Efficient Intracellular Delivery of a Protein and a Low Molecular Weight Substance via Recombinant Polyomavirus-Like Particles

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1 The abbreviations are used: GFP, green fluorescent protein; MTX, methotrexate; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; ESI-MS, electrospray mass spectrometry.

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Efficient encapsulation of foreign molecules like proteins and low molecular weight drugs into polyoma virus-like-particles (capsoids) was achieved by the development of an anchoring technique based on the specific interaction of the inner core protein VP2 with VP1 pentamers. A stretch of 49 amino acids of VP2 served as an anchor molecule, either expressed as a fusion protein with green fluorescent protein (GFP) or covalently linked to methotrexate (MTX). The average loading capacity was 64 GFP molecules or 462 MTX molecules per capsoid, respectively. The loaded capsoids showed regular morphology and stability for several months. GFP and MTX were internalized into cells in vitro, as was demonstrated by the detection of GFP and VP1 fluorescence in mouse fibroblasts and the cytostatic effect of intracellularly released MTX on leukaemia T cells.
The murine polyomavirus is a small, non-enveloped double stranded DNA virus. Its natural ability to infect a broad range of eukaryotic cells makes it potentially a useful vehicle for gene, protein or drug delivery. The outer shell is composed of the major capsid protein VP1: 360 VP1 molecules are arranged in 72 pentamers on an icosahedral lattice (1), which encloses the inner core proteins VP2 and VP3. The latter proved not to be essential for capsid formation, as was shown by formation of virus-like particles after expression of VP1 alone in insect cells (2). VP1 recombinantly expressed in *Escherichia coli* (3) spontaneously self-assembles into VP1 polyomavirus-like particles (capsoids) in the presence of Ca\(^{2+}\) ions (4). Posttranslational modifications found in the wild type virus, e.g. phosphorylation, are not required for capsid formation.

Other encapsulation strategies, like the liposome technology, have limited applicability for the delivery of low molecular weight substances such as methotrexate (MTX), due to leakage of enclosed but not covalently bound molecules (5, 6). For gene delivery, viral and non-viral vectors are almost exclusively used as vehicles where DNA is either unspecifically encapsulated or covalently bound to the vector’s surface (7). The major techniques to load viral or non-viral vectors with DNA fragments or low molecular weight substances are assembly in the presence of high concentrations, (8-10), application of osmotic shock (11, 12) or sonication (13).

The major advantage of the enclosure of macromolecules, especially proteins, is the protection against enzymatic cleavage. Recently, an anchoring technique to achieve a directed localization to the inward-oriented side of the capsid-forming subunits prior to assembly has been described. Schmidt et al. (14) employed WW domains, fused to the inward-oriented side of VP1, as small modules interacting specifically with proline-rich regions of the molecules to be encapsulated. In this study we established a new method to efficiently encapsulate proteins and low molecular weight substances by exploiting the ability of VP2 to bind tightly into the inward-facing VP1 pentamer cavity (15). We used a conserved stretch of 47 amino acids of VP2 for the attachment of model substances like green fluorescent protein (GFP) and MTX, allowing efficient encapsulation of both substances in VP1 capsoids. Due to the open issue of the hydrophobic interplay between VP1 and VP2 molecules we analyzed two VP2-derivates for their capacity to encapsulate attached ligands (GFP or MTX). It should be noted that both VP2-derivates have the same affinity in binding to VP1-pentamers.

Due to the fixed ratio of one VP2 binding site per VP1 pentamer, the amount of encapsulated molecules was well reproducible and their concentration could thus be standardized, which was essential for defined delivery of MTX in cell culture experiments. GFP fluorescence allows easy monitoring of the encapsulation efficiency during capsid production as well as of the cellular uptake. MTX is a well-known antifolate used in tumour therapy, which is usually taken up into cells by the reduced folate carrier (RFC) and converted to the intracellularly persistent MTX polyglutamates (MTXPG). MTX and MTXPG inhibit the folate dependent enzymes dihydrofolate reductase (DHFR) and thymidylate synthase (TS), thus abrogating DNA synthesis and cell division, resulting in cell death (16). A major mechanism of resistance to MTX (17) is a transport deficiency of the reduced folate carrier. We wanted to find out, whether MTX, when endocytosed inside a macromolecular carrier like capsoids and delivered to a different cell compartment, exhibits toxicity in the same way as the unconjugated drug and is able to overcome MTX transport resistance of cells.
EXPERIMENTAL PROCEDURES

Materials – Isopropyl-β-D-thiogalactoside (IPTG) was obtained from peqlab (Germany), and kanamycin sulphate from Calbiochem-Novabiochem (Germany). Amino acid derivatives were purchased from Calbiochem-Novabiochem AG (Switzerland), methotrexate, MESNA, ethanethiol, TBTU, EDCl, piperidin, DIPEA, DMSO and DMF from Sigma-Aldrich (Germany). DNase, water and acetonitrile were obtained from Merck (Germany). Restriction endonucleases were obtained from New England Biolabs or from MBI Fermentas, and Oligonucleotide Primers were synthesized by ThermoHybaid.

Cloning and vector construction – The complete mouse polyoma virus VP1 cDNA, obtained as an EcoRI - SdaI fragment from expression vector pHB17/6 (11), was subcloned into the corresponding restriction sites of LITMUS28 (New England Biolabs, Germany). After addition of a Ndel site comprising the start codon of VP1 and removal of a second endogenous Ndel site by site-directed mutagenesis with a modified QuickChange™ protocol (Stratagene, Netherlands), the VP1 cDNA was subcloned as a Ndel - BamHI fragment into the expression vector pSG1 (a pBR322 derivative containing an IPTG-inducible tac promoter) resulting in the final vector pSG1/VP1 used for expression of VP1. For construction of the expression vector pTXB1/GFP-VP2A1, the GFP-cDNA was amplified by PCR from pEGFP-N1 (BD Clontech, Germany) with primers generating a 5’ Ndel site comprising the start codon and 3’ SapI and EcoRI sites, and cloned into Ndel and EcoRI sites of pTXB1 (New England Biolabs, Germany). The resulting construct, termed pTXB1/C-GFP, was used for the fusion of the sequence coding for VP2(B) between the C-terminus of GFP and the N-terminus of the Mxe intein-CBD affinity tag by PCR-amplification of VP2(B) from vector pHB1 (11). The primers used for PCR amplification of VP2(B) generated inversely arranged terminal SapI sites, allowing directional cloning of the product into the corresponding restriction sites of the vector. The VP2(A) variant was generated by site-directed mutagenesis of VP2(B) as described above. The expression vector pTXB1/VP2A8 was obtained by PCR-amplification of the sequence coding for VP2(A) with primers generating 5’ Ndel and 3’ SapI sites, and subsequent cloning into the corresponding restriction sites of vector pTXB1.

Preparation of GFP-VP2(A) – GFP-VP2(A) was expressed as an N-terminal fusion with the Mxe intein/chitin-binding domain from expression vector pTXB1/GFP-VP2A1 in E.coli BL21(DE3) cells grown at 37°C in LB medium containing 100 µg/ml ampicillin. The expression of GFP-VP2(A) was induced at an OD 600 of 0.6-1.0 by the addition of IPTG to a final concentration of 1 mM, and cultivation was continued for 5 hours. After harvest, the cells were resuspended in 200 ml of buffer Mxel (20 mM Tris/HCl, pH 8.0; 500 mM NaCl; 1 mM EDTA) including 10 µl benzonase (25 U/µl; Merck, Germany) and broken by 5 passages through a gaulin LAB 1000 homogeniser at 800 bar. Cell debris was removed by centrifugation at 70.000 × g and 4°C for 1 hour, the clear supernatant was supplied with 10 ml chitin beads equilibrated in Mxel, and incubated for 2 hours at 4°C with constant agitation. After 10 minutes centrifugation at 3.000 × g, the slurry was transferred to a chromatography column and washed over night with buffer MxeW1 (20 mM Tris-HCl pH 8.0; 0.5 M Guanidin-HCl; 1 M NaCl; 0.1 % Triton-X100; 1 mM EDTA) at 4°C and a constant flow rate of 0.2 ml/min. After washing the column with 50 ml MxeW2 (20 mM Tris-HCl pH 8.0; 150 mM NaCl; 1 mM EDTA), the gel bed was flushed with MxeC buffer (20 mM Tris-HCl pH 8.0; 150 mM NaCl; 1 mM EDTA; 50 mM DTT) and incubated at 37°C for 3 hours to ensure complete cleavage of the Mxe intein-CBD moiety. The free GFP-VP2(A) fusion
protein was eluted with MxeC buffer, the peak fractions were pooled, and concentrated with Centriprep-10 centrifugal concentrators (Millipore, Germany). Finally, the protein solution was incubated for another 3 hours at 25°C to allow the complete formation of the GFP chromophore. Homogeneity of the protein was analyzed by SDS-PAGE and mass spectrometry, and concentration was determined by UV-Vis spectroscopy.

**Purification of recombinant polyoma VP1 – E.coli**

BL21 cells were grown at 37°C in LB medium containing 70 µg/ml kanamycin. Expression of VP1 was induced by the addition of 1 mM IPTG. After continued cultivation for 5 hours, the cells were harvested, resuspended in P-A buffer (20 mM Tris/HCl, 2 mM EDTA, 6 mM DTT, 5 % glycerol, pH 8.5) and lysed at 800 bar using a Gaulin LAB 1000 homogenizer. Cell debris was removed by centrifugation at 15,000 × g for 2 hours and 4°C, and VP1 was captured from the supernatant by an anion exchange column (Poros, Applied Biosystems, Germany) previously equilibrated with P-A buffer. After elution of VP1 from the column by a linear gradient of NaCl from 5 mM to 1 M, the protein was dialyzed against QS-A buffer (20 mM ethanol amine, 2 mM EDTA, 6 mM DTT, 5 % glycerol, pH 10.5) over night. The VP1-solution was applied to a Q-Sepharose column (Amersham Biosciences, Germany) and eluted again by a linear gradient of NaCl to 1 M. Afterwards, the protein was dialyzed against L1 buffer (50 mM sodium phosphate, 2 mM EDTA, 6 mM DTT, 150 mM NaCl, 5 % glycerol, pH 7.0). The final step of the VP1 purification was a size exclusion chromatography by which aggregates of VP1 were removed. Homogeneity of the protein was checked by SDS-PAGE, photon correlation spectroscopy (PCS) and mass spectrometry. Protein concentration was determined by UV-Vis spectroscopy.

**Expression and purification of VP2-SEt – E.coli**

VP2-SEt was expressed as an N-terminal fusion with the Mxe intein/chitin-binding domain from expression vector pTXB1/VP2A8 in E.coli BL21(DE3) as described for GFP-VP2(A). Cells were harvested and the pellets were stored at -80°C. After thawing cell pellets on ice, which were obtained from 12 litres of culture volume, cells were resuspended in 125 ml of buffer A (20 mM HEPES, 0.5 M NaCl, 1 mM EDTA, 10 mg/ml DNAse, pH 6.8) and broken by 5 passages through a Gaulin LAB 1000 homogenizer at 800 bar. The resulting suspension was centrifuged at 39000 × g for 45 minutes, the clarified supernatant was loaded onto a chitin column, washed with 10 CV buffer B (20 mM HEPES, 0.5 M NaCl, 1 mM EDTA, 0.1% Triton X100, pH 6.8) and cleaved with buffer C (2% [v/v] ethanethiol, 50 mM sodium phosphate, 200 mM sodium chloride, 1 mM EDTA, pH 6.8) over night at room temperature. After elution according to the instruction manual, the protein was isolated by preparative HPLC (Agilent SB C18 column, solvent system see above). In an HPLC-MS re-analysis of the product fraction, VP2-SEt was found to be > 98% pure.

**Synthesis of dipeptide linker**

MTX-Boc-Cys(Trt)-Lys(Fmoc)-OMe was synthesized according to a standard TBTU-peptide coupling protocol. The product could be isolated quite pure from the reaction mixture by precipitation with water, filtration and lyophilization. ESI-MS calculated m/z for C_{49}H_{53}N_{3}O_{7}S [M+Na]^+: 851.0; found: 850.5. Deprotection of the lysine side chain was accomplished by treatment of 50 mg Boc-Cys(Trt)-Lys(Fmoc)-OMe with 100 µl 20% piperidin in DMF at room temperature for 20 minutes. The product was purified by SPE on a RP-C18-cartridge with H_{2}O/acetonitrile and then lyophilized. After removal of the solvents, the raw product Boc-Cys(Trt)-Lys-OMe was obtained in quantitative yield as a colourless amorphous powder. ESI-MS calculated m/z for C_{34}H_{43}N_{3}O_{5}S [M+H]^+: 606.8; found: 606.2. A 1.5 molar excess of MTX (23.3 mg) was pre-activated with 8.5 mg EDCI × HCl and 15.3 µl of DIPEA in 400 µl DMF for 5 minutes and then added to a solution of 18.0 mg Boc-
Cys(Trt)-Lys-OMe in 400 µl DMF. After 30 minutes of incubation at room temperature, the reaction was quenched by the addition of 10 ml H₂O. The product was centrifuged (20000 × g, 1 minute), purified by preparative RP-HPLC and lyophilized to give Boc-Cys(Trt)-Lys(MTX)-OMe as a yellow amorphous powder. Yield: 28.4 mg (95 %); ESI-MS calculated m/z for C₅₄H₆₃N₁₁O₉S [M+H]⁺: 1043.2; found: 1043.3. Both protecting groups of the cysteine were removed by treatment with TFA:EtSH:H₂O:TIPS (50 : 10 : 4 : 2) for 5 hours at room temperature. The deprotected MTX-dipeptide conjugate was precipitated with ice-cold diethylether. After centrifugation, the raw linker-MTX-conjugate was isolated as an orange amorphous solid. ESI-MS calculated m/z for C₃₀H₄₁N₁₁O₇S [M+H]⁺: 700.8; found: 700.3 Da.

Synthesis of VP2-Cys-Lys(MTX)-OMe – 15.3 mg VP2-SEt and a threefold molar excess (6.9 mg) of Cys-Lys(MTX)-OMe were dissolved in 2.7 ml of 400 mM phosphate buffer, containing 10 mM EDTA and 10 % (v/v) DMSO. One equivalent of tris-carboxyethyl phosphine was added to establish reductive conditions, and MESNA (0.1 eq) was added as a catalyst. The reaction was monitored by analytical HPLC and showed a quantitative derivatization of VP2-SEt after 18 hours. After final reduction by addition of 2 equivalents of DTT at room temperature for 15 minutes, the solvents were removed in vacuum and the raw product was isolated by preparative HPLC (Agilent SB C18 column, solvent system see above). The HPLC-MS analysis showed a purity of >98 % for the obtained VP2-Cys-Lys(MTX)-conjugate.

Formation of capsoids from VP1 pentamers and VP2 anchor constructs containing GFP or MTX – For the assembly of capsoids, DTT was removed from the VP1 pentamer solution with a desalting column (HiPrep 26/10, Amersham Biosciences; column volume 53 ml, flow rate 2 ml/min) equilibrated with KB1 buffer (50 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, 5 % glycerol, pH 6.8). Structural integrity and concentration of the desalted pentamers were determined by PCS and UV-Vis spectroscopy. VP1 pentamers were incubated with a 7 fold molar excess of GFP-VP2(A) or with a 6 fold molar excess of VP2(B)-MTX for 1 hour at room temperature, respectively. Afterwards, assembly of the pentamers was initiated by adding KB2 buffer (10 mM Tris/HCl, 150 mM NaCl, 5 % glycerol, 3 M (NH₄)₂SO₄, pH 8.0) to a final ammonium sulphate concentration of 250 mM. After incubation for 30 minutes at 25 °C, the formed capsoids were oxidized by the addition of KB3 (400 mM GSSG in KB2) to a final GSSG concentration of 7.2 mM. Afterwards, the protein was dialyzed over night at 25 °C against PBS (Biochrom KG, Germany) containing 0.7 mM CaCl₂. The excess of non-assembled VP2-anchor construct was removed by size exclusion chromatography (SEC) using a Superdex 200HR 10/30 column (flow rate 0.5 ml, Amersham Biosciences). A precipitation with polyethylene glycole (10% PEG 3000, Sigma-Aldrich) was performed in order to concentrate the capsoids for cell culture experiments.

Photon correlation spectroscopy (PCS) – Particle sizing was performed at 25 °C using a High Performance Particle Sizer (ALV-NIBS, Germany). The particle size was calculated by the autocorrelation function of the ALV Sizer software.

Immunoprecipitation – 3.1 µM VP1 pentamers were co-incubated for 4 hours at 25 °C with a 10 fold molar excess of GFP-VP2(A) or GFP, respectively. VP1 pentamers, GFP and GFP-VP2(A) were also incubated and served as controls. Protein-A-sepharose (Amersham Biosciences) was equilibrated with KB1 buffer and monoclonal antibodies (mab) directed against a conformational determinant of VP1-specific pentamers and appropriate capsoids (mab #17 kindly provided by Dr. M.
Pawlita, Heidelberg, Germany) or GFP were coupled for 1 hour at 25 °C. After washing the sepharose two times with IP wash buffer (PBS, 0.1 % Triton X-100, pH 7.4), the above described protein samples were added and incubated for 1 hour at 25 °C. Unbound protein was removed by washing three times with IP wash buffer. Subsequently, the samples were analyzed by reducing 10% SDS-PAGE and Western-Blotting.

Fluorescence spectroscopy – To investigate the interaction between VP1 pentamers and the GFP-VP2(A) anchor construct, GFP fluorescence polarization measurements were carried out. GFP-VP2(A) was incubated with an increasing amount of VP1-pentamers for 2 hours at 25 °C. GFP fluorescence polarization was measured at 510 nm (excitation 488 nm) for 10 minutes in a thermostatically controlled cell using a Fluoromax-3 spectrofluorimeter with autopolarizer (Jobin Yvon, Germany). The average signal was plotted against the VP1 pentamer concentration [M] and the dissociation constant ($K_d$) calculated according to equations 1A to 1C:

$$c = V_0 + A_0 + K_d$$  
(1A)  
$$V_A = \frac{c}{2} - \frac{c^2}{4A_0} - V_0 \cdot A_0$$  
(1B)  
$$f = S_0 + \left( S_{max} - S_0 \right) \cdot \frac{V_A}{A_0}$$  
(1C)

$V_0$ is the initial concentration of the VP1 pentamer, $A_0$ the concentration of the VP2 anchor, and $VA$ the concentration of the complex. $S$ stands for the starting ($S_0$) and the maximum signal ($S_{max}$).

In addition, the assembly of capsoids was monitored by measurement of light scatter of the sample at an angle of 90° and a wavelength of 360 nm.

Analytical size exclusion chromatography – Analytical size exclusion chromatography was carried out to follow capsoid formation. Using a Superdex 200hr 10/30 column (Amersham Biosciences) (volume 23.5 ml, flow rate 0.5 ml).

High Performance Liquid Chromatography – Electrospray Mass Spectrometry (HPLC-ESI-MS) - HPLC analyses were performed on an Agilent Technologies 1100 MSD Series system equipped with diode array detector and coupled single quadrupole MS. HPLC solvents consisted of 1% acetonitrile in H$_2$O containing 0.1% trifluoroacetic acid (Solvent A) and 90% acetonitrile in H$_2$O containing 0.1% trifluoroacetic acid (Solvent B). Conditions: A Polymerlabs PLRP-S (300 Å, 4.6 x 150 mm) column was used at a flow rate of 0.8 ml/min. The column temperature was maintained at 25 °C. The gradient started with a solvent composition of 80% A and 20% B followed by a linear gradient in 20 minutes to 0% A and 100% B, after which the column was re-equilibrated.

Cell cultivation – Swiss mouse fibroblasts (Swiss 3T3) were grown in DMEM medium supplemented with 2 mM glutamine (Pan Biotech, Germany) and 10% fetal bovine serum (GIBCO Invitrogen Corp., Germany). The human T cell leukaemia line CCRF-CEM was cultivated in RPMI medium supplemented with 2.5% human serum, 4 mM glutamine, 25 mM HEPES buffer (all obtained from Pan Biotech) and 200 μg/ml human serum albumin (Pharma Dessau GmbH, Germany). The MTX transport resistant CCRF-CEM/MTX cells (18) were cultivated in the same medium with additional 1 μM MTX. For experiments, the cells were grown in medium without MTX for seven
passages. All cell lines were tested negative for mycoplasma contamination and grown at 37 °C in a 5% CO₂-air humidified incubator.

Fluorescence immunocytochemistry with Swiss 3T3 cells – Swiss 3T3 cells were grown on coverslips for 2 days. For the attachment of GFP-loaded capsoids or VP2-GFP(A) anchor to the cell surface, cells were incubated in DMEM containing 10 µg/ml GFP-loaded capsoids (approximately 4 x 10⁶ particles per cell) or 10 µg/ml VP2-GFP(A) anchor, respectively. After 1 hour incubation at 0 °C, some samples were immediately prepared for immunocytochemistry (attachment 0 h) by washing the cells in ice-cold medium for the removal of unbound GFP-loaded capsoids or VP2-GFP(A) anchor. For the uptake of GFP-loaded capsoids or VP2-GFP(A) anchor after attachment, some samples were washed in pre-warmed DMEM, transferred to 37 °C and incubated for 0.5 hours, 1 hour and 3 hours, respectively. After the indicated time points all samples were prepared for visualization of VP1 by immunofluorescence. The following steps were performed at room temperature. Cells were fixed with 1% paraformaldehyde (Sigma) for 30 minutes, rinsed twice with PBS and incubated with 80 mM glycine (Sigma) in PBS (pH 8) for 10 minutes to block free aldehyde functions. After washing twice with PBS, cells were permeabilized with 0.1% Triton-X100 (Sigma) in PBS for 4 minutes for intracellular staining of VP1, or remained unpermeabilized for VP1 staining on the cell surface by incubating with PBS only. All samples were rinsed twice with PBS. Blocking of unspecific binding was performed with 1% bovine serum albumin (BSA) in PBS (PBS/BSA; albumin fraction V, Roth, Germany) for 15 minutes followed by incubation with a mouse anti-VP1 mab #13 (undiluted hybridoma supernatant) for 1 hour, washing twice with PBS, and labelling with a Cy3-rabbit-anti-mouse polyclonal antibody (Dianova, Germany) diluted 1:300 in PBS/BSA. The anti-VP1 mab #13 kindly provided by Dr. M. Pawlita recognizes a conformational determinant of VP1 pentamers as well as VP1 capsoids. Denatured VP1 protein could not be detected with anti-VP1 mab #13 as shown by SDS-PAGE and subsequent Western-Blotting analysis (data not shown). It should be noted that anti-VP1 mab #13 and #17 could recognize similar epitopes of particulate VP1-structures (M. Pawlita, personal communication). Samples were rinsed twice with PBS, and cell nuclei stained for 5 minutes with 0.2 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS. After washing with PBS and H₂O, coverslips were mounted on microscope slides with anti-fading solution: 6 g glycerol, 2.4 g Mowiol (Calbiochem, U.S.A.) and 0.2% 1,4-diazabicyclo-[2.2.2]octane in 0.2 M Tris/HCl, pH 8.5. Two control conditions were examined: incubation of cells with VP2-GFP(A) anchor, and immunofluorescence staining with omission of primary antibody as negative control. None of these conditions resulted in cell staining. GFP fluorescence and the immunofluorescence signal of VP1/Cy3 were observed with a Zeiss Axiosplan 2 imaging fluorescence microscope (Zeiss, Göttingen, Germany) and fluorescence filters F11-013 (DAPI), F41-054 (GFP) and F41-007 (Cy3) (AHF Analysentechnik, Germany). Pictures were taken with a digital camera, model 2.3.0 (Diagnostic Instruments, U.S.A.) followed by digital image processing with Metamorph software, version 4.6 (VisiTech Systems, Germany).

Dose response assays with CCRF-CEM and CCRF-CEM/MTX cells – Exponentially growing CCRF-CEM cells were seeded in 96 well plates with 1.5 x 10⁴ cells in 200 µl RPMI per well containing different concentrations of MTX loaded capsoids, MTX (USP grade Sigma), capsoids (control), VP2(B)-MTX anchor (control) or medium only (untreated control). After 24, 48 and 72 hours a Calcein™ viability assay was performed. 100 µl of medium per well were aspirated and 100 µl of a 20 µM solution of Calcein™ (Molecular Probes Europe) in Dulbecco's phosphate buffered saline
(Biochrom KG, Germany) were added to a final Calcein® concentration of 10 µM. Cells were incubated for 1 hour at 37 °C and fluorescence was measured at 485 nm/528 nm (excitation/emission) with an FLx800T BIE-microplate reader (Bio-Tek Instruments GmbH, Germany). CCRF-CEM/MTX cells were grown in medium without MTX for seven passages and seeded in 96 well plates with 1.5 x 10^4 cells in 200 µl RPMI per well containing different concentrations of MTX loaded capsoids, MTX, capsoids (control), VP2(B)-MTX anchor (control) or medium only (untreated control). After 48 hours of incubation a Calcein® viability assay was performed as described above.

Transmission electron microscopy (TEM) of capsoids – Capsoids, GFP-loaded capsoids, or MTX loaded capsoids were attached on formvar-carbon coated grids (300 mesh; Plano GmbH, Germany) and contrasted by the single droplet negative staining technique modified after Harris. Briefly, grids were wetted for 10 seconds on a 20 µl droplet of an aqueous solution of 0.1% poly-L-lysine hydrobromide (Sigma), washed on a droplet of 20 µl H₂O and transferred to a droplet of 20 µl PBS containing 100 µg/ml capsoids, GFP-loaded capsoids or MTX-loaded capsoids. After 2 minutes of attachment, grids were washed twice on 20 µl H₂O and negative stained for 15 seconds on 20 µl of an aqueous solution of 5 % uranyl acetate (Plano GmbH, Germany). After drying for 5 minutes, samples were ready for immediate TEM study on a Zeiss LEO 906E (LEO Elektronenmikroskopie GmbH, Germany) operating at 80 kV.

RESULTS

Expression and purification of VP1 – The outer shell protein VP1 of murine polyomavirus was expressed in E. coli. The protein self-assembled into soluble pentamers in the cytoplasm of E. coli and was purified by ion exchange and size exclusion chromatography to homogeneity (data not shown). Neighbouring VP1 pentamers are covalently linked by disulfide bonds and calcium bridges in the capsid (20, 21), preventing disassembly of the structure in the absence of reducing and chelating agents. In order to avoid aggregation of VP1 pentamers by unspecific cross-linking, DTT and EDTA were present in all buffers throughout the purification process. Under these conditions, the protein was stable and could be stored at -80°C for several months.

VP2 anchor variants – Owing to the hydrophobic character of the anchor molecule, we chose different VP2 variants for the expression of the GFP-VP2(A) fusion protein and covalent linkage of MTX to the VP2(B) anchor, in order to obtain soluble proteins capable of interacting with VP1 pentamers. Variant VP2(A) is based on the VP2 sequence found in the mouse polyoma virus Py-2a strain (22). Variant VP2(B) was derived from polyoma virus strain 3. Both anchor molecules differ only in the PGGA or QVVS motifs corresponding to positions 276-279 of VP2 (Fig. 1). The X-ray structure of VP1 interacting with VP2 was performed with the VP2-derivate containing the QVVS motif (22). In contrast, our original VP2-derivate with PGGA residues showed non-conservative amino acid alterations at the same positions (276-279). Therefore and due to the open issue of the hydrophobic interplay between VP1 and VP2 molecules we analyzed both VP2-derivatives for their capacity to encapsulate attached ligands (GFP or MTX). It should be noted that both VP2-derivatives have the same affinity in binding to VP1-pentamers.

Expression and purification of GFP-VP2(A) – The GFP-VP2(A) protein was expressed in E. coli as a soluble fusion protein with the Mxe intein-CBD moiety as removable affinity tag (IMPACT™ system, New England Biolabs), and was obtained in highly purified form after affinity chromatography on chitin.
agarose and subsequent induction of the intein self-cleavage reaction (by addition of DTT). The fusion of GFP to the C-terminus of the anchor molecule VP2(A) resulted in poor solubility of the fusion protein VP2(A)-GFP, when expressed with the Mxe intein-CBD tag (data not shown).

Expression and purification of VP2(B) anchor and conjugation to MTX – A multistep synthesis strategy to conjugate MTX covalently to the VP2(B) anchor was developed. In order to prevent interference of the capsid assembly by VP2(B)-MTX, MTX was coupled to the C-terminus of the VP2(B) anchor molecule, which is positioned at the sterically free accessible site of the VP1 pentamer (22). This conjugation was realized by expressed protein ligation (23). The required VP2(B)-ethylthioester was generated from the recombinant fusion protein VP2(B)-intein-CBD by induction of the intein cleavage with ethanethiol. It was sufficiently soluble and stable to hydrolysis, which enabled isolation by preparative HPLC. The same procedure was performed with the VP2(A) anchor molecule, but interestingly the resulting VP2(A)-ethylthioester was only poorly soluble and could not be isolated. The functionalization of the dicarbonic acid MTX with the free amino acid cysteine affords a diamino linker unit, for which lysine was chosen (Fig. 1). Selective deprotection of the ε-amino group in Boc-Cys(Trt)-Lys(Fmoc)-OMe gave Boc-Cys(Trt)-Lys-OMe. Since the application of standard peptide coupling protocols leads to the formation of multiple undesirable side products, it was necessary to develop a special protocol for the subsequent coupling of MTX to the free ε-amino side chain group. Variation of reaction parameters and coupling agents (HBTU, TBTU, NHS active ester, EDCI, EDCI/HOBT) revealed that selective mono-functionalization of MTX is achieved by the following settings: i) MTX in 1.5 molar excess, ii) EDCI as coupling reagent, ii) DIPEA in a limited amount (2 eq.) and iv) quenching of the reaction after 30 minutes by addition of H2O. Consequent application of these parameters reduced the formation of side products to a minimum. Unmodified or bis-modified MTX as well as other common side products were not detectable and the desired product was isolated by preparative HPLC with a purity of > 98%. Finally, the N/S-protecting groups of Boc-Cys(Trt)-Lys(MTX)-OMe were cleaved in one step with TFA:EtSH:H2O:TIPS = 50:10:4:2. Diethylether precipitation of the reaction mixture gave the raw product, which was directly used for the recombinant protein ligation. For the ligation reaction VP2(B)-SEt was incubated with 3 eq of Cys-Lys(MTX)-OMe and a catalytic amount of MESNA in 10% DMF. After 16 hours, the conjugation of VP2(B) was quantitative and VP2(B)-Cys-Lys(MTX)-OMe [VP2(B)-MTX] was isolated by preparative HPLC with a purity of > 98%.

Interaction of the VP2 anchor constructs with the VP1 pentamer – The outer shell of the polyomavirus capsid is composed of 360 VP1 monomers, arranged in 72 pentamers on an icosahedral lattice (1). VP2 binds to the hydrophobic pocket of the VP1 pentamer on the inward-facing side (15). In order to evaluate, whether the C-terminal fragment of VP2 we used as an anchor molecule is able to interact with a VP1 pentamer, even when a protein like GFP is fused to the N-terminus, we performed gel filtration experiments. Free GFP-VP2(A) was clearly separated from the VP1 pentamer fraction. After incubation of GFP-VP2(A) with an equimolar amount of VP1 pentamer for 2 hours, both proteins co-eluted from the column (data not shown). To assess the specificity of the interaction between the VP1 pentamer and the VP2 anchor, we performed immunoprecipitation experiments. VP1 was co-incubated either with GFP-VP2(A) or GFP as a control, followed by immunoprecipitation with monoclonal antibodies against VP1 or GFP, respectively. SDS-PAGE and Western-Blot analysis of the precipitated samples showed that GFP-VP2(A) and VP1 co-precipitated in both cases, revealing a tight interaction between the VP1 pentamer and the anchor (Fig. 2).
contrast, no co-precipitation was observed after co-incubation of GFP and VP1, indicating that the attachment of GFP-VP2(A) to VP1 pentamers is highly specific and occurs via the C-terminal VP2 anchor. A corresponding fusion protein of GFP and the VP2(B) anchor gave similar results in analogous experiments (data not shown).

An important prerequisite for encapsulation of VP2 anchor constructs in VP1 capsoids is the sufficient stability of the interaction between the VP1 pentamer and the VP2 anchor over the time period which is required for complete capsid assembly. The GFP fluorescence enabled us to investigate the interaction between VP1 pentamers and GFP-VP2(A) in detail by fluorescence polarization. Binding of the VP2 anchor to VP1 restricts the freedom of motion of the molecule, causing an increase in GFP fluorescence polarization. Using a 10-fold molar excess of VP1 pentamer over GFP-VP2(A) in our measurements, we observed 100% binding (Fig. 3). We calculated a dissociation constant ($K_d$) of 190 ± 60 nM from the plot analysis, and a similar dissociation constant for the GFP-VP2(B) anchor (data not shown). The association of the anchor and the VP1 pentamer occurred very rapidly, with the polarization signal reaching 80% of the final value within 15 seconds after start of the reaction by manual mixing (data not shown). The interaction remained stable for at least 4 hours, as was concluded from subsequent immunoprecipitation experiments.

Taken together, our results clearly demonstrate that the VP2 anchor comprising amino acids 251-297 of VP2 efficiently binds to the VP1 pentamer, even if covalently linked to a heterologous protein. The association process is fast and results in a tight binding, which remains stable over a time range sufficient for capsid assembly.

**Encapsulation of VP2 anchor constructs** – In order to achieve the encapsulation of VP2 anchor-bound molecules into VP1 capsoids, a robust method allowing both the attachment of the VP2 anchor to the pentamer and the subsequent capsid assembly within a short time frame was developed. For capsid formation, DTT was removed from the pentamer solution to enable the formation of intrapentameric disulfide bonds, which stabilize assembled VP1 capsoids (14, 20, 21, 24). In contrast to the assembly method described by Stehle and co-authors (20, 25), we induced the assembly process by addition of high salt buffer to the pentamer solution. As shown in Figure 4A, this approach leads to capsid formation in a concentration dependent way. At the lowest pentamer concentration examined (0.9 µM), the pentamers associated with a half-time of 170 seconds and the entire process was completed after 5 minutes. Increase of the protein concentration resulted in a faster assembly reaction. The course of assembly was also followed by PCS measurement, which showed a particle size of 8-9 nm for VP1 pentamers at the start of the reaction (Fig. 4B) and a particle diameter of 35 nm for the resulting capsoids (Fig. 4C).

The same method was applied for encapsulation of the different VP2 anchor constructs. Since we observed that higher molar ratios of anchor to pentamer inhibit capsid assembly (data not shown), VP1 pentamers were incubated with a 6-fold molar excess of VP2(B)-MTX or a 7-fold molar excess of GFP-VP2(A), respectively (data not shown). After the formation of capsoids, free anchor was removed either by dialysis or size exclusion chromatography and homogeneously formed capsoids with a size of 35-40 nm were obtained (Fig. 5A). Furthermore, size exclusion chromatography experiments revealed that no aggregates were present in the capsid preparation (Fig. 5B).

The encapsulated heterologous molecules confer additional absorption maxima to the VP1 spectrum at 310/377 nm for MTX, and at 488 nm for GFP. From UV-Vis spectra analysed at 488 nm,
the number of GFP-VP2(A) molecules per VP1 capsoid was calculated to be about 104, corresponding to 144% of the theoretical loading maximum (data not shown). Analytical gel filtration indicated that GFP-VP2(A) was enclosed in the VP1 capsoid, since the anchor was found to co-elute with the VP1 peak (Fig. 5B). A minor amount of GFP anchor molecules - probably bound unspecifically to the capsid surface - eluted separately from the column (indicated by an arrow in Fig. 5B). Integration of both GFP signals revealed a ratio of 2.6 : 1 (corresponding to 64 and 40 anchor molecules, respectively). The calculated loading ratio of GFP molecules per capsid was 89% of the theoretical amount, assuming 72 VP1 pentamers per capsid.

Immunoprecipitation experiments confirmed that, apart from encapsulated GFP-VP2(A), additional molecules either occur freely or unspecifically bound to the surface of the VP1 capsoids (data not shown). The gel filtration experiments suggested a rather weak interaction which was easy to disrupt during the chromatography process. After re-chromatography or extended dialysis of the capsid fraction, no more free anchor was detectable by immunoprecipitation (data not shown).

Quantification of the encapsulated MTX by UV vis spectroscopy and gel filtration gave an average amount of 462 anchor molecules per VP1 capsid (data not shown), whereas free VP2(B)-MTX was not observed. In comparison, none-conjugated MTX could not be encapsulated during VP1 assembly due to the small molecule size and the cavity-containing morphology of the final capsoids (data not shown).

Electron microscopy of the loaded capsoids revealed a regular morphology, showing no significant differences to empty particles (Fig. 6A-C). Nevertheless, the capsoids GFP-VP2(A) showed minor particle formation in comparison to empty virus-like-particles and those that have encapsulated MTX ligands suggesting that the lower co-assembling efficiency within a given time-frame may depend on the charge distribution and the size of appropriate ligands, GFP versus MTX (Fig. 6C).

Taken together, we have shown that the VP2-derived anchor molecule allows highly efficient encapsulation of GFP or MTX in recombinantly produced VP1 capsoids.

Protein delivery into cells via capsoids – GFP encapsulated in mouse polyoma capsids was delivered to Swiss 3T3 cells. Incubation of the cells with GFP-loaded capsoids at 0°C led to evenly distributed attachment of capsoids to the cell surface. Immunofluorescence labelling of VP1 via a primary mab against VP1 recognizing a conformational determinant of pentamers and capsoids and a secondary Cy3-labeled rabbit anti-mouse polyclonal antibody showed the co-localization of GFP fluorescence and VP1/Cy3 (Fig. 7, A: overlay), which was not influenced by Triton permeabilization (Fig. 7, D: overlay). The cells were allowed to take up the attached GFP-loaded capsoids for 1 hour at 37°C. To demonstrate whether GFP was located intracellularly after delivery, immunofluorescence was performed either with or without previous Triton permeabilization of the cells. Non-permeabilized cells with intact membranes, which do not allow the entry of antibodies, showed vesicular structures containing GFP only (Fig. 7, B and C: overlays), while VP1/Cy3-fluorescence was restricted to the cell surface (Fig. 7, b and c). Permeabilized cells, which allowed entry of antibodies into the cytosol and intracellular membrane enclosed compartments, contained GFP and VP1/Cy3 partly co-localized in vesicular structures (Fig. 7, E and F: overlays). The insets e and f in Fig. 7 show the single fluorescence of VP1/Cy3, and a comparison with the overlays in Fig. 7, E and F, clearly demonstrates that vesicular structures contain both GFP and VP1/Cy3. Fig. 8, B, C and D overlays show a time course of the uptake of GFP-loaded capsoids in Swiss 3T3 cells at 37 °C. After the attachment of
GFP-loaded capsoids at 0 °C (Fig. 8, A: overlay) GFP fluorescence was evenly distributed on the cell surface and co-localized with VP1/Cy3.

After 30 minutes of uptake, GFP occurred in vesicular structures (Fig. 8, b), which were labelled by VP1/Cy3 to the same extent (Fig. 8, bb), showing that GFP and VP1/Cy3 were predominantly co-localized (Fig. 8, B). After 1 hour of uptake a redistribution occurred: Fig. 8, D is a phase contrast picture of the peripheral cytosol with 5 clearly distinguishable vesicles (see framed detail) with markedly different fluorescence intensities of GFP (Fig. 8, E) and VP1/Cy3 (Fig. 8, F) (Fig. 8, G shows the overlay of both fluorescence signals). This redistribution intensified after 3 hours of uptake (Fig. 8, C: overlay and Fig. 8, c: GFP and Fig. 8, cc: VP1/Cy3).

**Methotrexate delivery into cells via capsoids** – MTX encapsulated in mouse polyoma capsoids was delivered into MTX-sensitive CCRF-CEM cells. Two independently performed dose response assays are shown (Fig. 9, A and B). Free MTX in a concentration range of 1 µM to 40 µM was cytotoxic on these cells in a time- but not concentration-dependent manner as shown in Figure 10, A. Concentration dependence was only observed between 0.02 µM and 1 µM MTX. The median survival after 48 hours of treatment with 0.02 µM MTX was 47.4% and 23.2% for 1 µM MTX. MTX concentrations of 10 µM, 20 µM and 40 µM did not increase effectivity and led to a median survival after 48 hours of 19.8%, 19.9% and 18.4%, respectively. Differentiation between growth arrest and cytotoxicity was achieved by microscopic observation, because the Calcein™ assay is a measure for cell viability only. Cell populations treated with 0.02 µM MTX showed a clearly reduced density but only a few damaged or dead cells, as compared to the untreated control cells over the whole time period. Cells treated with cytotoxic MTX-concentrations above 1 µM predominantly appeared damaged or dead.

MTX delivered via capsoids in a concentration range of 1 µM to 40 µM was also cytotoxic. In the range of 1 µM to 10 µM MTX the effect was concentration dependent, whereas no difference was observed between the effect of 10 µM and 40 µM encapsulated MTX (Fig. 9, B). However, the time-span required for the occurrence of cytotoxic effects was markedly different from the experiments with free MTX. After 24 hours of treatment, MTX-loaded capsoids over the whole concentration range had only a low impact on cell growth and resulted in a median survival of 81.2% to 84.6%. The cytotoxic effect did not appear until 48 hours of incubation: the median survival after treatment with capsoids containing 10 µM, 20 µM and 40 µM MTX was 36.5%, 31.3% and 29.4%, respectively, and thus almost reached the effectivity of free MTX. The effect of 1 µM MTX delivered via capsoids corresponded to the impact of 0.02 µM free MTX. Two additional control conditions were examined to rule out effects not based on capsoid-mediated delivery of MTX: cells were treated with a) empty capsoids and b) VP2(B)-MTX-conjugate, which were both non-toxic (Fig. 9, B).

Empty capsoids with a concentration of 1.8 mg/ml, corresponding to the capsoid concentration of MTX-loaded capsoids containing 40 µM MTX, did not exhibit cytotoxic effects: no influence was seen on cell survival after 24 hours, a low impairment resulting in median survival of 80.1% after 48 hours and a recovery to 99.3% survival after 72 hours. The VP2(B)-MTX conjugate with 117 µM MTX (total protein content 0.7 mg/ml) had no influence on the cells after 24 hours and 48 hours and the median survival after 72 hours was only slightly reduced to 90.1%. The ineffectivity of this high MTX concentration is most probably due to the lack of entry of the drug into the cell because of its conjugation to VP2(B).
Methotrexate delivered via capsoids also circumvented the MTX transport resistance of CCRF-CEM/MTX cells (Fig. 10). When these cells were treated with 0.02 µM or 1 µM free MTX, the median decrease of survival after 48 hours of incubation was only 14.2% or 22.5% respectively, and thus comparable to the slight growth impairment of 13.1% caused by empty capsoids. In contrast, both 0.02 µM and 1 µM MTX were cytotoxic in the parental MTX-sensitive cell line CCRF-CEM causing a survival decrease of 59.0% and 83.1%, respectively.

When MTX transport resistant cells were treated with 1 µM encapsulated MTX, MTX became cytotoxic causing a 50.4% decrease of survival, which was comparable to the reduction of 67.2% observed with MTX-sensitive cells.

**DISCUSSION**

In the present study, we examined the transfer of GFP and MTX into eukaryotic cells via polyoma virus-like particles, termed capsoids. The capsoids were entirely assembled in vitro from the recombinantly expressed outer shell protein VP1 purified to homogeneity. In order to achieve efficient and directed encapsulation, GFP and MTX were conjugated to a conserved stretch of the inner core protein VP2, which tightly binds to the inward-oriented side of VP1 pentamers (15, 24) and thus served as a molecular anchor prior to capsoid assembly. This delivery approach of using the interaction of two polyomavirus-specific proteins, VP1 and VP2, clearly differs from encapsulation strategies described by Schmidt et al. (26). For packaging of foreign substances into VLPs, VP1 has to be engineered at an internal loop domain, whereas our approach left VP1 untreated and therefore offered more variability for the delivery of complete proteins independent of their structural interference with VP1-specific capsoid assembly. Purification of full-length VP2 of mouse polyomavirus is impossible because of the insolubility of the protein when expressed in *E.coli*. Refolding from inclusion bodies does not result in adequate amounts of soluble protein (22, 27). Chen and co-workers (22) also showed that the 45 C-terminal residues of VP2 are sufficient for stable binding to the inner surface of the VP1 pentamer, which forms a cup-like cavity. Only residues 269-296 of VP2, which are part of a highly conserved stretch among different polyomaviruses, were clearly visible in the electron density map, revealing a hydrophobic interaction between a short α-helix and the VP1 pentamer. Consequently, we decided to employ this fragment of VP2 as core of the anchor molecule. The residues N-terminal of Val 269, which is located at the top of the cavity formed by the VP1 pentamer, could not be resolved in the crystal structure, and the authors concluded that this part of VP2 might be somewhat flexible. We estimated that the VP2-mediated attachment of GFP to the inner surface of the VP1 pentamer would require a distance of at least 20 Å between Val 269 and the GFP-termini (located adjacent at one narrow side of the protein) in order to avoid steric hindrance. This notion favoured the fusion of GFP to the C-terminus of the VP2 anchor, as the corresponding Tyr 296 is located at the base of the pentamer. Since the C-terminal part of the resolved VP2 structure approaches the termini of VP1, which are very likely engaged in pentamer-pentamer interactions (20, 21), the fusion of a 27 kDa protein at this site could possibly interfere with capsoid assembly. Therefore, a GFP-fusion to the N-terminus of the anchor molecule was considered as the better alternative, but obviously required an additional polypeptide stretch between Val 269 and GFP as a (preferably flexible) spacer. Based on
the average pitch of an α-helix (presumed as the worst case for the spacer) of 5.4 Å per winding, we calculated that the inclusion of the 18 adjacent residues N-terminal of Val 269 in the anchor molecule would provide a spacing of at least 27 Å, thus preventing any steric hindrance with capsoid assembly.

In spite of a highly hydrophobic stretch present in the VP2 anchor, the resulting fusion protein GFP-VP2(A) was solubly expressed, showing that solubility is mainly determined by the larger protein domain. Furthermore, binding of the VP2 anchor to the VP1 pentamer was not hindered by GFP, indicating that the crucial hydrophobic patch of VP2 is still accessible. The strength of the hydrophobic interaction between the anchor and the pentamer is thermodynamically comparable to that between proline-rich sequence motifs and the WW domain derived from the mouse formin binding protein 11 (FBP11). Schmidt et al. (26) inserted this WW domain into an inward-facing loop of VP1 in order to bind and encapsulate GFP containing a PPLP ligand. The interaction between the GFP-VP2(A) molecule and VP1, which was not observed between free GFP and VP1, proved the physicochemical polarity of the GFP-VP2(A) fusion protein and the specificity of its binding to VP1. The orderly binding of the anchor moiety of GFP-VP2(A) to the inward-oriented cavity of VP1 pentamers was further confirmed by the virtually unchanged assembly into regularly formed capsoids.

For the coupling of the VP2 anchor with a low molecular weight substance such as MTX, another approach was made. Native chemical ligation (NCL) is a well-established method for covalent and regioselective binding of various substances to recombinantly expressed proteins (28). This reaction affords a free cysteine residue in the molecule to be coupled. Therefore, the new dipeptide linker Boc-Cys(Trt)-Lys-OMe was used allowing an universal and rapid modification of carboxylic acids for NCL. MTX has frequently been coupled to macromolecular carriers such as antibodies or synthetic polymers, using carbodiimides and N-hydroxysuccinimide (NHS) (29). This active ester method, however, does not only lead to the desired MTX α- or γ-isomers, but also to bis-derivatives, MTX oligomers and several by-products, e.g. N-acylisourea derivatives due to rearrangement reactions (30). Especially, the bis-derivatives have to be avoided, since bis(VP2-Cys-Lys-OMe)-MTX would be able to crosslink VP1, presumably causing problems in capsoid assembly. Formation of the described by-products was avoided by modifying a recently published coupling protocol of Riebeseel et al. (31).

In case of loading substances to the interior of VP1 capsoids, an incubation and binding step, respectively, precedes capsoid assembly. The thermodynamic and kinetic stability of the binding of the anchor to the VP1 pentamer is a prerequisite for directed encapsulation of proteins and low molecular weight substances and in the end for an efficient loading of the capsoids. Aside, the sterical properties of the molecule to be encapsulated directly influence the encapsulation capacity. The interior of the capsoids has a volume of about 7150 nm³, which is enough space for a theoretical loading of 408 molecules of GFP. From this point of view, an amount of encapsulation near 100% should be achievable for the GFP-anchor molecule, assuming that a single capsoid is composed of 72 VP1 pentamers and one anchor binds to one pentamer.

In consideration of the determined dissociation constant of the VP1 pentamer anchor complex, a complete loading of the VP1 capsoid should be achievable employing a 10-fold molar access of the anchor. However, under these conditions, capsoid formation was not observed, presumably an indication for sterical influences and/ or an alteration of the VP1 pentamer VP1 pentamer interaction via an allosteric mechanism. Finally, 64 GFP molecules were enclosed in VP1 capsoids, which is lower than achieved using the VP1-WW system (14), but does not require molecular engineering of
VP1 and, therefore, leaves the delivery and cell entry properties of VP1 untouched. Beside loading capsoids with recombinant proteins, the VP2 anchor is distinguished to enable the encapsulation of low molecular weight substances, e.g. MTX. Interestingly, in this case, the loading rate of the VP1 capsoids is significantly higher than in the case of the GFP anchor. 462 MTX anchor molecules could be enclosed into the virus-like particles indicating that besides specific interactions unspecific hydrophobic interactions must occur due to the increased hydrophobicity of the anchor. None-conjugated MTX was not found to be encapsulated showing that the conjugation with the VP2 anchor is crucial for the hydrophobic interaction. Since hydrophobicity is found to be the driving force of the anchor-VP1 interaction and VP1 shows a distinct polarity (charged on the surface, hydrophobic on the inward-facing site) (22), efficient and directed encapsulation of proteins and low molecular weight substances is ensured.

Beyond the mere delivery of a macromolecule like GFP, we wanted to show the effectivity of an encapsulated cytostatic drug in case of intracellular degradation of drug-loaded capsoids after uptake by the cell and release of the drug into the cytoplasm. For this purpose we chose MTX, since it is an inhibitor of cytoplasmic folate dependent enzymes. Furthermore, an MTX transport deficient cell line is available (32) to demonstrate the circumvention of resistance by an alternative uptake route for MTX, as was already shown for an MTX-albumin conjugate with the same cells (33). MTX-albumin was taken up via endocytosis, degraded lysosomally and the liberated MTX caused cytotoxicity in the MTX transport resistant cells, which are usually unaffected by free MTX at the same concentration.

We also tried to gain information about the intracellular fate of GFP- and MTX-loaded capsoids. An approach similar to our model was developed by Guenther et al. (34), who produced VP1 virus-like particles containing a WW domain fused to the N-terminus of VP1 for ligand binding. The VP1-WW virus-like particles containing GFP were efficiently delivered into NIH 3T3 cells, and both capsoids and GFP were found in endocytic vesicles. But only weak co-localization with lysosomes was found, making lysosomal degradation unlikely, although at least a partial release of GFP from the capsoids occurred (34).

A study by Richterova et al. (32) showed the entry of VP1 pseudo-capsoids into NIH 3T6 mouse cells in smooth non-clathrin-coated monopinocytic vesicles in the proximity of larger, caveola-like invaginations. They observed intracellular transport of viral particles in larger endosomes, created by fusions of monopinocytic vesicles with caveola-derived vesicles, in which viral particles might have been partially disassembled. They also found an accumulation of VP1 around the nuclear membrane, but no significant VP1 signal in the cell nucleus. They suggested that only a few intact virions entered the cell nucleus, whereas the majority became disassembled and subsequently degraded in the cytoplasm. We assume that this also applies to most of the internalized GFP- and MTX-loaded capsoids in our experiments.

The release of GFP from the capsoids was shown by the increasing separation of the GFP and VP1/Cy3 fluorescence signals into different vesicular structures 3 hours after uptake, suggesting a kind of sorting mechanism. Endosomal localization of GFP-containing capsoids could affect anti-VP1 antibody binding due to acidification of the endosomes. Thus, the observed phenomenon of augmented separation of the GFP and VP1/Cy3 fluorescence signals could be also attributed to an impaired intracellular staining procedure. However, we can neither confirm nor exclude that functional GFP, or at least the intact chromatophore of the molecule, is released from vesicles. Although we
noticed a diffuse distribution of GFP in the cytoplasm at later time points, these fluorescence signals were hardly distinguishable from GFP-loaded capsoids eventually remaining on the cell surface. Neither GFP nor VP1/Cy3 fluorescence was found in the nuclei. Delivery of encapsulated molecules into the cytoplasm, however, was clearly demonstrated by the cytotoxic effect of MTX-loaded capsoids. The toxicity was time and concentration dependent as expected for an endocytotic uptake mechanism with subsequent endosomal degradation and sufficient release of MTX into the cytoplasm. The high loading rate of the capsoids and the presence of VP2 anchor molecules may account for slight differences in intracellular processing, possibly through subtle changes in the particle surface conformation and/or a contribution of VP2 to viral entry (22). An et al. (35) showed that recombinant virus-like particles assembled from different structural polyomavirus proteins i.e. VP1, VP1/2, VP1/3 or VP1/2/3, were equally internalized into 3T6 mouse cells and localized in the cytoplasm. The decision about the intracellular fate of the particles might be taken during and/or after internalization, when, depending on capsoid composition and structure, different intracellular pathways are addressed or initiated. It seems likely that there is no exclusiveness for one particular pathway, but maybe a shift of preferences occurs.

From an immunological point of view, we present here a device, which is very suitable for the in vivo delivery of full-length proteins like tumour antigens because heterologous polyoma capsoids displaying a CD8 T cell epitope were already able to protect inbred mice from lethal challenge with melanoma cells that express this relevant protein antigen (36). For this reason, these capsoid-based immunotherapeutics may offer new opportunities for the treatment of cancer patients.

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REFERENCES

FIGURE LEGENDS

Fig. 1: VP2-sequence used for construction of molecular anchors VP2(A) and VP2(B) and protection group scheme of the dipeptide linker for the coupling of the VP2 anchor and MTX and the chemical structure of methotrexate (MTX). (A) VP2 sequence from position 251 to 297 of mouse polyoma PY-2a strain (VP2(A)) and mouse polyoma 3 strain (VP2(B)). (B) The Lys-side chain of the dipeptide linker was selectively deprotected by 20% piperidin in DMF. After attachment of MTX to the free amino function both protection groups of Cys are cleaved by treatment with TFA:EtSH:H$_2$O:TIPS = 50:10:4:2 to generate free Cys necessary for native chemical ligation. (C) Methotrexate (MTX).

Fig. 2: Analysis of the interaction between VP1 pentamers and GFP-VP2(A) by immunoprecipitation (IP), SDS-PAGE and Western Blot (WB). VP1-pentamers and a 10-fold molar excess of GFP-VP2(A) or GFP were co-incubated for 4 h at 25 °C (VP1+GFP-VP2(A); VP1+GFP). Incubations of VP1 pentamers, GFP and GFP-VP2(A) served as controls. The samples were immunoprecipitated with monoclonal antibodies against VP1 (anti-VP1) or GFP (anti-GFP), respectively. (A) SDS-PAGE (reduced): Lanes 2-7: IP with anti-VP1. Lanes 12-17: IP with anti-GFP. Lanes 1, 11 and 20: molecular weight standard. Reference samples (ref.): Lanes 8 and 18 - VP1 pentamer (VP1 ref.); lanes 9 and 19 - GFP ref.; lane10 - GFP-VP2(A) ref. (B) Western-Blot analysis: The appropriate samples were immunoprecipitated with anti-VP1 mab #17, blotted and immunostained with polyclonal anti-VP1 antibodies recognizing denatured VP1 or anti-GFP, respectively using a secondary swine α-rabbit antibody detection system. Lane 2, VP1 + GFP-VP2(A); lane 3, VP1 + GFP; lane 4; GFP-VP2(A) reference; lane 5, VP1 reference; lane 6, GFP reference. Abbreviation: WB, Western-Blot.

Fig. 3: Determination of the dissociation constant of the protein complex GFP-VP2(A)/VP1 pentamer by fluorescence polarization. The GFP-VP2(A) concentration was kept constant at 198 nM whereas the VP1 pentamer concentration was increased stepwise to the molar ratio of 1:15. The samples were incubated for 2 h and measured in KB1 buffer at 25 °C. The excitation wavelength was 488 nm, emission was monitored by the averaged signal at 510 nm.

Fig. 4: Assembly of VP1 pentamers to capsoids. (A) Capsoid formation kinetic was initiated by the dilution of KB2 buffer into the protein solution reaching a final ammonium sulphate concentration of 250 mM. The protein concentration was 194 µg/ml (Δ) (0.9 µM VP1 pentamer), 387 µg/ml (□) (1.8 µM pentamer) and 775 µg/ml (○) (3.6 µM pentamer) in KB1 buffer.
buffer. The assembly process was followed by light scattering at 25 °C. Photon correlation spectroscopy (PCS) measurements done at the same temperature at the beginning (B) (corresponds to VP1 pentamers) and at the end of the capsoid assembly process (C) (corresponds to VP1 capsoids).

**Fig. 5: Encapsulation of GFP-VP2(A) in VP1 capsoids.** (A) Photon correlation spectrum of GFP-VP2(A) loaded VP1 capsoids. The molar ratio of GFP-VP2(A) to VP1 pentamer was 7:1. (B) Analytical size exclusion chromatogram of GFP-VP2(A) VP1 capsoids. The absorption was simultaneously recorded at 280 nm (—) and 488 nm (---). Beside the loaded capsoids, a minor amount of free GFP anchor molecules (38% of the entire GFP anchor amount) eluted separately from the column (arrow).

**Fig. 6: Electron microscopy.** Capsoids (A), MTX-loaded capsoids (B) or GFP-loaded capsoids (C) were attached to formvar-carbon coated grids, contrasted by negative staining and visualized by electron microscopy.

**Fig. 7: Fluorescence microscopy of Swiss 3T3 mouse fibroblasts treated with GFP-loaded capsoids and immunocytochemistry of VP1.** Co-localization of GFP- and VP1-Cy3-fluorescence after the attachment of GFP-loaded capsoids at 0 °C to the cell surface without (surface - 0 h ⊕ Triton; A) or with permeabilization by Triton X 100 (surface - 0 h + Triton; D). Uptake of GFP-loaded capsoids was performed for 1 h at 37 °C, followed by immunofluorescence labelling of VP1 by Cy3 (VP1-Cy3) and staining of cell nuclei with DAPI as described under „Experimental Procedures“. In unpermeabilized cells (B and C), VP1-Cy3-fluorescence is restricted to the cell surface (b and c), while GFP is found in intracellular vesicular structures (B and C). Intracellular GFP and VP1-Cy3-fluorescence occurred in permeabilized cells (E and F). VP1-Cy3-fluorescence (e and f) was co-localized with GFP in intracellular vesicular structures.

**Fig. 8: Time course of uptake and intracellular localization of GFP-loaded capsoids combined with immunocytochemistry of VP1.** Co-localization of GFP and VP1-Cy3-fluorescence after the attachment of GFP-loaded capsoids at 0 °C to the cell surface (surface - 0h; A) as described under „Experimental Procedures“. Intracellular localization of GFP (b, c; E) and VP1-Cy3 (bb, cc; F) (arrows indicate areas of insets), and their co-localization in vesicular structures (framed detail in phase contrast; D) after 0.5 hours (intra - 0.5 h; B), 1h (intra - 1 h, GFP/VP1-Cy3; G) or 3 hours (intra - 3 h; C) of uptake at 37 °C. After 0.5 hours of uptake, similar fluorescence intensities for GFP and VP1-Cy3 (B) were found in vesicular structures, whereas at the later time points, the fluorescences of GFP and VP1-Cy3 become partly distributed to different vesicular compartments (C, E, and F).
Fig. 9: Effect of capsoid delivered MTX on CCRF-CEM cells. Dose response assay of CCRF-CEM cells treated with different concentrations of MTX (Fig. 3A), VP2(B)-MTX, capsoids, and MTX-loaded capsoids (Fig. 3B). Viability of cells was determined by a fluorescence assay with Calcein^AM as described under „Experimental Procedures“. Dose response is expressed in growth over concentration curves as % survival compared to the untreated control cells taken as 100%. MTX exhibits cytotoxicity in a predominantly time dependent manner (A). VP2(B)-MTX conjugate and empty capsoids exhibit a low impact on cell viability, whereas MTX-loaded capsoids cause cytotoxicity in a time and partly concentration dependent manner (B). After 48 h 0.02 µM MTX decreases survival to 55.8% compared to untreated control cells (= 100%). 1 µM MTX trapped inside and intracellularly delivered via capsoids is needed to achieve a similar effect of 60.3% survival decrease. Two independently performed experiments (open and solid symbols) are shown both in (A) and (B) (except for VP2(B)-MTX). Results are expressed as means ± SD from n = 4.

Fig. 10: Effect of capsoid delivered MTX on MTX transport resistant CCRF-CEM/MTX cells. CCRF-CEM and CCRF-CEM/MTX cells were treated with capsoids [0.9 mg/ml], [0.02 µM], [1 µM] and MTX-loaded capsoids [0.023 mg/ml capsoids + 1 µM MTX]. Viability of cells after 48 h of incubation was determined by a fluorescence assay with Calcein^AM as described under „Experimental Procedures“. Dose response is expressed in growth over concentration as % survival decrease compared to the untreated control cells taken as 0% decrease. Results are expressed as means ± SD from n = 4. Empty capsoids lead to a minor impairment of growth of 13.1% in CCRF-CEM/MTX cells and 19.5% in CCRF-CEM cells. In MTX-sensitive CCRF-CEM cells 0.02 µM and 1 µM free MTX are cytotoxic and lead to a concentration dependent decrease of survival of 59.0% and 83.1% respectively. In CCRF-CEM/MTX cells treated with 0.02 µM and 1 µM free MTX this decrease is only 14.2% and 22.5%. 1 µM MTX delivered via capsoids leads to a survival decrease of 50.4% in these MTX transport resistant cells, which is comparable to the reduction of 67.2% found in the MTX-sensitive cells.
Efficient intracellular delivery of a protein and a low molecular weight substance via recombinant polyomavirus-like particles
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