeted to tumor cells by the antibody fragment and then internalized via the endocytotic pathway (Brinkmann and Pastan, 1994, Kreitman, 1999). Whereas these immunotoxins are released from the endosomes as a result of the molecular function of the toxin (Brinkmann and Pastan., 1994), no such mechanism is known for the polyoma-VLPs. Instead, polyomavirus as well as VLPs of VP1 are internalized via caveolae (Richterova et al., 2001). Thus, VLPs of VP1-E8C are not adapted to surmount the endosomal membrane and hence persisted in endosomes and are finally degraded in lysosomes without achieving a functional transfection of the respective cells. In addition the capacity of VLPs of VP1-E8C to package/transport DNA is quite low. VLPs of wtVP1 are known to tightly associate with plasmid DNA either by incubating the VLPs directly with DNA or by using a osmotic shock procedure (Barr et al., 1979). However, these methods presumably do not lead to an incorporation of plasmid DNA into the VLPs but to a tight binding of DNA on the surface of the VLPs. Since the surface of the VLPs of VP1-E8C possesses a high density of

negatively charged residues the electrostatic repulsion of VLPs and DNA prevents an efficient DNA binding.

Taken together we characterized the mechanism of internalization of VLPs of VP1-E8C coupled with dsFv-B3-R8C. In contrast to VLPs of wtVP1 the mutant form is internalized by the respective antigen exposing cells depending on the metabolism of the antigen, in this case the dsFv-B3-R8C mediated targeting leads to a clathrin-dependent endocytosis of the VLPs and furtheron a degradation of these particles in lysosomes. Together with the low capacity of these VLPs to transport DNA, these are major reasons for the low transfection efficiency of cells using VP1-E8C. These results are very important for designing the next generation of vector systems based on VP1-E8C. The lysosomal degradation of the VLPs may be obviated by establishing an endosomal release mechanism. Intracellular parasites, such as *Listeria*, possess a lipase that degrades the endosomal membrane of the host cell. This lipase, fused to a polyionic peptide as shown for dsFv-B3-R8C, may be coupled to the VLPs, thus enabling the release of the VLPs from the endosomes after cellular uptake. Furthermore, the DNA as therapeutic should not be associated with the vector system after forming the VLPs but should be bound to the capsomers in vitro before the assembly takes place. This is even more reasonable because VP1 exhibit a DNA binding site at its N-terminus, which is located inside the VLPs after assembly. Since the assembly is prohibited by the presence of long DNA fragments (Moreland et al., 1991), therapeutic nucleic acids, transported with these VLPs should consist of short functional fragments such as ribozymes, DNazymes or RNAi. These two approaches, deduced from the results of this study, should permit the development of modified polyomaVLPs as valuable vector system for cell-type specific gene therapy.

Acknowledgments

We thank Jan Hoffmann for advice on laser scanning microscopy and Kay Stubenrauch for helpful discussions. This work was supported by a grant of the Deutsche Forschungsgemeinschaft to HL and a scholarship of the European Union to SG.

Abbreviations

AAV, adeno-associated virus; dsFv-B3-R8C, disulfide-stabilized Fv fragment of the monoclonal antibody B3 fused to the peptide Arg₈Cys; GFP, green fluorescent protein; GSH, reduced glutathion; GSSG, oxidized glutathione; ib, inclusion bodies; PBS, phosphate buffered saline; VH, variable domain of the heavy chain of antibodies; VL, variable domain of the light chain of antibodies; VLP, virus like particle; VP1, major coat protein of the murine polyoma virus; VP1-E8C, VP1 mutant containing the peptide Glu₈Cys in the HI-loop; wt, wild type

References

- Barr, S.M., Keck, K., and Aposhian, H.V. 1979. Cell-free assembly of a polyoma-like particle from empty capsid and DNA. *Virology* 96, 656-659.
- Brinkmann, U., Pai, L.H., FitzGerald, D.J., Willingham, M., and Pastan, I. 1991. B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. *Proc. Natl. Acad. Sci. USA* 88, 8616-8620.
- Brinkmann, U., Reiter, Y., Jung, S.H., Lee, B., and Pastan, I. 1993. A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc. Natl. Acad. Sci. USA* 90, 7538-7542.
- Brinkmann U., und Pastan I. 1994. Immunotoxins against cancer. *Biochem Biophys Acta* 1198, 27-45.
- Engelstadter, M., Bobkova, M., Baier, M., Stitz, J., Holtkamp, N., Chu, T.H., Kurth, R., Dornburg, R., Buchholz, C.J., and Cichutek, K. 2000. Targeting human T cells by retroviral vectors displaying antibody domains selected from a phage display library. *Hum. Gene Ther.* 11, 293-303.
- Forstova, J., Krauzewicz, N., Sandig, V., Elliott, J., Palkova, Z., Strauss, M., and Griffin, B.E. 1995. Polyoma virus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells. *Hum. Gene Ther.* 6, 297-306.
- Freund, R., Garcea, R.L., Sahli, R., and Benjamin, T.L. 1991. A single-amino-acid substitution in polyomavirus VP1 correlates with plaque size and hemagglutination behavior. *J. Virol.* 65, 350-355.
- Gleiter, S., Stubenrauch, K., and Lilie, H. 1999. Changing the surface of a virus shell fusion of an enzyme to polyoma VP1. *Protein Sci.* 8, 2562-2569.
- Gleiter, S., and Lilie, H. 2001. Coupling of antibodies via protein Z on modified polyoma virus-like particles. *Protein Sci* 10, 434-444.
- Henke, S., Rohmann, A., Bertling, W.M., Dinger, T., and Zimmer, A. 2000. Enhanced in vitro oligonucleotide and plasmid DNA transport by VP1 virus-like particles. *Pharm. Res.* 17, 1062-1070.
- Jiang, A., Chu, T.H., Nocken, F., Cichutek, K., and Dornburg, R. 1998. Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies. *J Virol.* 72, 10148-10156.
- Johnson, J.E., Schnell, M.J., Buonocore, L., and Rose, J.K. 1997. Specific targeting to CD4+ cells of recombinant vesicular stomatitis viruses encoding human immunodeficiency virus envelope proteins. *J. Virol.* 71, 5060-5068.
- Konishi, H., Ochiya, T., Chester, K.A., Begent, R.H., Muto, T., Sugimura, T., Terada, M., and Begent, R.H. 1998. Targeting strategy for gene delivery to carcinoembryonic antigen-producing cancer cells by retrovirus displaying a single-chain variable fragment antibody. *J. Virol.* 72, 10148-10156.

- Krauzewicz, N., Cox, C., Soeda, E., Clark, B., Rayner, S., and Griffin, B.E. 2000. Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids. *Gene Ther.* 7, 1094-1102.
- Kreitmair, J.R. 1999. Immunotoxins in cancer therapy. *Curr Opin Immunology* 11, 570-578.
- Leavitt, A.D., Roberts, T.M., and Garcea, R.L. 1985. Polyoma virus major capsid protein, VP1. Purification after high level expression in *Escherichia coli*. *J. Biol. Chem.* 260, 12803-12809.
- Moreland, R.B., Montross, L., and Garcea, R.L. 1991. Characterization of the DNA-binding properties of the polyomavirus capsid protein VP1. *J. Virol.* 65, 1168-1176.
- Morizono, K., Bristol, G., Xie, Y., Kung, S.K., and Chen, I.S. 2001. Antibody-Directed Targeting of Retroviral Vectors via Cell Surface Antigens. *J Virol.* 75, 8016-8020.
- Nguyen, T.H., Pages, J.C., Farge, D., Briand, P., and Weber, A. 1998. Amphotropic retroviral vectors displaying hepatocyte growth factor- envelope fusion proteins improve transduction efficiency of primary hepatocytes. *Hum.Gene Ther.* 9, 2469-2479.
- Ohno, K., and Meruelo, D. 1997. Retrovirus vectors displaying the IgG-binding domain of protein A. *Biochem. Mol. Med* 62, 123-127.
- Ohno, K., Sawai, K., Iijima, Y., Levin, B., and Meruelo, D. 1997. Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A. *Nat. Biotechnol.* 15, 763-767.
- Richter, S., Stubenrauch, K., Lilie, H., and Rudolph, R. 2001. Polyionic fusion peptides function as specific dimerization motifs. *Prot. Engineering* 14, in press.
- Richterova, Z., Liebl, D., Horak, M., Palkova, Z., Tokrova, J., Hozak, P., Korb, J., and Forstova J. 2001. Caveolae are involved in the trafficking of mouse polyoma virions and artificial VP1 pseudocapsids towards cell nuclei. *J.Virol.*, in press.
- Robbins, P.D., Tahara, H., and Ghivizzani, S.C. 1998. Viral vectors for gene therapy. *Trends Biotechnol.* 16, 35-40.
- Rudolph, R., Boehm, G., Lilie, H., and Jaenicke, R. 1997. Folding proteins. In *Protein function* (Creighton T.E., ed.), pp. 57-99, IRL PRESS, Oxford.
- Salunke, D.M., Caspar, D.L., and Garcea, R.L. 1986. Self-assembly of purified polyomavirus capsid protein VP1. *Cell* 46, 895-904.
- Salunke, D.M., Caspar, D.L., and Garcea, R.L. 1989. Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophys J.* 56, 887-900.
- Schnierle, B.S., Stitz, J., Bosch, V., Nocken, F., Merget-Millitzer, H., Engelstadter, M., Kurth, R., Groner, B., and Cichutek, K. 1997. Pseudotyping of murine leukemia virus with the envelope glycoproteins of HIV generates a retroviral vector with specificity of infection for CD4-expressing cells. *Proc. Natl. Acad. Sci USA* 94, 8640-8645.

- Soeda, E., Krauzewicz, N., Cox, C., Stokrova, J., Forstova, J., and Griffin, B.E. 1998. Enhancement by polylysine of transient, but not stable, expression of genes carried into cells by polyoma VP1 pseudocapsids. *Gene Ther.* 5, 1410-1419.
- Stehle, T., Yan, Y., Benjamin, T.L., and Harrison, S.C. 1994. Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* 369, 160-163.
- Stehle, T., and Harrison, S.C. 1996. Crystal structures of murine polyomavirus in complex with straight- chain and branched-chain sialyloligosaccharide receptor fragments. *Structure* 4, 183-194.
- Stubenrauch, K., Bachmann, A., Rudolph, R., and Lilie, H. 2000. Purification of a viral coat protein by an engineered polyionic sequence. *J Chromatogr. B Biomed. Sci Appl.* 737, 77-84.
- Stubenrauch, K., Gleiter, S., Brinkmann, U., Rudolph, R., and Lilie, H. 2001. Conjugation of an antibody Fv fragment to a virus coat protein: cell- specific targeting of recombinant polyoma-virus-like particles. *Biochem J* 356, 867-873.
- Wang L.H., Rothberg K.G., und Anderson R.G.W. 1993. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J Cell Biol* 123, 1107-1117.
- Yerushalmi, N., Brinkmann, U., Brinkmann, E., Pai, L., and Pastan, I. 2000. Attenuating the growth of tumors by intratumoral administration of DNA encoding *Pseudomonas* exotoxin via cationic liposomes. *Cancer Gene Ther.* 7, 91-96.

Figure legends**Fig.1:** DNA binding to VLPs of VP1-E8C

The plasmid pELI92 at a concentration of 2 nM was incubated with 10 nM VLPs of VP1-E8C for at least 1 h at 25°C. The reaction was performed in either 20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM CaCl₂, 5 % glycerol or 10 mM Na-acetate, pH 5, 150 mM NaCl, 1mM CaCl₂, 5 % glycerol. The samples were analysed by analytical ultracentrifugation at 10 000 rpm and 260 nm. Radial scans were taken every 10 min (displayed every 20 min).

A) DNA in the absence of VLPs at pH 7.4

B) VLPs in the absence of VLPs at pH 7.4

C) DNA and VLPs at pH 7.4

D) DNA and VLPs at pH 5

Fig. 2:

DNA of different length (0.8 µg) was incubated with 10 µg VLPs for 1 h at 25°C. The DNA-protein interaction was analysed by a gel shift assay using a 1 % agarose gel in 40 mM Tris, 20 mM acetate, pH 7.4. The DNA was stained with ethidium bromide. Lane M, 0.8 µg DNA; lane 1, DNA plus VLPs of VP1-E8C at pH 5, lane 2, DNA plus VLPs of VP1-E8C at pH 7.4; lane 3, DNA plus VLPs of wtVP1 at pH 7.4.

Fig. 3: Targeting of VLPs of VP1-E8C/dsFv-B3-R8C to MCF 7 cells

MCF 7 cells, grown on cover slips, were incubated with 1.4 µg Texas Red labelled VP1-E8C/dsFv-B3-R8C for 1 h at 37°C. Afterwards the cells were extensively washed and preserved.

A) Fluorescence view of cells at 200fold magnification.

B) Transmission view of the same detail as in A.

C) Color-coded picture of a 3D-image of a cell cluster.

Fig. 4: Co-localization of VLPs of VP1-E8C/dsFv-B3-R8C and transferrin.

MCF 7 cells were incubated for 20 min at 37°C with both 1.4 µg Texas Red labelled VLPs of VP1-E8C/dsFv-B3-R8C and 40 µg Oregon Green labelled transferrin. Afterwards the cells were preserved and analysed by fluorescence microscopy. VLP staining is shown in red, transferrin in green. Co-localization results in yellow staining. Individual spots, marked by an arrow, were shown to be located inside the cells by laser scanning microscopy.

Fig. 5: Transfection of MCF 7

MCF 7 cells were incubated with 25 µg VP1-E8C/dsFv-B3-R8C, loaded with 5 µg plasmid pEGFP, for 30 min in medium, pH 5. Subsequently, the solution was removed and fresh medium, containing 50 µM chloroquine was added. After 48 h incubation the medium was replenished by fresh medium without chloroquine and the cells were further incubated for 12h.

A) Transmission view at 200fold magnification.

B) Fluorescence view of the same detail.

Figure 1

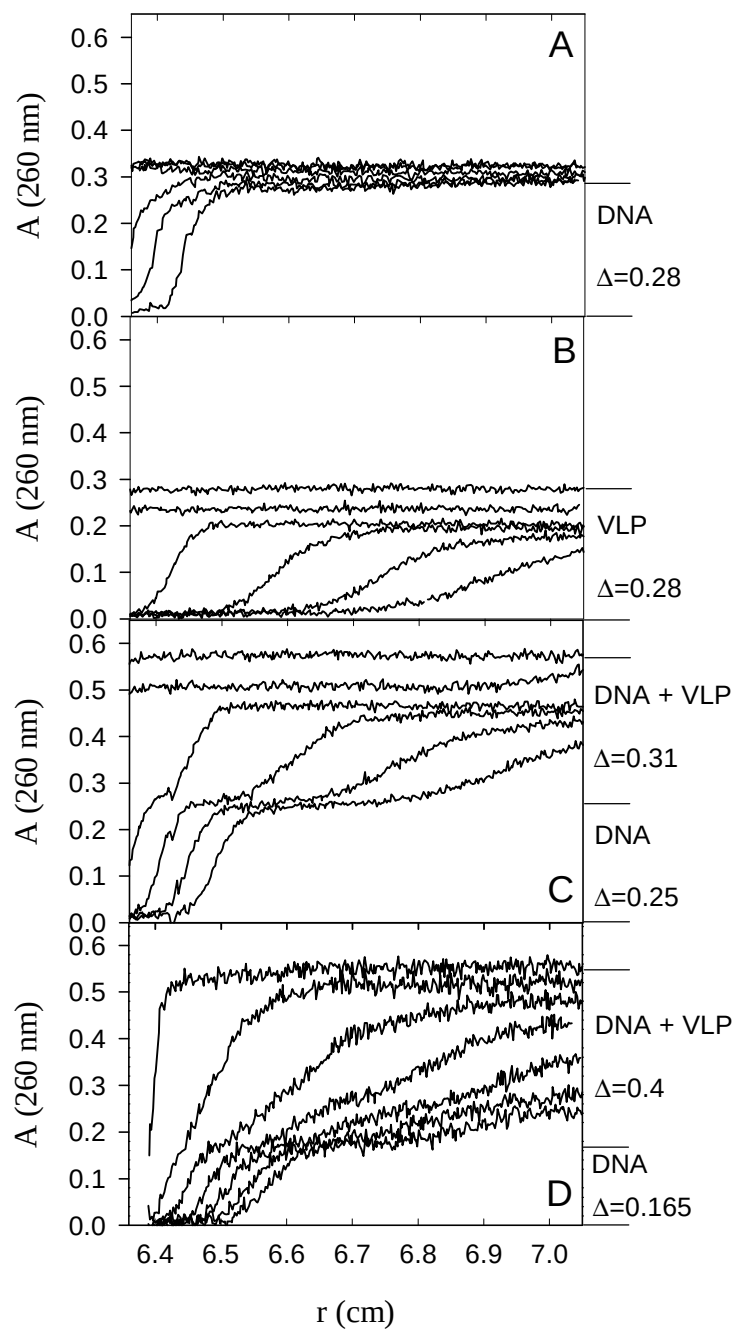


Figure 2

M 1 2 3

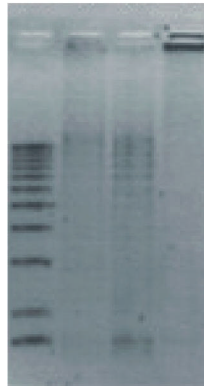


Figure 3

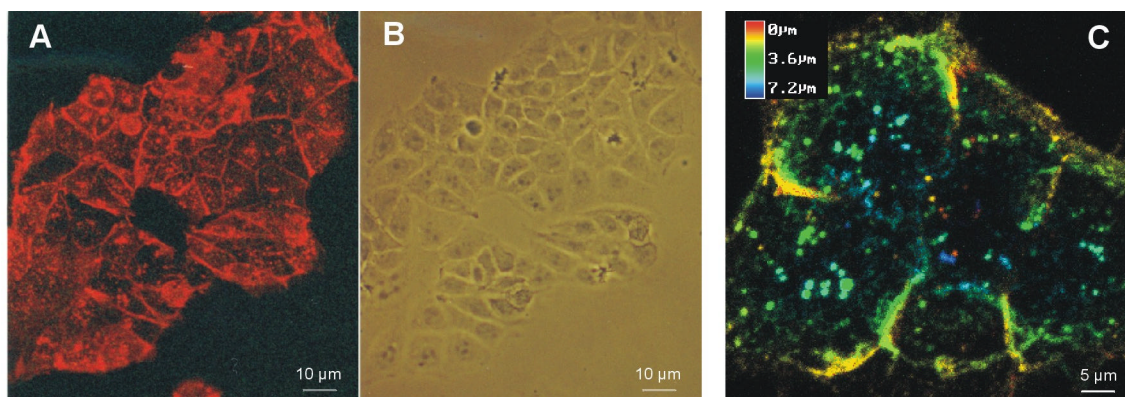


Figure 4

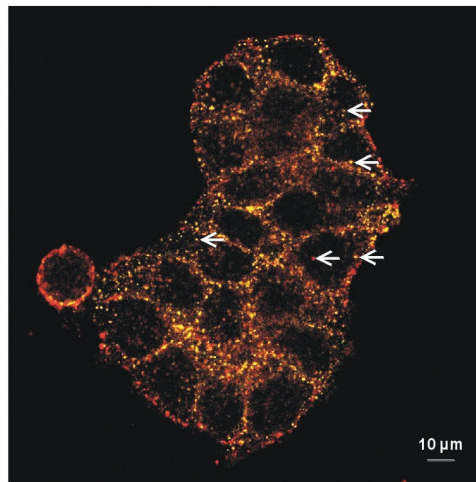
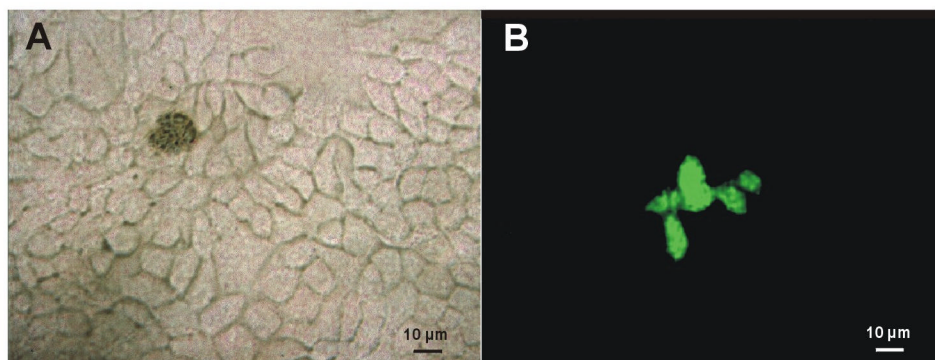


Figure 5



8 Anhang

Lebenslauf

Name: Stefan Gleiter
Geburtsdatum: 20.09.1972
Geburtsort: Laupheim (Baden-Württemberg)
Familienstand: ledig
Eltern: Doris Gleiter, geb. Hein
Willi Gleiter

Ausbildung

1979-1983	Besuch der Grundschule Laupheim
1983-1992	Besuch des Gymnasiums Laupheim
1992	Erwerb der allgemeinen Hochschulreife
1992-1993	Wehrdienst im Topographiezug 200 bzw. 500
1993-1998	Studium an der Martin-Luther-Universität Halle-Wittenberg im Fach Biochemie (Diplom)
1995	Vordiplom in Biochemie
1997-1998	Diplomarbeit mit dem Titel: Strategien zur funktionellen Modifikation des Polyomahüllproteins. Angefertigt am Institut für Biotechnologie im Fachbereich Biochemie/Biotechnologie der Martin-Luther-Universität Halle-Wittenberg unter Leitung von Prof. Dr. Rainer Rudolph und Dr. Hauke Lilie.
Seit Okt. 1998	Promotion am Institut für Biotechnologie im Fachbereich Biochemie/Biotechnologie der Martin-Luther-Universität Halle-Wittenberg unter der Leitung von Prof. Dr. Rainer Rudolph und Dr. Hauke Lilie.

Hiermit erkläre ich an Eides statt, daß ich die vorliegende Arbeit selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Die aus fremden Quellen entnommenen Gedanken sind als solche kenntlich gemacht.

Die vorliegende Arbeit wurde bisher noch keiner anderen Prüfungsbehörde vorgelegt.

Halle an der Saale,