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Binding of external ligands onto an engineered virus capsid

Uli Schmidt[1,3,4], Rainer Rudolph[1] and Gerald Böhm[1,2]

[1]Institut für Biotechnologie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Str. 3, 06120 Halle and [2]ACGT Progenomics AG, Weinbergweg 22, 06120 Halle, Saale, Germany

[3]Present address: WAIMR Cancer Biology Division, Institute for Child Health Research, 100 Roberts Road, Subiaco, WA 6008, Australia

[4]To whom correspondence should be addressed.
E-mail: ulis@ichr.uwa.edu.au

The development of novel delivery systems for therapeutic substances includes targeting of the carriers to a specific site or tissue within the body of the recipient. This can be accomplished by appropriate receptor-binding domains and requires linking of these domains to the carrier. We have used recombinantly expressed polyomavirus-like particles as a model system and inserted the sequence of a WW domain into different surface loops of the viral capsid protein VP1. In one variant, the WW domain maintained its highly selective binding properties of proline-rich ligands and showed an increased affinity but also an accelerated association/dissociation equilibrium compared to the isolated WW domain, thus allowing a short-term coupling of external ligands onto the surface of the virus-like particles.
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Introduction

In recent years, there have been intense efforts to generate targetable, injectable vectors for gene and drug delivery based on a variety of viral and non-viral systems (Anderson, 1998). It is envisaged that the ideal targeted vector would be administered by intravenous infusion or injection, and then concentrates in the tissues and organs harboring the targeted cells (Peng and Russel, 1999). Strategies to achieve a cell-type specific targeting often use direct genetic modification of the coat proteins with receptor-binding domains or sequences, e.g. the insertion of integrin-binding RGD sequences into an adenoviral capsid (Wickham *et al.*, 1997; Vigne *et al.*, 1999). Another approach is the use of soluble bifunctional crosslinkers that bind both to the vector and to a cell-surface receptor (Douglas *et al.*, 1996). A combined approach is to display an immunoglobulin-binding domain of protein A on the vector as a genetic fusion to the coat protein, and then use a monoclonal antibody to crosslink the vector with the targeted cell (Ohno and Meruelo, 1997; Ohno *et al.*, 1997). Here, we investigate a more general strategy using a genetic fusion of a viral capsid protein with a WW domain, in order to bind any recombinantly produced polyproline-tagged protein which can be, for example, an antibody or a receptor-binding domain.

Surface loops of viral capsid proteins often show a high sequence variability due to the influence of the immune system in viral life-cycles which makes these loops susceptible to the insertion of foreign sequences (Stirk and Thornton, 1994). Recombinantly expressed polyomavirus-like particles were used as a model for a viral protein shell. The outer capsid protein VP1 of murine polyomavirus can be expressed from recombinant *Escherichia coli* (Leavitt *et al.*, 1985; Schmidt *et al.*, 2000; Stubenrauch *et al.*, 2000). It is a pentameric protein which forms virus-like particles *in vitro* consisting of 72 pentamers (Salunke *et al.*, 1986; Salunke *et al.*, 1989). The *in vitro* assembly process was studied in great detail and is thought to involve a pre-existing equilibrium between free capsomeres and capsids which is completely shifted to capsids upon oxidation of a single disulfide bridge (Schmidt *et al.*, 2000). The crystal structures of the capsid and of truncated pentameric VP1 have been reported (Stehle *et al.*, 1994; Stehle and Harrison, 1996, 1997). Polyomavirus VP1 gained attention for the *in vitro* packaging of plasmid DNA and oligonucleotides for the development of non-viral gene transfer vectors (Forstova *et al.*, 1995; Braun *et al.*, 1999).

In order to specifically bind proline-rich ligands onto the outer surface of VP1 capsids, the sequence of a WW domain was genetically fused into β-turns of VP1. WW domains are the smallest protein domains known so far and were first discovered in the Yes-kinase associated protein (YAP) of *Saccharomyces cerevisiae* (Bork and Sudol, 1994; Sudol *et al.*, 1995). They were named after two conserved tryptophan residues which are essential for the maintenance of the native fold and for ligand binding (Koepf *et al.*, 1999). Until now, WW domains were found in several proteins of different species where they contribute to signal transduction processes or to protein–protein interactions in general. In analogy to SH3 domains, WW domains bind proline-rich peptide sequences which differ in their consensus sequences (Pawson and Scott, 1997). WW domains can either be subdivided into four classes according to their binding specificity (Bedford *et al.*, 2000) or into three subclasses according to sequence similarity (Macias *et al.*, 2000). The NMR structures of WW domains representing all three sequence subtypes have been reported, including the YAP WW domain in complex with a proline-rich peptide (Macias *et al.*, 1996) and the FBP28 and YJQ8 WW domains (Macias *et al.*, 2000). The WW domain contains a three-stranded antiparallel β-sheet; the proline-rich peptide is mostly bound by hydrophobic interactions. In this study the first of two WW domains of the mouse formin-binding protein 11 (FBP11) was used which binds PPLP-motifs (Chan *et al.*, 1996). The affinity of this WW domain to its ligand is the highest reported so far for WW domains. Its equilibrium dissociation constant K_D is 21 nM (Bedford *et al.*, 1997).

Materials and methods

Protein modeling

The structure of VP1-WW150 was modeled using the program Modeller 4 (Sali and Blundell, 1993) based on the published structures of VP1 at 1.9 and 3.65 Å (Stehle and Harrison, 1996, 1997). The inserted FBP11 WW domain was modeled

using the NMR structure of the YAP WW domain (Macias *et al.*, 1996). Figures were generated by MolScript (Kraulis, 1991) and Raster3D (Merritt and Bacon, 1997).

Cloning and vector construction

The first FBP11 WW domain was genetically fused into the VP1 sequence as shown in Figure 2 using the oligonucleotides FBP11-WWa-5′ (5′-ATACTCTTCA GGCAGCGGCT GGACAGAACA TAAATCACCT GATGG-3′), FBP11-WWa-3′ (5′-ATACTCTTCT ACCACTACCA TCATCCGGCT TTTCCCAGGT AGACTG-3′), VP1-150-WWaC (5′-ATACTCTTCA GGTAGCGGCG TAAACACAAA AGGAATTTCC ACTCCAG-3′), VP1-150-WWaN (5′-ATACTCTTCAGCCGCTGCCTGTATCTGTCGGTTTGTTGAACCCATG-3′), VP1-292-WWaC (5′-ATACTCTTCA GGTAGCGGCG TTACAAGAAA CTATGATGTC CATCAC-3′), VP1-292-WWaN (5′-ATACTCTTCA GCCGCTGCCC CAGCCCATTA TATCTACGCT CGAG-3′), VP1-Nde I-5′ (5′-TATACATATG GCCCCCAAAA GAAAAAGC-3′) and VP1-Sma I-3′ (5′-ATATCCCGGG AGGAAATACA GTCTTTGTTT TTCC-3′). The resulting final PCR product was cloned via introduced *Nde*I and *Sma*I restriction sites into the plasmid pET21-Int which contained a T7lac-promoter for high level expression in *E.coli*, and a C-terminal fusion with an intein and a chitin-binding domain for affinity chromatography (Schmidt *et al*., 2000).

Protein expression and purification

All proteins were expressed from recombinant *E.coli* as C-terminal fusion proteins with modified intein and chitin-binding domains and were purified as described before (Chong *et al.*, 1997; Schmidt *et al.*, 2000).

In vitro assembly and size-exclusion chromatography

Particles were assembled *in vitro* according to Salunke *et al.* (Salunke *et al.*, 1986, 1989). For the binding onto virus-like particles the proline-rich ligands were added after completion of the assembly process. The capsid assembly was quantitatively analyzed by size-exclusion chromatography using 14 ml TSKgel 5000/6000PW_{XL} columns (Tosoh Biosep, Stuttgart, Germany) as described before (Schmidt *et al.*, 2000).

Surface plasmon resonance

In order to determine the affinity of the VP1-WW fusion proteins to polyproline sequences, surface plasmon resonance was measured using a Biacore X (Biacore AB, Uppsala, Sweden) and a CM5 sensorchip which was coated with PPLP peptide (sequence: $CSGP_6PPLP$) following the manufacturer's protocol. The protein concentrations were varied between 5 and 50 nM. For the screening of different buffers and additives the protein solution was diluted in the buffer which was also used for the measurement. Kinetic parameters were calculated with the BIAevaluation software using a simple Langmuir binding model.

Circular dichroism (CD) spectroscopy

Far-UV CD spectra of VP1 variants were measured from 195 to 260 nm in 0.1 mm cuvettes. The proteins (concentration: 0.5 to 1.0 mg/ml) were dialyzed against a buffer containing 10 mM HEPES, 100 mM NaCl, pH 7.2. The secondary structure contents of the proteins were calculated with the program CDNN (Böhm *et al.*, 1992) from the buffer-corrected spectra.

Electron microscopy

For electron microscopy studies, an EM 912 instrument (Zeiss) was used with a magnification factor of 63 000. Staining of the specimen was performed with uranyl acetate on bacitracin-incubated (0.1 mg/ml, 1 min) copper–carbon-grids according to standard protocols.

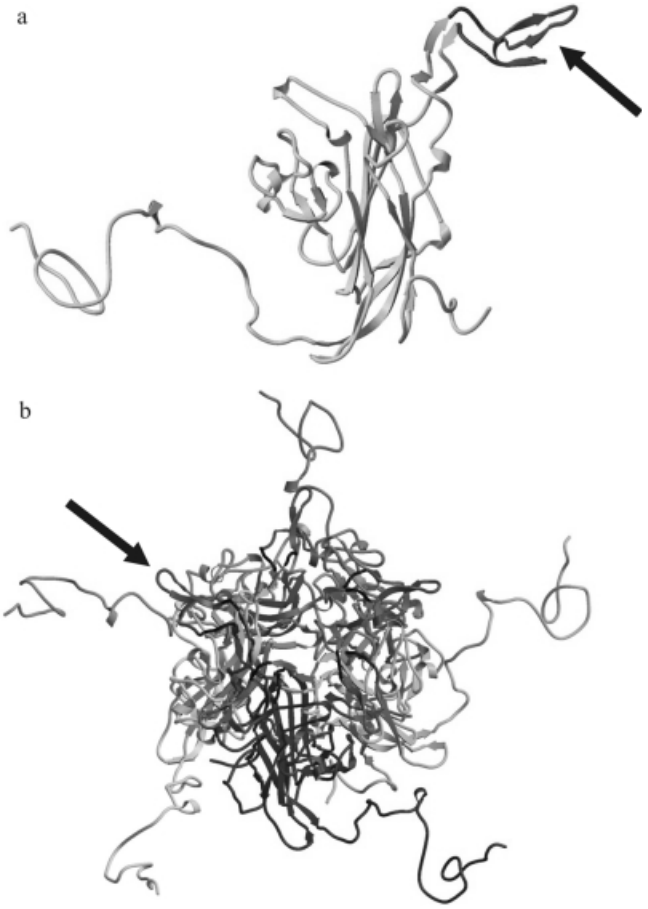

Fig. 1. Model structure of VP1-WW150: (**a**) monomeric subunit and (**b**) pentameric capsomere. The WW domain is represented in purple and the serine/glycine-linker in blue. The WW domain folds towards the central hole of the capsomere and does not interfere with the pentameric structure.

Results

Protein design and model building

Two variants of the fusion protein were designed for the presentation of the WW domain on the surface of polyomavirus-like particles. The first incorporates the WW domain in the DE loop at position 150 in the VP1 sequence and the second in the HI loop at position 292. Both loops are well accessible from the outer surface and are flexible according to the temperature factors determined from crystallographic data (Stehle and Harrison, 1996). These positions are also distant from the CD loop and C-terminal sequences which are required for the formation of virus-like particles. Therefore, insertions at these positions should not inhibit the formation of virus-like particles. A sequence alignment did not reveal any conservation in those regions between polyomavirus strains from different species (data not shown). The WW domain insert was flanked by spacers of five amino acids consisting of serine/glycine repeats to allow maximal flexibility and solubility. The overall size of the inserted sequence was 38 amino acids. A model of the VP1-WW150 structure is presented in Figure 1.

Fig. 2. Cloning of the fusion constructs VP1-WW150 and VP1-WW292. The strategy avoids changes in the original sequences and involves the ligation and reamplification of three separate fragments that were generated by PCR. The recognition sequence of the endonuclease *Eam*1 104 I is printed in bold letters.

Cloning and protein expression

For the genetic fusion of the WW domain into the VP1 sequence, PCR products with overhangs containing the serine/glycine linker sequences and recognition sites for the type IIs restriction endonuclease *Eam*1 104 I were generated (Figure 2). *Eam*1 104 I cleaves outside its recognition sequence and therefore allows ligation of fragments without the introduction of suitable restriction sites.

The expression of both proteins as C-terminal fusions with intein and chitin-binding domains allowed a single-step purification (Chong *et al.*, 1997; Schmidt *et al.*, 2000) and yielded approximately 6 mg of purified protein per liter of culture medium.

CD analysis reveals increased β-sheet contents

Far-UV CD spectra were recorded and compared with the authentic VP1 in order to verify the native fold of the VP1-WW fusion proteins (Figure 3). The spectra of VP1 and VP1-WW292 had similar shapes, whereas the VP1-WW150 spectrum had a significantly increased negative ellipticity difference (Δε) below 207 nm, indicating a higher portion of β-sheet secondary structure. Secondary structure deconvolution of the spectra revealed an increase of antiparallel β-sheets in VP1-WW150 and VP1-WW292, which was, however, significantly higher for VP1-WW150. The difference spectra of VP1-WW150/VP1-WW292 minus VP1 should represent the spectrum of the single WW domain (Figure 3). The difference CD spectrum of VP1-WW150 had a maximum at 225 nm and an intense negative ellipticity at 198 nm. This spectrum corresponds to a typical WW domain spectrum that is shifted approximately 5 nm towards shorter wavelengths (Macias *et al.*, 2000), indicating that the WW domain obtains its native fold in the fusion protein VP1-WW150. In contrast, the difference spectrum of VP1-WW292 showed a much smaller amplitude, suggesting that the WW domain is not or only partially folded in this construct.

Ellipticity changes during thermal denaturation yielded a T_m of 51°C for the VP1 capsomer (Figure 3). The thermal transition midpoint for VP1-WW150 was lowered by 6°C to 45°C. This value corresponds well to the typical midpoint transition temperature determined for a prototype WW domain (44°C; Macias *et al.*, 2000). The denaturation of VP1-WW292 did not have a sigmoidal shape and began to decrease already at 40°C and increased again beyond 60°C, probably due to aggregation followed by sedimentation of the protein that may be caused by large unfolded regions in the protein. The starting aggregation at 40°C was therefore set to be the starting point of thermal denaturation of VP1-WW292.

VP1-WW150 specifically binds proline-rich ligands

For an analysis of the binding properties of the inserted WW domains, a proline-rich peptide with the sequence $^{+}H_3N$-CSGP$_6$PPLP-COO^{-} was immobilized via its N-terminal cysteine residue on the surface of a sensorchip for surface plasmon resonance measurements. This peptide contains the PPLP consensus motif that makes it an ideal ligand for binding of the FBP11 WW domain used in this study (Bedford *et al.*, 1997). The sensorchip was tested with a linear construct of the WW domain as an N-terminal fusion with glutathione-*S*-transferase (GST-WW). For this protein, the equilibrium dissociation constant K_D was determined to be 18 nM (Table I), in good agreement with the value of 21 nM published earlier (Bedford *et al.*, 1997).

The kinetic parameters of the interaction of the WW domains within the VP1 sequence with the peptide were determined with serial dilutions of the fusion proteins under different buffer conditions. VP1 without a WW domain did not interact with the sensorchip surface or with the immobilized peptide (data not shown). The protein VP1-WW292 did not bind to the ligand under any condition tested so far, indicating that the WW domain in this construct did not obtain a native fold or had a dramatically lowered affinity which, therefore, could not be measured using surface plasmon resonance.

However, VP1-WW150 showed a high affinity for the ligand (Figure 4). Using the same conditions as for GST-WW, the equilibrium dissociation constant K_D of VP1-WW150 was increased to 7.7 ± 5 nM (Table I). VP1-WW150 exhibited a high affinity for the proline-rich ligand in the range of K_D = 4–15 nM under all buffer conditions tested. In order to mimic a cell culture or physiological system, the measurement was carried out in phosphate buffered saline (PBS) and in Dulbecco's modified Eagle cell culture medium supplemented with 10% fetal calf serum. The serum proteins did not compete with the binding sites on the VP1 surface and their presence did not inhibit the specific binding of VP1-WW150 to its PPLP-ligand, a prerequisite for therapeutic applications. Although the equilibrium constants were similar for the inserted and the linear WW domain, the interaction of the inserted WW domain is accompanied by an accelerated exchange of the ligands (Table I). Association and dissociation reactions (represented by the association/dissociation rate constants k_a and k_d in Table I) of VP1-WW150 were approximately 10 times faster than the respective values of GST-WW.

Altered in vitro assembly properties of VP1-WW fusion proteins

An essential function of VP1 is its ability to form virus-like particles *in vitro*. For easier handling, the VP1-WW fusion

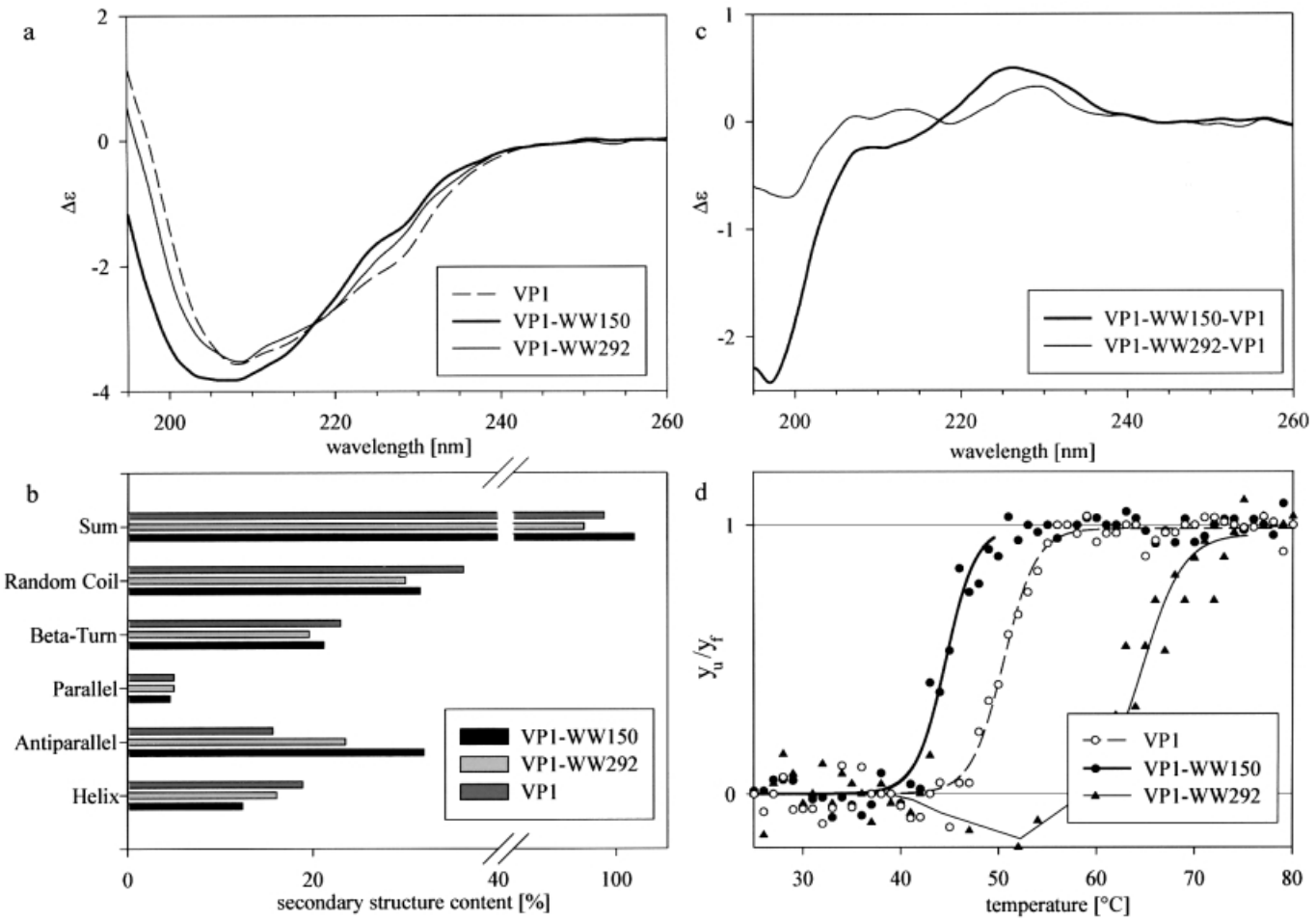

Fig. 3. Circular dichroism analysis. (**a**) CD spectra of pentameric VP1, VP1-WW150 and VP1-WW292. (**b**) Deconvolution with the program CDNN (Böhm *et al.*, 1992) indicates an increased secondary structure content of antiparallel β-sheet in the fusion constructs. (**c**) Difference spectra of VP1-WW150/VP1-WW292 minus VP1, representing the spectra of the WW domains in the fusion proteins as described in the literature. (**d**) Thermal denaturation experiments demonstrate that the thermal stability of VP1-WW150 is 6°C lower than that of VP1 (51°C). VP1-WW292 starts to aggregate at 40°C.

Table I. Kinetic parameters of the binding reaction of the WW domain to a proline-rich peptide using a Langmuir binding model

Protein	Buffer	k_a (1/M s)	k_d (1/s)	K_A (1/M)	K_D (M)
GST-WW	20 mM HEPES, 1 mM EDTA, 200 mM NaCl, pH 7.2	5.3×10^{4}	9.6×10^{-4}	5.5×10^{7}	$1.8 \pm 5\times10^{-8}$
VP1-WW150, w/o C19, 114	20 mM HEPES, 1 mM EDTA, 200 mM NaCl, pH 7.2	4.3×10^{5}	3.3×10^{-3}	1.3×10^{8}	$7.7 \pm 5\times10^{-9}$
	PBS	4.4×10^{5}	1.8×10^{-3}	2.4×10^{8}	$4.2 \pm 5\times10^{-9}$
VP1-WW150, incl. C19, 114	20 mM HEPES, 1 mM EDTA, 200 mM NaCl, pH 7.2	2.1×10^{5}	1.9×10^{-3}	1.1×10^{8}	$9.0 \pm 5\times10^{-9}$

proteins used so far did not contain cysteine residues and were therefore unable to form an intrapentameric disulfide bridge which is needed for a complete capsid assembly. However, upon addition of Ca^{2+} ions an equilibrium with 55% reduced capsids and 45% free capsomeres should be reached which is an early step during the assembly process (Schmidt *et al.*, 2000). However, removal of EDTA and the addition of Ca^{2+} resulted in a loss of 90% of the protein due to significant aggregation of VP1-WW150; this indicated a strong decrease of the solubility of the reduced capsid species which resulted in precipitation until the protein concentration fell below a critical limit. This was not due to aggregation via the WW domain in the presence of Ca^{2+} since the solubility of similar concentrations of the GST-WW protein did not decrease upon addition of $CaCl_2$ (data not shown). Also, in contrast to VP1-WW150, the protein VP1-WW292 did not aggregate and remained in solution.

The *in vitro* assembly can be quantitatively analyzed by size-exclusion chromatography (Schmidt *et al.*, 2000). Analysis of the assembly of the VP1-WW proteins revealed that VP1-WW150 that still remained in solution assembled to only 15% (Figure 5). VP1-WW292 did not form capsids at all and represents the first described variant of VP1 which is totally blocked in capsid formation, although the C-terminal domain for the interaction of capsomeres is present (Figure 5). These results demonstrate that large inserts, like the 38 amino acids containing the WW domain, into the viral capsid protein had long-distance effects on parts of the protein which are essential for capsid formation.

An intrapentameric disulfide bridge between cysteines 19

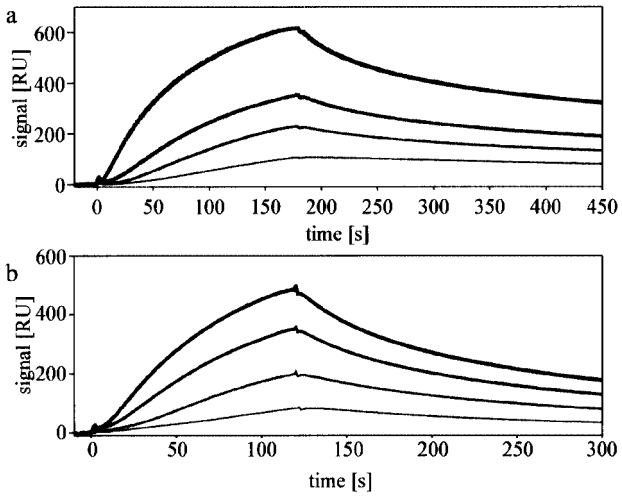

Fig. 4. Surface plasmon resonance with an immobilized PPLP ligand. Analysis with WW domain concentrations of 20, 15, 10 and 5 nM, respectively, in a buffer containing 20 mM HEPES, 1 mM EDTA, 200 mM NaCl, pH 7.2 of (**a**) cysteine free VP1-WW150 and (**b**) VP1-WW150 containing cysteines 19 and 114.

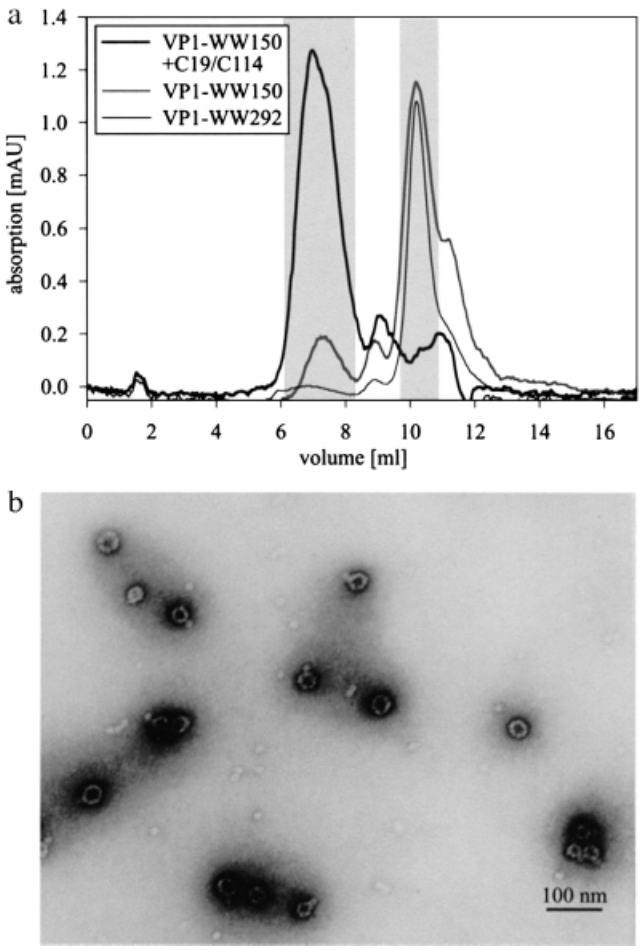

Fig. 5. *In vitro* assembly of VP1-WW fusion proteins. (**a**) Size-exclusion chromatography (TSKgel 5000PW$_{XL}$) demonstrates that only VP1-WW150, which contains cysteines 19 and 114, forms quantitatively virus-like particles. Elution volumes of capsids (6–8 ml) and free capsomeres (10–11 ml) are marked. (b) Electron microscopy shows an homogeneous population of disulfide-stabilized VP1-WW150 capsids.

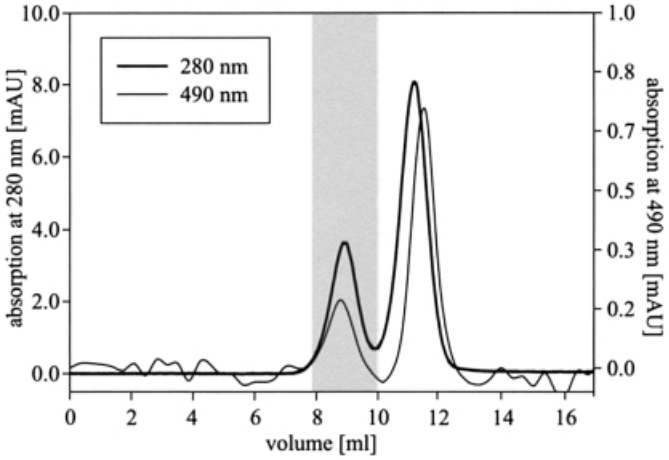

Fig. 6. Coupling of polyproline-tagged GFP onto the surface of virus-like particles. GFP-loaded capsids (elution volume 8–10 ml) are separated from excess GFP and minor impurities by size-exclusion chromatography (TSKgel 6000PW$_{XL}$).

and 114 of neighboring monomers is needed for a complete capsid assembly of the wild-type protein (Stehle *et al.*, 1994; Schmidt *et al.*, 2000). In order to improve the inefficient cysteine-free *in vitro* assembly and to test whether oxidized, disulfide-linked VP1-WW150 capsids are also prone to aggregation, cysteines 19 and 114 were reintroduced into VP1-WW150. The disulfide bridge forming variant of VP1-WW150 had no differences in the binding properties of proline-rich ligands (Figure 4; Table I) and did not aggregate upon initiation of *in vitro* assembly by dialysis. Size-exclusion chromatography demonstrated that the assembly efficiency of the modified protein was improved to >95%, similar to the wild-type protein. Electron micrographs also showed an homogeneous population of virus-like particles consisting of VP-WW150 (Figure 5). This single intrapentameric disulfide bridge could therefore completely restore the assembly properties of the wild-type protein.

VP1-WW150 capsids can bind external ligands in solution

In order to test the ability of polyomavirus-like particles to bind external ligands in solution, as would be necessary for therapeutic applications, completely assembled disulfide stabilized capsids of VP1-WW150 were mixed with an equal molar amount of green fluorescent protein (GFP) that was tagged with a PPLP motif at its C-terminus. The formation of the complex consisting of capsids and GFP was analyzed by size-exclusion chromatography (Figure 6). GFP could be detected by its specific absorbance at 490 nm while detecting the protein absorbance at 280 nm for both capsids and GFP in parallel. Absorbance at 490 nm at the elution volume of the capsids represented GFP bound to the particle's surfaces. Control experiments with VP1 wild-type capsids showed no co-elution of GFP with the capsids (data not shown). Integration of the peak areas and calculation of the molar ratio of capsids and GFP, respectively, indicated that 25 ± 5 molecules of GFP were bound to the capsid surface, demonstrating the general applicability of our approach. The binding ratio was probably limited by the rapid dissociation reaction of the inserted WW domain.

Discussion

In this work polyomavirus-like particles were used as a model for viral protein shells in order to study the influence of

insertion mutants with a module for the binding of external ligands onto the particle's surfaces. The binding module must fulfil certain criteria: It should specifically bind peptide ligands with a high or regulatable affinity. Preferably, it should also have a small, compact structure to avoid interferences with other functions of the protein shell. A module which combines many of these properties is the WW domain, the smallest, independently folding protein domain known to date. The highest affinity has been reported for the first FBP11 WW domain to a proline-rich sequence with a PPLP consensus motif (Bedford *et al.*, 1997).

Although theoretical considerations and calculations suggested that the WW domain could fold independently in both β-turns, which we chose for insertion into the VP1 sequence, only one variant, VP1-WW150, could specifically interact with proline-rich ligands. This was expected from CD and thermal denaturation experiments that demonstrated that the WW domain is only folded in VP1-WW150. Probably, the conformation of the DE loop of VP1 is more compatible with the WW domain structure than the alternative HI loop. This might not be a general conclusion since previous experiments demonstrated that insertion of an enzyme, namely dihydrofolate reductase, into the polyomavirus HI loop, yielded pentamers with enzymatic activity; however, these pentamers formed smaller virus-like particles than the VP1-wild-type protein (Gleiter *et al.*, 1999). In contrast to these data we did not observe morphological differences between VP1-WW150 and VP1-wild-type capsids; the assembly efficiency of the modified capsomeres was similar to the wild-type protein, provided that an intrapentameric disulfide bridge could be formed during *in vitro* assembly.

The binding kinetics of the WW domain within the fusion protein has implications for the use of external ligand binding for therapeutic applications. The binding of a PPLP motif by the integrated WW domain (VP1-WW150) compared to a linear construct (GST-WW) resulted in an increased affinity in equilibrium while the ligand exchange was accelerated 10 times (Table I). Nevertheless, it could be demonstrated that a complex of polyproline-tagged GFP and virus-like particles can be isolated and that this complex is stable during this procedure (Figure 6). However, the coupling efficiency as well as the stability of the complex was limited by the fast dissociation reaction that would possibly reduce the effectiveness of the bound ligand *in vivo*. This is a general problem of non-covalently linked external ligands and was also discussed for the IgG-binding protein A fusion constructs with viral vector surfaces (Wickham, 1997).

In order to extend the stability of the complexes, modified WW domains were designed that allow the formation of a disulfide bridge with the ligand, and the analysis of binding and covalent linking looks very promising (C.Parthier and U.Schmidt, own unpublished results). It has also been proposed that the WW domain fold may constitute a template into which binding sites from unrelated proteins may be introduced in order to mimic proteins for use in drug design because of the high degree of sequence variability allowed by WW domains (Macias *et al.*, 2000). Therefore, random mutagenesis of WW domains, selection for a desired binding activity and reintroduction of the altered domain into the protein shell could result in viral particles with a new cellular tropism without the need for external receptor-binding domains or antibody fragments. Alternatively, a two-step therapeutic approach would be conceivable that marks in a first step the target cells with an antibody fragment presenting a proline-rich sequence, thereby directing the WW domain to the target cells in a second step. Similar strategies involving biotin/streptavidin interactions were used for a tissue specific radiation therapy (Paganelli *et al.*, 1999).

In summary, our experiments underline the applicability of protein design for the modification and incorporation of new functions into viral capsid proteins. WW domains as modules for the binding of external ligands constitute a flexible tool for future investigations in this direction. These or similar approaches will be useful for individual vector targeting systems and could have a broad range of applications in the drug delivery field.

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