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Highly efficient transport of carboxyfluorescein diacetate succinimidyl ester into COS7 cells using human papillomavirus-like particles

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Abstract Human papillomavirus virus-like particles (VLPs) have recently been used to deliver genes into mammalian cells in vitro and in vivo. Here, we investigated whether VLPs may serve as an efficient carrier of low molecular weight compounds (e.g. hormones, vitamins, peptides etc.) into cells. COS7 cells were incubated with recombinant HPV-16L1/L2 VLPs labelled with the fluorescence dye carboxyfluorescein diacetate succinimidyl ester. Using flow cytometry, we demonstrate that labelled VLPs can specifically bind to the cell surface followed by their complete internalisation. Our results indicate that VLPs are promising vehicles for highly efficient delivery of low molecular weight compounds into cells.

Key words: Human papillomavirus; Virus-like particle; Fluorescence labelling; Drug delivery system

1. Introduction

Human papillomaviruses (HPV) are small, non-enveloped DNA viruses comprising a large group with more than 100 subtypes described so far. HPV exclusively infect basal cells of epithelia in skin and mucosa in vivo, causing mild pathogenic lesions such as benign genital warts and more severe diseases like pre-malignant cervical/penile intraepithelial neoplasia and/or invasive carcinoma (reviewed in [1]).

HPV capsids show a $T=7$ eicosahedral symmetry about 55 nm in diameter containing a single molecule of circular double-stranded DNA of approximately 8000 bp in length [2]. Each virion contains 360 molecules of the major capsid protein L1 forming 72 pentamers, the so-called capsomers [3]. In addition, around 12 molecules of the minor capsid protein L2 are included in the virus shell [4].

Upon synthesis in eukaryotic cells using various expression systems, L1 and L2 proteins spontaneously self-assemble into recombinant virus-like particles (VLPs) [5–8]. Comparative studies of native virions and recombinant VLPs produced in *Escherichia coli* [9], yeast [8] and insect cells [5–7] showed that they are similar with regard to the morphological structure and cell binding [10,11]. Therefore VLPs represent a useful model system to study different aspects of the HPV infection cycle, in particular their cell binding and uptake. It has recently been shown that VLPs bind to the cell surface via heparan sulphate followed by their internalisation [12,13].

Earlier, VLPs were used to generate pseudovirions containing non-HPV DNA molecules. Pseudovirion studies demonstrated that different genes of interest can be transferred in vitro in a wide range of cell types by HPV VLPs leading to the production of heterologous proteins [14–17].

In order to elucidate whether VLPs can be used as a general carrier vehicle to transport low molecular weight cargo into target cells in vitro, we chose carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) as a model substrate. After labelling of HPV-16L1/L2 VLPs with CFDA-SE, COS7 cells were incubated with these VLPs. Our results demonstrated that HPV-16L1/L2 VLPs represent a useful carrier system to deliver low molecular weight molecules into mammalian cells.

2. Materials and methods

2.1. Cell culture conditions

All cell culture media and supplements were obtained from Invitrogen (Karlsruhe, Germany). COS7 cells were cultured as adherent monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 1 mM sodium pyruvate at 37°C under 5% CO_2 atmosphere.

2.2. Antibodies

The following antibodies were used: (i) mouse monoclonal antibody (mAb; clone 33L1-7) recognising linear epitope aa 303–313 (ESQLFNKPYWL) of HPV-33 capsid protein L1 [18], (ii) goat anti-mouse IgG horseradish peroxidase-conjugated antiserum (Sigma, Taufkirchen, Germany), (iii) polyclonal rabbit antiserum K75 raised against recombinant HPV-16L1 protein containing neutralising antibodies and (iv) mAb (clone 16L1-56E) raised against HPV-16L1 VLPs recognising the native capsid conformation.

2.3. Production and purification of HPV-16L1/L2 VLPs

VLPs were generated in Sf9 insect cells by co-infection with recombinant baculoviruses carrying the L1 and L2 gene of HPV-16 kindly provided by J.T. Schiller and purified according to Volpers et al. [7].

2.4. CFDA-SE labelling of HPV-16L1/L2 VLPs

Purified HPV-16L1/L2 VLPs were diluted in phosphate-buffered saline (PBS, pH 8.5) to 0.5 mg/ml, and then CFDA-SE (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 10 μM or 100 μM. VLPs were omitted from control reactions. After incubation for 1 h at room temperature in the dark, reactions were subjected to centrifugation in a sucrose density gradient containing 30% (w/w) and 70% (w/v) steps (2 h at 42 000 rpm in a SW60 rotor) to separate VLPs from free CFDA-SE. Under these conditions CFDA-SE remains on the top of the gradient as visualised by fluorescent

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CFSE, which is formed due to spontaneous decay in aqueous solutions. 200 μl fractions were collected from the bottom, aliquots of each fraction were subjected to 12.5% SDS–PAGE and analysed by Western blot ECL detection using primary mAb 33L1-7 (1:500) and horseradish peroxidase-conjugated goat anti-mouse secondary antiserum (1:5000). The integrity of VLP conformation was tested by enzyme-linked immunosorbent assays (ELISA) using heparin-coated and uncoated microtitre plates in combination with mAbs 16L1-56E and 33L1-7 as described by Giroglou et al. [13].

2.5. Cellular uptake assay

The assay was performed according to Unckell et al. [15] with our modifications. Briefly, 1–2 μg of labelled VLPs in PBS (pH 6.8, 0.1 mg/ml bovine serum albumin, BSA) was incubated with 1.5×10^5 COS7 cells either as a monolayer or as a suspension culture for 1 h at 4°C. In the case of monolayer culture, the supernatant containing VLPs was removed; cells were washed twice with PBS followed by incubation at 37°C in DMEM for the indicated periods of time. COS7 cells grown as suspension cultures were treated with VLPs for 1 h at 4°C, then directly transferred into 6-well plates and incubated in DMEM at 37°C for the indicated periods of time.

2.6. Flow cytometric analysis

COS7 cells infected by CFSE-labelled VLPs were washed and resuspended in FACS buffer (2% foetal calf serum in PBS, pH 7.2). Measurements were performed using FACSCalibur (excitation laser frequency was 488 nm) and CELLQuest software (Becton Dickinson, Heidelberg, Germany). Typically, 20 000 events were collected. Recordings were made at green fluorescence (FL1) and data were expressed as histograms.

2.7. Fluorescence microscopy

COS7 cells grown on glass coverslips were incubated with CFSE-labelled VLPs and CFDA-SE for 1 h at 4°C followed by 18 h at 37°C. Cells were washed with PBS and fixed with isopropanol for 30 min. They were examined with a Zeiss 510 laser scan microscope.

3. Results

3.1. Generation and purification of CFSE-labelled HPV-16L1/L2 VLPs

To test whether HPV-16L1/L2 VLPs can effectively deliver low molecular weight compounds into mammalian cells, we used CFDA-SE as a fluorescent marker to label VLPs recombinantly produced in Sf9 cells. After purification of HPV-16L1/L2 VLPs by two subsequent centrifugations in cesium chloride density gradient [7], the fractions were tested using Western blot analysis to confirm the co-assembly of L1 and L2 proteins in VLPs. We demonstrated that both capsid proteins co-migrated at a density of 1.29 g/cm^3 suggesting that L1 and L2 were incorporated into VLPs (data not shown). Two different CFDA-SE concentration (10 and 100 μM) were used to label purified HPV-16L1/L2 VLPs resulting in a covalent amide bond between fluorescence dye and lysine residues of L1 and L2 proteins [19]. The reactive fluorochrome is generated by conversion of carboxyfluorescein diacetate to anionic carboxyfluorescein through intracellular esterases after the entry into cells [19]. Control reactions in the absence of VLPs were performed in addition. To obtain highly purified fluorescent VLPs, non-bound CFDA-SE was separated from labelled VLPs by centrifugation in a sucrose step gradient followed by 12.5% SDS–PAGE. Under the conditions chosen CFDA-SE does not migrate into the gradient whereas Western blot analysis revealed that fractions 5 and 6 from the 30–70% sucrose interface contained a strong 55 kDa band corresponding to L1 protein of VLPs (Fig. 1). Results shown in Figs. 2–5 demonstrate that these VLPs indeed contain the label. The two peak fractions were pooled and used in all subsequent experiments. In addition, the corresponding fractions from the control gradients loaded with CFDA-SE alone were also pooled. Intact conformation of generated labelled VLPs was checked using a heparin–BSA-based ELISA method [13] with mAb 16L1-56E. This experiment clearly showed that the labelled VLPs had a similar reactivity with mAb 16L1-56E as unlabelled ones (data not shown).

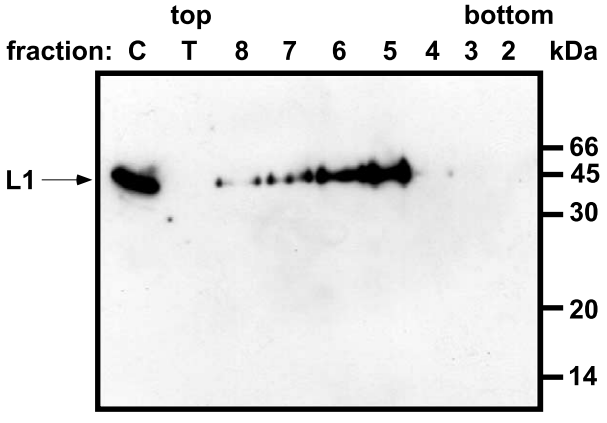

Fig. 1. Purification of CFSE-labelled HPV-16L1/L2 VLPs. Purified CFSE-labelled VLPs were subjected to centrifugation in a sucrose density gradient (30% (w/w) and 70% (w/v) sucrose steps). Aliquots of displayed fractions were separated by 12.5% SDS–PAGE and analysed by Western blotting using the mAb 33L1-7. C, control (HPV-16L1 VLPs); T, top fraction.

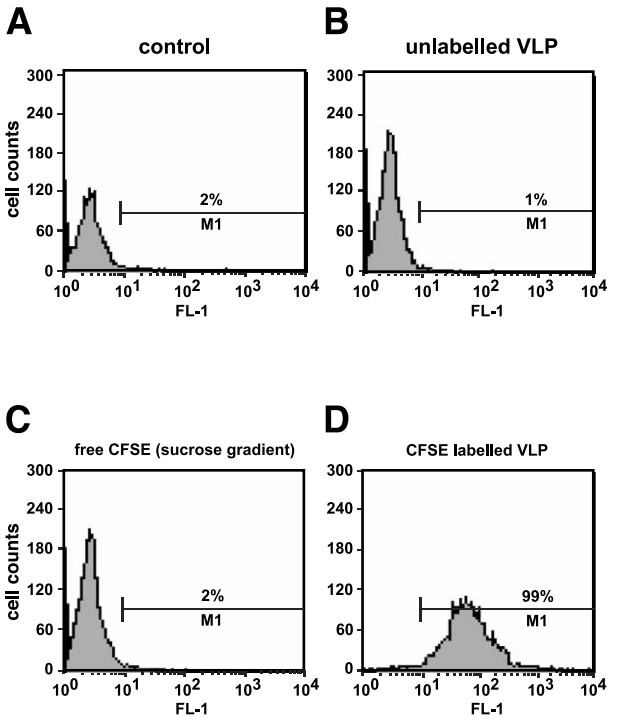

Fig. 2. Flow cytometric analysis of HPV-16L1/L2 VLP association with COS7 cells. COS7 cells were incubated with the indicated compounds in suspension and subsequently analysed by flow cytometry after 18 h. A: Control (autofluorescence of COS7 cells). B: Incubation with 2 μg unlabelled VLPs. C: Incubation with free CFDA-SE (100 μM). D: Incubation with 1–2 μg VLPs labelled with 100 μM CFDA-SE.

3.2. VLP-mediated delivery of fluorescence dye CFSE to COS7 cells

To measure the delivery of CFSE-labelled HPV-16L1/L2 VLPs to COS7 cells, we incubated the cells with CFSE-labelled VLPs first for 1 h at 4°C, then for 18 h at 37°C followed by a flow cytometric analysis. As a negative control, COS7 cells were treated in the same manner with unlabelled VLPs or without them. It was demonstrated that 99% of COS7 cells being cultured with CFSE-labelled HPV-16L1/L2 VLPs were CFSE-positive (Fig. 2D). In negative control probes, only 1–2% cells showed background fluorescence (Fig. 2A,B). These data were reproduced in several independent experiments. Results were essentially the same when 10 or 100 μM CFDA-SE was used for labelling. To exclude the possibility that the determined fluorescence is caused by non-bound CFDA-SE dye co-migrating with the labelled VLPs in fractions 5 and 6 of the sucrose gradient during the purification procedure (see Fig. 1), COS7 cells were also incubated with the corresponding fractions from the control gradient. As shown in Fig. 2C, the number of CFSE-positive cells (2%) is similar to that in negative controls.

From these results we conclude that HPV-16L1/L2 VLPs may be a highly efficient transport vehicle delivering low molecular weight compounds to cells.

3.3. Specific blockage of HPV-16L1/L2 VLP binding to COS7 cells by anti-L1 polyclonal antiserum

To answer the question whether the binding of labelled VLP to cells is a specific process, we performed an inhibition study using different polyclonal antisera. CFSE-labelled HPV-16L1/L2 VLPs were incubated with the neutralising antiserum K75 or with pre-immune serum as a negative control followed by culturing with COS7 cells for 18 h, and flow cytometric analysis. Antiserum K75, which was generated in rabbits against HPV-16L1 VLPs, neutralises HPV-16 pseudoinfection and blocks VLP interaction with the cell surface (data not

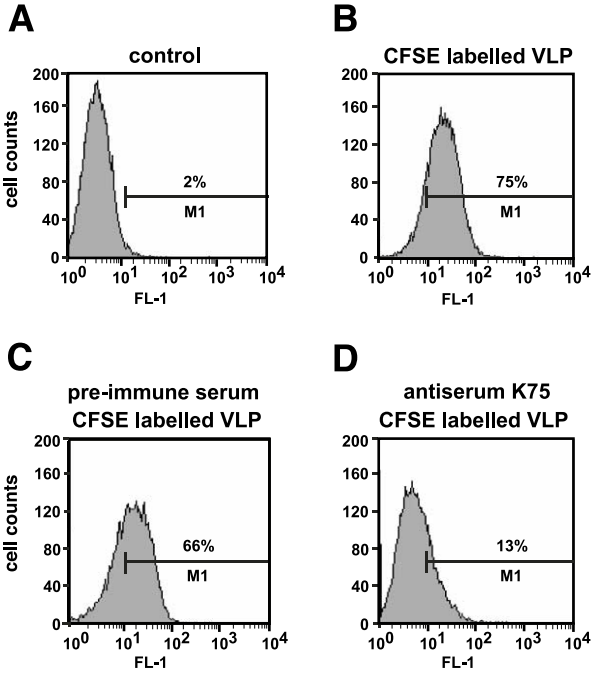

Fig. 3. HPV-16L1/L2 VLP binding is inhibited by VLP-specific antiserum. Approximately 250 ng CFSE-labelled HPV-16L1/L2 VLPs (10 μM CFDA-SE) was pre-incubated for 1 h with the indicated polyclonal antisera (1:100). Then COS7 cells were incubated with these VLPs for 18 h and subsequently analysed by flow cytometry. A: Control (autofluorescence of COS7 cells). B: Incubation with CFSE-labelled VLPs. C: Incubation with CFSE-labelled VLPs pre-incubated with pre-immune serum. D: Incubation with CFSE-labelled VLPs pre-treated with VLP neutralising antiserum K75.

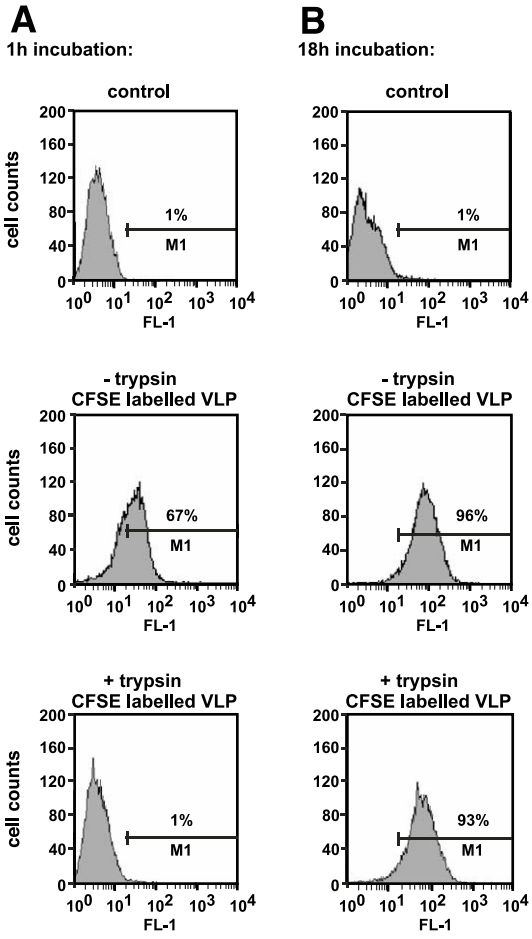

Fig. 4. Effect of trypsin treatment on the uptake of CFSE-labelled VLPs into COS7 cells. COS7 cells were incubated with 1–2 μg CFSE-labelled VLPs (10 μM CFDA-SE) in suspension followed by a flow cytometric analysis. A: After a 1 h incubation at 4°C, the cells were treated as indicated. B: The cells were incubated for 1 h at 4°C, then 18 h at 37°C followed by the treatment as shown. Controls represent the autofluorescence of COS7 cells.

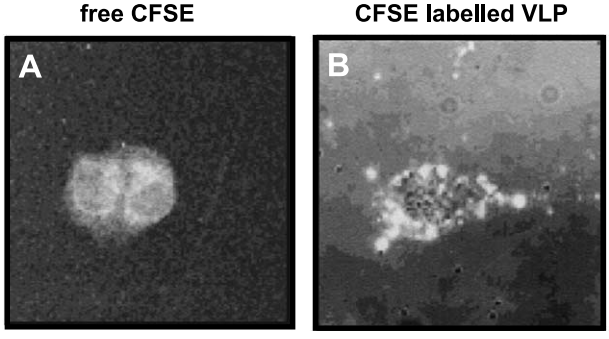

Fig. 5. Uptake of CFSE-labelled VLPs in vesicular structures. Labelled VLPs were incubated with COS7 cells for 1 h at 4°C followed by 18 h at 37°C. A: COS7 cells+free CFSE. B: COS7 cells+CFSE-labelled VLPs. Cells were fixed and analysed by confocal microscopy using a Zeiss 510 laserscan microscope.

shown). Fig. 3 shows one representative experiment out of several independent inhibition tests. Pre-incubation of labelled VLPs with antiserum K75 strongly decreased the number of CFSE-positive cells in comparison to untreated labelled VLPs (from 75 to 13%; mean fluorescence level from 31 to 5%; Fig. 3B,D). In contrast, pre-treatment with the control antiserum caused only slight reduction of the number of CFSE-positive cells (Fig. 3B,C).

Taken together, these data underline the specific binding of labelled VLPs to the cell surface.

3.4. Uptake of CFDA-labelled HPV-16L1/L2 VLPs into COS7 cells

CFDA-SE is stable in aqueous solutions only for a short time since the diacetate ester bonds of CFDA-SE can be spontaneously hydrolysed by water and converted into the active fluorochrome without the action of intracellular esterases. Therefore we could not exclude that a fraction of CFSE-labelled VLPs is activated before entering the cells. To prove that CFSE-labelled VLPs are indeed internalised into COS7 cells, protease protection experiments were performed. COS7 cells were incubated for 1 h at 4°C with labelled VLPs followed by removal of unbound VLPs. The cells were then either directly treated with trypsin or incubated for 18 h at 37°C followed by protease treatment. As shown in Fig. 4A, trypsin addition after 1 h incubation at 4°C resulted in a reduction of the number of CFSE-positive cells to the background level (from 69 to 1%). In contrast, after 18 h incubation the fluorescence label was resistant to trypsin (95 and 97% CFSE-positive cells with and without protease treatment, respectively; Fig. 4B). These data suggest that the vast majority of VLPs were internalised by the cells and no longer served as a substrate for the extracellularly added trypsin.

Intracellular localisation of the labelled VLPs was confirmed by confocal laser microscopy. COS7 cells treated with CFSE-labelled VLPs and free CFDA-SE dye, respectively, showed that labelled-VLPs are localised in vesicular structures, whereas free CFDA-SE is homogeneously distributed in the cell (Fig. 5).

In summary, these data underline the uptake of VLPs by mammalian cells and their possible use as a carrier vehicle to introduce low molecular weight compounds into cells.

4. Discussion

In the last 10 years many virus-based gene transfer systems have been established and used in different approaches for DNA vaccination and gene therapy in vitro and in vivo (reviewed in [21]). Recently papillomavirus VLPs of different genotypes consisting of capsid proteins L1 and L2 [15,17] or only L1 [18] were packed with non-viral DNA and successfully transferred into a wide variety of cell types in vitro and in vivo. In addition, papillomavirus VLPs consisting of a fusion between the major capsid protein L1 and proteins or peptides of interest (chimeric VLPs) were applied in prophylactic and therapeutic vaccination studies [20,22]. However, little is known about the field of application of papillomavirus VLPs to transport low molecular weight substances into target cells in vitro and in vivo. Successful encapsidation of fluorescence dye propidium iodide into polyomavirus VLPs was recently shown by Goldman et al. [23], but the transfer of generated fluorescence particles into the cells was not investigated. Using CFDA-SE as a test molecule, we have now demonstrated that VLPs can serve as an efficient carrier for uptake of low molecular weight compounds into cells.

After incubation of COS7 with CFSE-labelled VLPs, we measured their internalisation by flow cytometry. Our results demonstrate (i) a specific binding of labelled VLPs to the cell surface and (ii) an efficient uptake of fluorescently labelled particles into COS7 cells. Attachment of HPV-11L1 VLPs, HPV-16 and -33 pseudovirions to the cell surface was shown to be mediated by an interaction between the major capsid protein L1 of the viral particle and heparan sulphate molecules exposed extracellularly [12,13]. It is very likely that the labelled particles also use proteoglycans for their cell attachment. It is not known so far whether these cell surface molecules also directly mediate the uptake of VLPs or whether transfer to a secondary receptor is required.

Using a neutralising polyclonal antiserum, we were able to inhibit the specific cell binding of labelled VLPs which was reflected by a strong decrease of CFSE-positive cells (Fig. 3). Since our experimental system could not distinguish between interaction of VLPs with the cell surface and their uptake into the cells, we performed protease protection experiments. During the 1 h incubation at 4°C, CFSE-labelled VLPs were bound extracellularly to their primary receptor and did not enter the cells because of endocytosis blockage at 4°C [14]. In contrast, all fluorescent particles were resistant to trypsin treatment after incubation for 18 h at 37°C suggesting an entry of CFSE-labelled VLPs into target cells.

Therefore, HPV VLPs could be an efficient alternative to commonly used synthetic polymer- and liposome-based delivery systems sharing with the latter such properties as (i) a high transfer rate, (ii) a target specificity, and (iii) a protection against degradation (reviewed in [24]). Importantly, the target specificity of HPV in vivo to epithelial cells of the skin and mucosa [1] offers the opportunity to use these carriers as an attractive transepithelial delivery system.

Taken together, the data presented in this report suggest that HPV VLPs are promising vehicles for delivery of biologically active low molecular weight compounds into mammalian cells.

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