Protein and peptide delivery via engineered polyomavirus-like particles[1]

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SPECIFIC AIMS

A system for the delivery of peptides and proteins into eukaryotic cells based on the directed encapsidation of polyproline-tagged compounds into modified polyomavirus-like particles was developed. The properties of capsomeres fused to a WW domain were investigated with respect to their physicochemical properties, ligand encapsidation, and uptake into mammalian cells.

PRINCIPAL FINDINGS

1. Polyomavirus-like particles can encapsidate protein ligands

The inner surface of capsids was modified in order to achieve a specific interaction of the capsomeres with the ligands. During in vitro assembly, this interaction results in encapsidation of ligands like proteins and peptides into the forming polyomavirus-like particles. The first WW domain of the mouse Formin binding protein 11 (FBP11) was fused to the NH_2 terminus of VP1 (which faces the interior of the capsid) as a module to facilitate this interaction. WW domains are small protein domains that were named after two conserved tryptophan residues that are essential for maintenance of the native fold and for ligand binding. The FBP11 WW domains specifically bind proline-rich ligands that bear the consensus motif PPLP.

Protein modeling studies of the VP1-WW fusion protein revealed there is sufficient space inside the capsid to allow proper folding and arrangement of the WW domains. Given that there are 360 ligand binding sites (72 pentameric VP1 molecules per capsid), it was calculated that 360 globular proteins with an average size of up to 17 kDa could theoretically be encapsidated.

2. VP1-WW capsomeres obtain a native fold and bind PPLP sequences

The fusion protein VP1-WW, expressed and purified from recombinant *Escherichia coli* in a soluble form, was characterized with respect to correct protein folding and ligand binding of the WW domain. To confirm the native fold, far UV circular dichroism spectra of the VP1-WW fusion protein were recorded and compared to the spectrum of the wild-type protein. The VP1-WW spectrum had a significantly increased negative ellipticity difference ($\Delta\varepsilon$) below 207 nm, indicating an additional portion of β-sheet secondary structure as expected from the addition of the three-stranded antiparallel β-sheet of the WW domain. The difference spectrum of VP1-WW minus VP1 represents the spectrum of the single WW domain. The integrity and functionality of the fused WW domain were further verified by surface plasmon resonance using an immobilized PPLP peptide ligand. Determination of the kinetic parameters yielded an equilibrium dissociation constant $K_d = 40 \pm 5$ nM. These results are in good agreement with earlier surface plasmon resonance data using a fusion protein of glutathione-S-transferase and the FBP11 WW domain. The observed features (i.e., correct protein fold and specific binding of PPLP ligands) are important prerequisites for a directed encapsidation of PPLP-tagged peptides and recombinant proteins into the virus-like particles.

3. VP1-WW capsomeres encapsidate PPLP-tagged peptides and proteins during in vitro assembly

Either fluorescence-labeled PPLP peptides or PPLP-tagged green fluorescent protein (GFP) were used as model ligands for optimization of their encapsidation. Encapsidation rates were determined by size exclusion chromatography, which separates virus-like particles from free capsomeres and free ligands (**Fig. 1**). The absorptions of the proteins at 280 nm and of the chromophores at 490 nm and 583 nm, respectively, were recorded simultaneously; after integration of the

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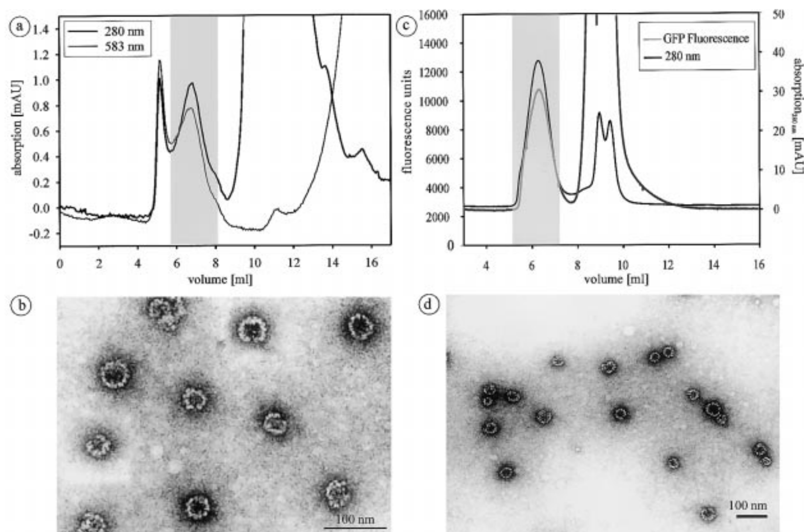

Figure 1. In vitro assembly and ligand encapsidation of VP1-WW. Size exclusion chromatography profiles show that capsids contain the model ligands, either fluorescence-labeled PPLP peptide (*a*) or GFP (*c*). Electron micrographs reveal a homogeneous population of PPLP peptide (*b*) or GFP (*d*) containing capsids that are indistinguishable from wild-type capsids.

respective peak areas, the in vitro assembly efficiencies and the encapsidation rates were calculated. It was found that the maximum encapsidation rate was 230 peptide molecules and 260 GFP molecules per virus-like particle.

4. Model peptides and proteins are efficiently delivered into NIH 3T3 cells

The ability of polyomavirus-like particles to transfer encapsidated molecules into eukaryotic cells was examined in tissue cultures in vitro. Cultures of NIH 3T3 mouse fibroblasts were incubated with virus-like particles containing either fluorescence-labeled peptides or PPLP-tagged GFP and subsequently analyzed using confocal laser scanning microscopy. Significant fluorescence was detected within all cells even after 30 min incubation time (**Fig. 2**). The fluorescent molecules were localized near the cellular membrane and eventually were distributed more throughout the entire cells with longer incubation times, indicating that encapsidated ligands were rapidly and efficiently delivered.

To determine the distribution of capsids and encapsidated ligands individually, the capsid protein VP1 was labeled with a different fluorescence dye at a specific cysteine residue in addition to the fluorescent ligands. During uptake experiments into NIH 3T3 cells, the capsid proteins and ligands could be clearly detected and distinguished. Within the time frame of the experiments, most of the ligands were still colocalized with the capsid proteins (Fig. 2). However, there was also a portion of noncolocalized fluorescence indicating that at least partial release of the ligands from the capsids occurred within the period of the experiment. Although capsids and ligands were also found in endocytotic vesicles, simultaneous staining of lysosomes revealed only weak colocalization with the peptides. Therefore, lysosomal degradation of the ligands seems unlikely.

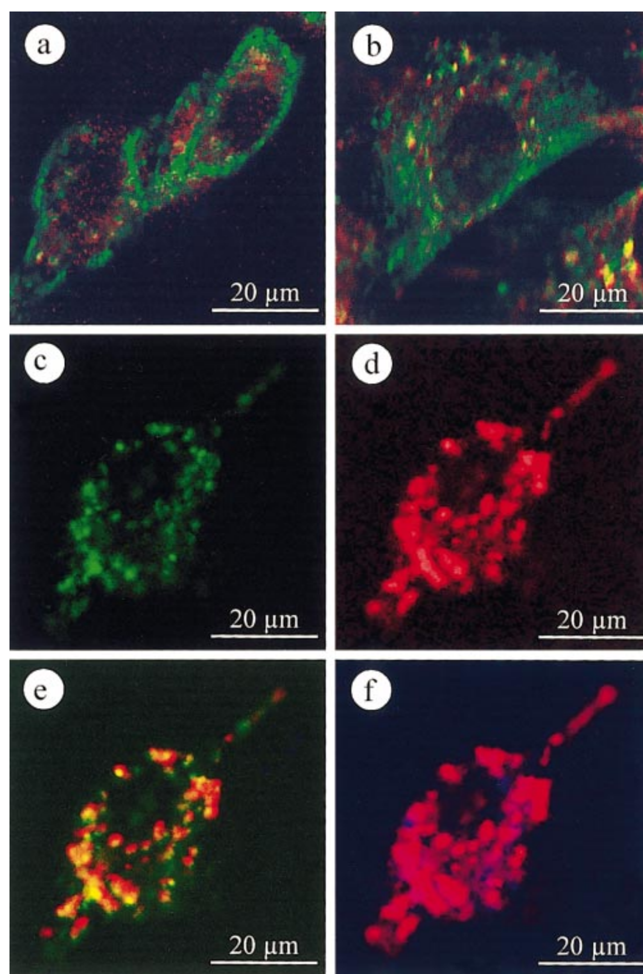

Figure 2. Delivery of fluorescence-labeled PPLP peptide into NIH 3T3 cells. *a)* The peptide-loaded particles are found mainly in the membrane area and the outer cytoplasm. *b)* After further incubation, the particles migrate mainly into the cytoplasm and are in part taken up into lysosomes, which results in yellow staining due to the colocalization of blue (lysosomes) and green (ligand inside capsids) dyes. Differential staining of capsids (c), ligands (*d*), and lysosomes (*f*, blue) reveals colocalization of ligands and capsids (*e*) and partial uptake of the loaded capsids into lysosomes (*f*).

CONCLUSIONS

With an increasing understanding of the pathogenesis of many diseases at a molecular level, it is possible to use recombinant proteins for the functional substitution of defective or missing proteins or for the modulation of metabolic or signal transduction pathways. However, most proteins are unable to enter cells and reach intracellular targets, and are therefore limited to extracellular applications. Recently, fusions of target proteins with viral protein sequences of the HIV TAT and the Herpes simplex virus VP22 were found to mediate transfer of the target protein across the plasma membrane into cells.

In a novel approach, we used polyomavirus-like particles that are able to enter mammalian cells for the delivery of peptides and proteins. The icosahedral viral shell of murine polyomavirus is composed of 72 pentameric subunits of the major capsid protein VP1 and an inner layer of the minor capsid proteins VP2 and VP3. The outer capsid protein VP1 can be expressed and purified from recombinant *E. coli* and spontaneously forms virus-like particles in vitro in the presence of Ca^{2+} ions without the need for VP2 or VP3.

The major focus of the present study was the directed encapsidation of model ligands into the particles. We focused on peptides and recombinant proteins as prospective therapeutic molecules and demonstrated that capsomeres fused to a WW domain can be used for an efficient directed encapsidation of peptides and proteins tagged with a proline-rich sequence (**Fig. 3**). The observed maximum encapsidation rates of fluorescence-labeled PPLP peptide (230) and GFP (260) were significantly higher than the statistical inclusion of less than three molecules calculated for the capsid and ligand concentrations used here, although the theoretical limit of encapsidation was not reached, probably due to a fast association/dissociation equilibrium between the proline-rich ligands and the WW domain.

Cell culture experiments demonstrated an efficient uptake of the vector and the delivery of the encapsidated substances. There is approximately an equal distribution of the ligands within the cytoplasm and ligands remaining in endosomes. Since there is not much known about endosomal release of polyomavirus-like particles, these observations cannot be fully explained yet. For the native virus, myristylation of the minor coat protein VP2 may have a crucial function for endosomal release; empty wild-type polyomavirus-like particles consisting only of VP1 are mostly targeted to lysosomes.

The drug delivery system presented here should be applicable in general for the delivery of a wide range of peptides, small PPLP-tagged compounds, and proteins. Possible limitations for the encapsidation of proteins could arise for large proteins, which may decrease the in vitro assembly of the capsids due to steric hindrance. This effect could possibly be avoided by reducing the number of binding sites per particle using mixed capsids (Fig. 3).

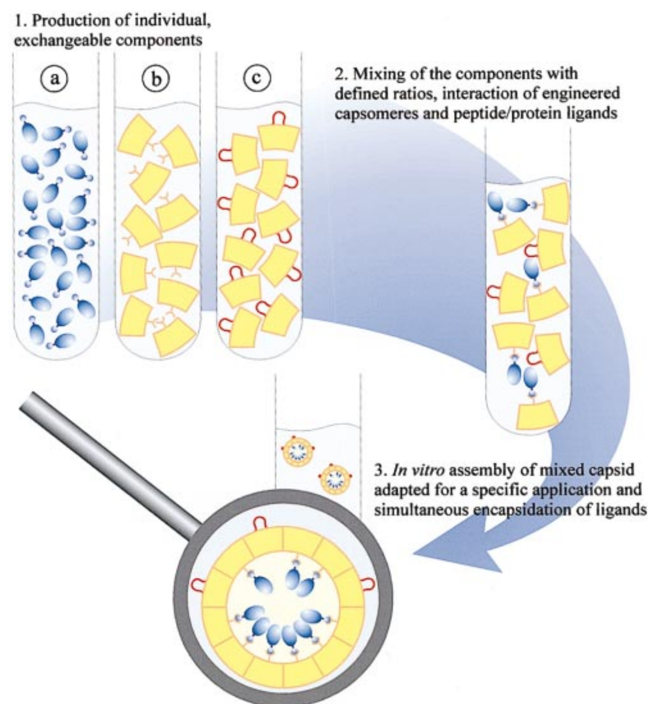

Figure 3. Schematic diagram of peptide/protein delivery using virus-like particles. All components are produced separately, including the PPLP-tagged therapeutic peptide or protein (*a*), VP1-WW capsomeres (*b*), and VP1 capsomeres with additional functions (*c*)—for example, sequences to target the assembled particles to specific cellular receptors. The components are then mixed and virus-like particles are generated by in vitro assembly, yielding tailored therapeutics.

Compared to fusion proteins with viral sequences, encapsidation into virus-like particles has the advantage that the ligands are protected inside a stable protein shell from external proteases. The strategy of mixed capsid assembly may also be exploited to combine different functions within the vector (Fig. 3). For example, capsomeres that facilitate encapsidation of the ligands could be combined with capsomeres modified on the outer surface so as to allow targeting of specific cell types. It is also possible to fuse other WW domains to the VP1 capsomeres that recognize different proline-rich sequences. During in vitro assembly with varying VP1-WW fusion capsomeres, different ligands could be encapsidated and administered simultaneously with an exactly defined ratio. For example, emerging resistance of tumor tissue to a single drug could be circumvented by applying a mixture of diverse drugs.

In summary, it is envisaged that engineered virus-like particles as a basis of a modular delivery system may greatly enhance the use of therapeutic peptides and recombinant proteins. Such multifunctional drug delivery systems may be useful for treating human diseases. Future studies will focus on the delivery of biologically active compounds and their distribution and effects in tissue and animal models. FJ