



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Virus-Like Particles: Models for Assembly Studies and Foreign Epitope Carriers

ANDRZEJ PALUCHA,[°]
 ADRIANNA LONIEWSKA,[°]
 SUBBIAN SATHESHKUMAR,[†]
 ANNA M. BOGUSZEWSKA-
 CHACHULSKA,[°] MAHADEVAIAH
 UMASHANKAR,[†] MALGORZATA
 MILNER,[°]
 ANNE-LISE HAENNI,^{°,‡,§} AND
 HANDANAHAL SUBBARAO
 SAVITHRI[†]

[°]*Institute of Biochemistry and Biophysics,
 Pawinskiego 5a, 02-106 Warszawa, Poland*

[†]*Department of Biochemistry, Indian
 Institute of Science, Bangalore,
 560012 India*

[‡]*Institute Jacques Monod,
 CNRS—University of Paris VI and VII, 2
 place Jussieu, 75251 Paris Cedex 05, France*

[§]*Laboratorio de Immunovirologia, Faculty
 of Medicine, University of Antioquia, A. A.
 1226 Medellin, Colombia*

I. Introduction	136
II. Requirements for VLP Assembly.....	136
A. Protein Requirements for the Assembly of Spherical VLPs	137
B. Protein Requirements for the Assembly of Rod-Shaped VLPs	141
C. Role of Viral Scaffolding Proteins	142
D. Protein–Nucleic Acid Interactions.....	144
E. Cell Membranes.....	146
F. Disulfide Bonds and/or Metal Ions.....	148
G. Role of Proteolytic Maturation in VLP Formation.....	150
H. Host Factors Supporting VLP Formation	151
III. VLPs as Epitope Carriers and Foreign Antigen Presentation Systems.....	152
IV. Concluding Remarks.....	158
References.....	159

I. Introduction

Virus-like particles (VLPs), formed by the structural elements of viruses, have received considerable attention over the past two decades. The number of reports on newly obtained VLPs has grown proportionally with the systems developed for the expression of these particles. When expressed in a suitable heterologous system, viral structural proteins involved in capsid or envelope formation often self-assemble into VLPs in the absence of other viral components usually required for virus assembly, such as multiple structural or nonstructural proteins and viral genomes. Protein–protein interactions in VLPs are relatively strong and can result in the formation of stable structures. Several experiments have been reported that may help answer questions regarding the requirements for VLP formation. Knowledge on the assembly process of VLPs is crucial to define the usefulness of such particles for the presentation of their own or foreign epitopes as carriers for transiently expressed proteins as a means of vaccine production. The aim of the present chapter is to outline recent achievements in two important fields of research brought about by the availability of VLPs produced in a foreign host. These are (1) the requirements for VLP assembly and (2) the use of VLPs as carriers for foreign epitopes. To date, reviews in these areas have mainly focused on results obtained with a specific virus genus or family of viruses (1–5) and the reader is advised to refer to these reviews for complementary information.

II. Requirements for VLP Assembly

Most viruses and VLPs have either a helical or an icosahedral symmetry and are composed of a single layer of viral proteins. Others are double-layered, possessing an outer and an inner layer, whereas others still are triple-layered. Some additionally possess an envelope. Virus assembly involves bringing together many protein subunits, packaging the genome, and for some viruses, ultimately maturing them into an infective particle. The assembly pathway is difficult to establish because polymerization occurs rapidly, yet with high fidelity and generally without the accumulation of intermediates (6). However, assembly is not always a single high-order reaction and can include a cascade of low-order, intermediate reactions (7). One of the ways of trapping intermediates at different stages of assembly is by mutagenesis of the structural protein followed by expression in a suitable heterologous system.

VLPs serve as excellent model systems to study assembly of macromolecular complexes. The pathway of VLP assembly can differ depending on the nature of the stabilizing interactions such as protein–protein, protein–nucleic acid, and metal ion or disulfide bond-mediated interactions. In more

complex viruses, assembly can also be mediated by proteolytic maturation of viral proteins, by expression of scaffolding elements, or by cell membranes, which make up viral envelopes. The available data on the assembly and structure of VLPs are discussed in the following sections and summarized in [Table I](#).

A. Protein Requirements for the Assembly of Spherical VLPs

For a number of eukaryotic viruses, structural proteins like coat proteins (CPs) or capsid proteins alone are competent to assemble into VLPs, even when expressed in heterologous systems without any of the remaining viral components. Structural requirements for the formation and stability of VLPs are carried by one, two, or more viral proteins and correspond to their different domains. To investigate the main driving forces responsible for VLP formation, the assembly of several viral structural proteins was studied.

Norwalk virus (NV; family *Caliciviridae*) is unique among animal viruses in possessing a single structural capsid protein and, hence, shares the structural simplicity of plant viruses. Expression of the capsid protein (VP1) in heterologous systems results in the formation of empty VLPs (8–10). A detailed study of the ability of a set of deletion mutants to assemble into VLPs revealed the regions involved in capsid formation. The NV VP1 contains two domains, a shell (S) domain at the N-terminus and a protruding (P) domain at the C-terminus, in which two subdomains (P1, P2) can be distinguished. The S domain alone can initiate VLP assembly whereas the P domain controls the stability and size of the particle when expressed in insect cells (11). Similar results were obtained for the VLPs of Venezuelan equine encephalitis virus (*Togaviridae* family) expressed in mammalian cells in which mutations ablating VLP assembly are located in the N-terminal and P1 domains (12). For NV VLPs, it was also demonstrated that a minor structural protein VP2 may be encapsidated by the particles when coexpressed in insect cells and cell-free systems (13,14). This small basic protein increases the expression and stability of VP1, protecting it also from disassembly and protease degradation (15). VP1 of another member of the *Caliciviridae* family, the Snow Mountain norovirus, expressed by a recombinant baculovirus, also self-assembles into VLPs: assembly analyses of the wild type and mutant VLPs expressing VP1 indicate that a single histidine residue at position 91 (S domain) is crucial for VLP formation (16).

The VP1, VP2, VP3, and VP4 polypeptides of Junonia coenia densovirus (family *Parvoviridae*) expressed by multiple overlapping translational initiation events at in-frame AUG codons and composing the viral capsid can assemble into VLPs when expressed independently by recombinant baculovirus in insect cells. No significant differences are observed between VLPs composed of any

TABLE I
VIRUSLIKE PARTICLE ASSEMBLY: STRUCTURAL REQUIREMENTS FOR DIVERS VIRUS FAMILIES^a

Family/Genus	Virus	VLP shape	Requirements for VLP formation	References
<i>Parvoviridae</i>	Junonia coenia	Spherical	VP4	(17)
	Adeno-associated (2)	Spherical	VP2 and VP3	(18)
<i>Caliciviridae</i>	Norwalk	Spherical	S and P domain of VP1	(8, 9, 10, 11)
	Snow Mountain	Spherical	His residue in the S domain of VP1	(16)
<i>Togaviridae</i>	VEEV	Spherical	P1 domain of VP1	(12)
	Sindbis	Spherical	Coiled coil structure of the capsid protein	(34)
	Sindbis, Ross River	Spherical	Viral RNA, tRNA ssDNA; nucleic acid-bound dimers	(81, 82, 83)
	Rubella	Enveloped	Coexpression of E1 and E2	(93, 94, 95, 96)
<i>Comoviridae</i>	Cowpea mosaic	Spherical	Coexpression of L and S CPs	(19, 20)
<i>Reoviridae</i>	Rice dwarf	Spherical	N-terminus of P3	(22, 23, 24)
<i>Tymovirus</i>	Physalis mottle	Spherical	C-terminus of CP	(25, 26, 27, 28)
<i>Papovaviridae</i>	Polyoma	Spherical	C-terminus of VP1	(30)
<i>Bromoviridae</i>	Alfalfa mosaic	Spherical	CP dimer	(31, 32)
	Brome mosaic		tRNA-like structures and tRNA	(75)
<i>Polerovirus</i>	Potato leafroll	Spherical	N-terminal modification on CP	(33)
<i>Tobamovirus</i>	Tobacco mosaic	Rod	CP and viral OAS on ssRNA	(36, 37)
<i>Sobemovirus</i>	Sesbania mosaic	Spherical	N-terminal residues of CP and structured RNA	(72, 73, 74)
			Calcium-binding	(113)
<i>Tetraviridae</i>	Thosea asigna	Spherical	Cleavage between L and S fragments of CP	(119, 120)
<i>Pecluvirus</i>	Peanut clump	Rod	CP and mRNA of CP gene	(38)
<i>Potyviridae</i>	Johnsongrass mosaic	Rod	Third and fifth helices of viral CP	(39, 40)
	Pepper vein banding	Rod	N- and C-terminus of the viral CP	(42, 43)
	Tobacco etch virus	Rod	N- and C-terminus of the viral CP conserved residues	(41)

TABLE I (Continued)

Family/Genus	Virus	VLP shape	Requirements for VLP formation	References
<i>Herpesviridae</i>	Herpes simplex (1)	Spherical	Scaffolding protein VP22a	(45, 46, 47, 48, 49)
<i>Reoviridae</i>	Simian rotavirus, Bovine rotavirus	Spherical	VP2, VP6—double layered VLPs; VP2, VP4, VP6 and VP7—triple layered VLPs	(50, 51, 52, 53, 54, 55)
	Bluetongue	Spherical	VP3, VP7—inner scaffold proteins	(56, 57, 58, 59, 60, 61)
<i>Nodaviridae</i>	Flock house	Spherical	N-terminal residues of the capsid protein and viral RNA	(76, 77, 78)
	MGNNV	Spherical	Capsid protein and random cellular RNAs	(79, 80)
<i>Birnaviridae</i>	Infectious bursal disease	Spherical	VP3 and pVP2; C-terminal glutamic acid of VP3	(62, 63, 64)
			Polyprotein maturation	(122)
<i>Flaviviridae</i>	Hepatitis C	Spherical	N-terminal 120–124 aa and folded RNA	(69, 70, 71)
<i>Retroviridae</i>	Rous sarcoma	Spherical	Nucleic acid-driven dimerization and VLP assembly	(84, 85, 86, 87)
	Simian foamy	Enveloped	Env protein	(92)
	SIV, HIV	Enveloped	Pr57gag and Pr55gag	(88, 89, 90, 91)
<i>Coronaviridae</i>	Infectious bronchitis	Enveloped	Cytoplasmic tails of M and E proteins	(97, 98, 99, 100)
<i>Orthomyxoviridae</i>	Influenza	Enveloped	Matrix protein M1	(101)
<i>Paramyxoviridae</i>	Human parainfluenza (1)	Enveloped	Matrix protein M	(102)
	Simian 5	Enveloped	Matrix protein M with HN or F proteins and the NC	(107)
<i>Filoviridae</i>	Ebola, Marburg	Enveloped	Matrix protein VP40 alone or VP40 and GP	(103, 104, 105, 106)
<i>Polyomaviridae</i>	BKPyV, MPyV	Spherical	Disulfide bonds and metal ions	(108, 109, 110)
	Simian 40	Spherical	Calcium ion binding	(111, 115)
	JC polyomavirus	Spherical	Calcium ion binding	(110, 112)
<i>Papillomaviridae</i>	Human papillomavirus	Spherical	Disulphate bonds	(116, 117, 118)

^aNote: VEEV, Venezuelan equine encephalitis virus; MGNNV, Malabaricus grouper nervous necrosis virus; SIV, Simian immunodeficiency virus; HIV, Human immunodeficiency virus; BKPyV, BK polyomavirus; MPyV, Murine polyomavirus; OAS, origin of assembly sequence.

of the four polypeptides. VP4 alone is sufficient to form VLPs and different ratios of the wild-type nucleocapsid mixtures of VP2, VP3, and VP4 can assemble into indistinguishable VLPs (17).

The capsid of Adeno-associated virus 2 (family *Parvoviridae*) is composed of three proteins, VP1, VP2, and VP3, of which only the last two are necessary for capsid formation. VP2 and VP3 expressed in insect cells generate VLPs, regardless of the ratio of the expressed proteins (18). In contrast, the Cowpea mosaic comovirus large (L) and small (S) coat proteins (CPs) assemble into VLPs only if both capsid components are expressed from the same transcription unit, i.e., a single plasmid expressing both proteins L and S in equimolar amounts from separate promoters (19, 20).

The Rice dwarf reovirus (RDV) icosahedral particle contains at least six structural proteins, P1, P2, P3, P5, P7, and P8 (21). The structural requirements leading to the formation of VLPs are carried by P3 and P8. Coexpression in transgenic rice plants of P3 and P8, but not expression of P8 alone, results in the formation of double-shelled VLPs similar to native RDV particles. Hence, these two proteins appear sufficient for the structural integrity of the VLPs in plants (22). Double-shelled VLPs were also observed upon coexpression or mixing of P3 and P8 *in vitro*. The structural protein P7 expressed in a similar manner was located inside the VLPs (23). Expression of the structural protein P3 in insect cells resulted in the formation of single-shelled core-like particles (CLPs). Self-assembly of P3 CLPs is driven by the N terminus of P3, and proteins with serial amino-terminal deletions form particles with gradually decreased stability. After removing the 52 N-terminal residues, CLPs are no longer formed (24).

The *Physalis mottle tymovirus* (PhMV) CP can assemble into VLPs when expressed in *Escherichia coli*. Up to 30 amino acids can be deleted from the N-terminus of the CP without hampering assembly. Moreover, addition of 41 amino acid residues to the N-terminus of the wild-type CP does not alter VLP formation. Comparison of the crystal structure of the empty T = 3 VLPs with that of the native T = 3 virus shows that the N-terminus provides the structural dynamism required for virus disassembly (25, 26). In contrast, deletion of one, five, or ten residues from the C-terminus alters VLP formation, and deletion of the C-terminal asparagine residue produces unstable VLPs that disassemble to form discrete intermediates (27, 28). Mutation of crucial interfacial residues identified on the basis of crystal structure of native virus and PhMV VLPs resulted in essentially partially folded monomers, suggesting that subunit folding and assembly are concerted (29). Similarly in Polyomavirus (family *Polyomaviridae*), truncation of the C terminus of VP1, the major structural protein, results in the formation of pentameric capsomers but not of VLPs, suggesting that the C terminus is involved in specific association of the capsomers (30).

In contrast to the majority of members of the *Alfamovirus* genus, most strains of Alfalfa mosaic virus (AMV) are composed of baciliform particles. Expression of the AMV CP in *E. coli* produces $T = 1$ icosahedral VLPs in the absence of viral RNA (31). Analysis of the crystal structure of the VLPs suggests that dimers are formed by interaction between the N- and C-termini. Deletion of the last 18 amino acids or introduction of point mutations into the C-terminus of the CP results in failure to form dimers or VLPs. Thus, the CP dimer is the minimum building block of the VLP (32).

In insect cells, the Potato leafroll polerovirus (PLRV) CP is unable to assemble into VLPs when expressed without modifications. This can be overcome by addition of 6 histidines at the N-terminus; the resulting icosahedral VLPs co-sediment in sucrose gradients with PLRV particles, suggesting that the VLPs contain RNA (33).

A well-established *in vitro* assembly system for Sindbis alphavirus (SINV) CLPs was used to explain the role of a coiled coil structure, designated helix I, spanning residues 38 to 55 of the viral capsid protein. As the analysis of mutants showed, three conserved leucine residues L38, L45, and L52, are crucial for the function of helix I, a leucine zipper-type determinant of CLP assembly (34). Assembly studies of chimeric capsid proteins with helix I substituted by the unrelated coiled coil of the yeast transcription activator GCN4 showed a similar ability to form CLPs, as have the wild-type proteins. Electron microscopy indicated that the chimeric particles were not as spherical as those observed for the unmodified particles and could be influenced by the difference in length between helix 1 and the helix of GCN4 (35).

B. Protein Requirements for the Assembly of Rod-Shaped VLPs

Basically, two types of rod-shaped viruses exist, the rigid rod-shaped viruses such as the tobamoviruses (ex., Tobacco mosaic tobamovirus; TMV) and the flexible filamentous viruses such as the potyviruses (ex., Johnsongrass mosaic potyvirus; JGMV). In both cases, the capsids generally consist of a single protein species.

The CP of TMV has long served as model in various kinds of structural studies. Depending on the conditions (pH, temperature, ionic strength, protein concentration), the TMV CP forms several types of specific ordered aggregates in solution (36). Native TMV CP expressed in *E. coli* forms non-helical, stacked-disc aggregates after dialysis against pH 5 buffer and is inactive for *in vitro* assembly with TMV RNA. However, when coexpressed *in vivo* with foreign single-stranded RNAs (up to about 2 kb) containing the TMV origin-of-assembly sequence, high yields of helical VLPs of the predicted length are obtained (37). A model was proposed for the assembly of TMV or VLPs

containing foreign RNA in which 20S protohelix CP aggregates with single-stranded RNA transcripts (including the origin-of-assembly sequence) are the starting point for the formation of virions or VLPs.

cDNA copies of the Indian Peanut clump virus (genus *Pecluvirus*) CP gene were expressed in *E. coli* and transgenic *Nicotiana benthamiana* plants, giving rise to rod-shaped VLPs. Depending on the source of extracts examined (either from *E. coli* or from *N. benthamiana*), VLPs of different lengths between 30 and 120 nm were identified. Most of them were about 30 nm long and immunocapture RT-PCR experiments confirmed that the VLPs contained the mRNA of the expressed CP gene. Such results suggest the presence of an origin-of-assembly motif or a specific sequence in the mRNA (38).

Systems in which JGMV CP or its mutated versions are expressed and assembled into VLPs provide a means of identifying regions of the protein crucial for filamentous particle assembly. Mutations of highly conserved amino acids in the third and fifth helices based on the anticipated structural model preclude VLP assembly (39). Site-directed mutagenesis of two charged residues previously proposed to represent a pair in the construction of a salt bridge crucial for the assembly process of the virus suggests that these two residues are required for assembly but not necessarily as a pair in a common salt bridge (40). Similar results were obtained when assembly of Tobacco etch virus (TEV) coat protein was studied in *E. coli* (41). Mutation of conserved amino acids revealed residues required for TEV VLPs assembly *in vitro*. Moreover, truncation of 28, 63, or 112 N-terminal amino acids resulted in assembly of VLPs that are more rigid and progressively smaller in diameter.

Expression of the CP of Pepper vein banding potyvirus (PVBV) resulted in the formation of flexuous rods of heterogeneous lengths encapsidating the viral RNA (42). *In vitro* assembly studies of the overexpressed and purified CP showed that VLPs of PVBV are formed via a ring-like intermediate and that electrostatic interactions may be important for stabilizing the particles. By progressive deletions at the N- and C-terminus of the recombinant CP, it was demonstrated that N terminal 53 and C terminal 23 residues are crucial for the inter subunit interactions involved in the initiation of assembly. Based on the results obtained, a working model for flexuous virus assembly was proposed (43).

C. Role of Viral Scaffolding Proteins

Scaffolding proteins play an essential structural role in ensuring correct assembly of large viruses. They have been described for various virus systems and are found in most double-stranded DNA phages (44).

Herpes simplex virus (family *Herpesviridae*) capsids are composed of an outer icosahedral shell of four proteins (VP23, VP5, VP26, and VP19C) and an inner core of three proteins (VP21, VP24, and VP22a), of which principally

VP22a serves as core scaffolding protein in particle assembly (45). The scaffolding protein is required for the correct assembly of the outer shell of the capsids. Coexpression of all seven components in insect cells results in the formation of VLPs indistinguishable from native particles. VLP assembly does not take place in the absence of VP23, VP5, or VP19C, whereas lack of VP26 is without effect on VLP formation. Partial and deformed capsid shells are formed in the absence of VP21, VP24, and VP22a (46). The C-terminal 25 amino acids of VP22a are cleaved after assembly or when complexed with one or more capsid shell proteins (47). However, expression of the C-terminally truncated VP22a leads to aberrant, incomplete capsid structures, suggesting a role of the C-terminal region in the formation of sealed VLPs (45, 48). In addition, the concentration of the scaffolding protein VP22a affects the structure of the nascent procapsids (49).

In the case of the triple-layered particles of members of the *Rotavirus* genus, such as those of the Simian rotavirus and consisting of the four major structural proteins VP2, VP4, VP6, and VP7, as well the two minor proteins VP1 and VP3 responsible for initial transcription of the genome, different variations of VLPs are formed, depending on the protein composition (50). VP2 by itself forms CLPs and, in particular, its N-terminus is responsible for binding and encapsidation of VP1 and VP3 (51). A detailed analysis showed that VP2 with any N-terminal truncation between amino acids 1 and 25 fails to bind VP1, yet that VP2 lacking the first 92 amino acids is still able to form single- and double-layered VLPs (52). VP2/VP6 interactions are sufficient to form double-layered VLPs, while addition of the other major capsid components VP4 and VP7 leads to the formation of triple-layered particles resembling native rotavirus particles. The Bovine rotavirus VP2 also can serve as a scaffold for the assembly of the other capsid proteins originating from the human virus (50). Based on the innermost core VP2, components of the second (VP6) and third (VP7) layers may be derived from viruses of different serotypes, thus forming heterotypic (chimeric) particles (53, 54, 55).

Bluetongue virus (BTV; family *Reoviridae*) as well as other orbiviruses possess four major structural proteins VP2, VP3, VP5, and VP7; they also possess three minor structural proteins VP1, VP4, and VP6 that have RNA-transcriptase and RNA-modifying properties. VP3 and VP7 are sufficient to form single-shelled CLPs in a baculovirus expression system (56). Deletion, point mutation, and domain switching analyses of the two proteins VP3 and VP7 helped to identify certain sequences that are essential for the formation of CLPs (57). Simultaneous coexpression of all the major structural proteins resulted in the assembly of double-shelled VLPs (58, 59). Identification of regions in the inner scaffold proteins VP3 and VP7, involved in CLP formation (60, 59), has made it possible to assemble VLPs using capsid components from different orbiviruses. The nature of the interactions between the innermost

VP3 and the intermediate VP7 layer were studied by analysis of VLP assembly of VP7 single or multiple site mutants of VP7. It was clearly shown that both the position and the nature of single residues are critical for the formation of VLPs with VP3 and VP7 (61).

Assembly studies of the Infectious bursal disease virus (family *Birnaviridae*) structural proteins expressed in a heterologous system showed that formation of VLPs depends on coexpression of VP3 and pV2, an immature precursor of VP2. It was also demonstrated that the 71 C-terminal amino acids of pVP2 are essential for the establishment of VP2–VP3 interactions (62). A critical role in VLP formation is played by the C-terminal oligomerization domain of the VP3 scaffold protein: a product lacking the 13 C-terminal residues as a result of proteolysis was unable to form VLPs. Prevention from protease activity by coexpression of VP1 which binds to the C terminus of VP3 led to efficient VLP formation (63). The C-terminal residue of VP3, glutamic acid, controls the assembly process. Deletion or replacement of the five terminal amino acids, or fusion with small proteins such as the green fluorescent protein (GFP) or truncated ovalbumin, as well as deletion of the C-terminal glutamic acid residue promoted VLP formation, suggesting that assembly requires screening of the negative charges at the C terminus of the scaffold protein VP3 (64). The fused polypeptides mimicked the function of VP1, which interacts with VP3 to promote VLP assembly.

D. Protein–Nucleic Acid Interactions

The main role of the various viral structural proteins is protection of the viral genetic material from degradation and formation of a relatively durable carrier/delivery system. Coevolution of viral genomes with their own structural elements has also led to interactions in which nucleic acids are the main driving force for VLP formation.

Hepatitis C flavivirus (HCV; family *Flaviviridae*) VLPs have been detected in various samples of infectious sera as well as in bacterial, insect, yeast, and other eukaryotic cell expression systems (65–68). However, attempts to obtain sufficient amounts of VLPs failed in most cases when using the core and envelope (E1 and E2) protein genes in the absence of other viral components that might enhance the assembly process. The first report on structural proteins assembled into VLPs with a bilayer envelope indicated the requirement of at least part of the 5' untranslated region of the viral genome (69). HCV CLP formation depends on the highly basic N-terminal 120 (70) or 124 (71) amino acids of the core protein and on oligonucleotides corresponding to the 5' untranslated region that harbors predicted secondary structural elements (71). Assembly of symmetrical nucleocapsid-like particles was highly dependent on the formation of secondary structures within the RNA molecule

that was co-incubated with the 124 amino acids of the core protein. Unfolded RNA obtained by treatment with EDTA could not lead to core formation.

The role of the N-terminal domain of the *Sesbania mosaic sobemovirus* (SeMV) CP in the assembly of VLPs was elucidated from the analysis of VLPs formed from wild-type or N-terminally deleted CPs. When expressed in *E. coli*, the recombinant CP, as well as a CP mutant whose first 22 amino acids had been deleted, self-assembled similarly to native virion. When 36 residues comprising the basic arginine-rich motif were removed, only $T = 1$ and pseudo $T = 2$ particles were observed. Hence, the arginine-rich motif can act as a molecular switch in $T = 3$ assembly (72). Deletion of the N-terminal 65 amino acid residues resulted in the formation of a $T = 1$ particle. Comparison of the crystal structure of this $T = 1$ particle with native SeMV showed that the major structural difference was in the icosahedral dimers (73, 74). Interestingly, all the VLPs encapsidated 23S rRNA or its degraded fragments, emphasizing the importance of RNA-protein interactions during the formation of these VLPs.

The role of RNA in viral assembly has been demonstrated in Brome mosaic bromovirus (BMV). The 3' terminal tRNA-like structure (TLS) is necessary for capsid assembly, since BMV genomic RNA lacking the 3' TLS failed to assemble into mature capsids in the presence of CP. However, VLPs were formed when tRNAs or short RNAs containing the TLS were included, although these additional RNAs were not encapsidated with the TLS-deprived BMV genomic RNA. Such tRNAs and TLS acted as chaperones in VLPs assembly (75).

The Flock house nodavirus (FHV) CP cleavage product CP-alpha, deleted of the N-terminal 50 amino acid residues containing positively charged amino acids, failed to assemble into VLPs, whereas removal of the first 31 residues resulted in heterogeneous particles with heterologous RNA (76-78). When expressed in the presence of replicating FHV RNAs, uniform particles were formed, suggesting that particle polymorphism was imposed by the type of RNA selected for packaging (79). Similarly, expression of the capsid protein of a fish nodavirus, Malabaricus grouper nervous necrosis virus in insect cells, resulted in VLP formations that were similar in size and geometry to native virus and were packed by random cellular RNAs (80).

Nucleic acids play an important role in the assembly of alphaviruses such as SINV and Ross River virus (family *Togaviridae*). The purified capsid proteins expressed in *E. coli* could form corelike particles in the presence of single-stranded but not double-stranded nucleic acids (81). It was shown that the predominantly monomeric CP rapidly forms CLPs of regular size and shape following the addition of an appropriate nucleic acid substrate such as viral RNA, yeast tRNA, or single-stranded DNA. Further nucleic acid-dependent cross-linking experiments of the nucleocapsid protein suggest

assembly models involving nucleic acid-bound dimers of the capsid protein in early steps of the CLP assembly pathway (82). Analyses of dimerization and *in vitro* assembly of CLPs from truncated and mutant CPs of SINV support the proposed model of assembly and suggest a possible role for the N-terminal region of the protein in bridging the space between two monomers and stabilizing the interaction of the intercapsomer dimer (83).

The purified retrovirus Gag proteins or Gag protein fragments of Rous sarcoma virus (RSV) are capable of assembling into VLPs *in vitro* in the presence of RNA (84). Soluble Gag protein lacking its N- and C-terminal domains was used to study the requirements for VLP formation. The results indicated that VLPs were formed independently of the length or type of nucleic acid used and that the ratio of protein mass to nucleic acid mass in purified particles was constant. Furthermore, it was shown that the dimerization process of Gag molecules is crucial for *in vitro* formation of VLPs. This process may be driven by nucleic acids interacting with the nucleic acid binding domain of Gag proteins or of foreign dimerization domains replacing the functional site of protein–nucleic acid interaction (85, 86). Finally, a model was proposed in which two adjacent Gag molecules bound on a stretch of nucleic acid can dimerize and expose a surface hidden in monomers, which allows formation of spherical shells (87).

E. Cell Membranes

Enveloped viruses form their proteolipid envelope by budding through cell membranes and particle formation is concomitant with budding. Two mechanisms can be distinguished in the assembly of such viruses. The first mechanism depends on the presence of the capsid protein. This structural compound of retroviruses is sufficient for budding of VLPs from the cell membrane, whereas togaviruses require both capsid protein and envelope proteins. The second mechanism is capsid protein-independent and budding occurs by formation of capsidless envelopes.

The native Pr57gag precursor of Simian immunodeficiency virus (SIV; family *Retroviridae*), when expressed and myristoylated in a baculovirus system, assembles into 100 to 120 nm VLPs that bud from the cell membrane into the environment (88). Similarly, the unprocessed Gag precursor (Pr55gag) of Human immunodeficiency retrovirus 1 (HIV-1) forms VLPs. Deletion of the C terminus, together with the finger domain of the Gag precursor, abolishes assembly of VLPs, whereas membrane targeting and evagination are still possible (89). Expression of the entire Gag-Pol protein in mammalian cells by way of a Vaccinia virus expression vector leads to the formation of immature and mature retrovirus-like particles budding from the cell surface (90), whereas expression of Gag in yeast cells does not lead to budding unless the yeast cell wall has been removed, forming spheroplasts (91). Unique among retroviruses,

transient expression of the Env protein of Simian foamy virus in BHK-21 cells leads to efficient budding and formation of VLPs that appear normal in size and morphology. This finding and other similarities align foamy viruses with Hepatitis B virus, despite all the significant structural differences that exist between viruses of these families (92). Rubella virus (family *Togaviridae*) forms VLPs by a budding mechanism, which involves coordinated expression in mammalian cells of the capsid protein and the two envelope glycoproteins E1 and E2 (93–95). Each glycoprotein contains two domains, the transmembrane and the cytoplasmic domain, required in early and late steps of the assembly pathway, respectively. E2 acts as a support recruiting newly synthesized E1 molecules. The E2 transmembrane domain contains a Golgi retention signal and its cytoplasmic domain is required for interaction with the capsid proteins when VLPs are secreted (96).

Unlike retrovirus requirements for VLP formation, members of the family *Coronaviridae* assemble their viral envelopes into VLPs independently of the capsid protein. Two factors, the viral membrane glycoprotein (M) and the envelope protein (E), are required for assembly of coronavirus VLPs resembling native virions (97). The E protein occurs in trace amounts in VLPs whereas the M glycoprotein, that spans the membrane bilayer three times, is abundant. Detailed mutation analyses and co-assembly of mutated M protein with assembly-competent (wild-type) M protein demonstrated that transmembrane domains play a crucial role in homotypic interactions between particular M proteins (98, 99). A detailed study of VLP formation driven by the M and E proteins of Infectious bronchitis virus (IBV; family *Coronaviridae*) showed that proteins correctly targeted to the Golgi complex require their cytoplasmic tails to assemble into VLPs (100). To study the major driving forces in budding of Influenza virus (family *Orthomyxoviridae*) VLPs, a number of plasmids expressing the viral structural components in COS-1 cells were constructed. Co-transformation experiments, followed by identification of VLPs by biochemical assays, immunoblotting, and electron microscopy, indicated that extracellular membranous spikeless particles similar in size and shape to those of true virions were formed in cell cultures expressing exclusively the viral matrix protein M1. Therefore, the M1 protein has all the structural information required to induce efficient formation of VLPs that bud from the cell membranes (101). Similarly, the human parainfluenza virus type 1 (hPIV-1; family *Paramyxoviridae*) matrix protein M alone, when expressed from a plasmid in mammalian cells, induced budding of VLPs from the plasma membrane. Furthermore, coexpression of the nucleoprotein, which only leads to the formation of intracellular nucleocapsid-like structures, resulted in budding of VLPs enclosing the nucleocapsid-like structures. This observation suggests that the M protein of hPIV-1 has the ability to induce the formation of VLPs and to incorporate nucleocapsid-like structures into these vesicles (102).

The viral protein 40 (VP40) of Ebola virus and Marburg virus (family *Filoviridae*) appears to be equivalent to the matrix protein of other viruses. VP40 of Ebola virus was widely studied to elucidate its function and clarify its role in the formation of membrane-bound particles when expressed in mammalian cells (103, 104). The expression of VP40 in the absence of any other Ebola virus protein leads to budding and formation of characteristic filamentous spikeless VLPs. The central role of VP40 in this process is supported by the fact that coexpression of the viral glycoprotein (GP), which is found in pleomorphic particles budding from the plasma membrane when expressed alone, leads to incorporation of the GP into filamentous VLPs (105). Such a mechanism of VP40–GP–membrane interaction seems to be common to all the members of the *Filoviridae* family. Coexpression of the GP and matrix protein of Marburg virus in mammalian cells also results in the formation of filamentous VLPs (106).

The requirements for budding of the paramyxovirus Simian virus 5 (SV-5) VLPs seems to be more complex. The matrix protein expressed in mammalian cells was not sufficient to direct vesicle budding and was not secreted from the cell. Only the M protein in the form of VLPs was efficiently secreted when expressed with one of the two viral glycoproteins, hemagglutinin-neuramidase (HN) and fusion protein (F), together with the nucleocapsid protein (NP) (107). Detailed analyses of deletion mutants of particular VLP components suggest that multiple viral components are important for the budding process of SV-5.

F. Disulfide Bonds and/or Metal Ions

As shown for several viruses, not only the viral structural components are important for VLP formation. Experiments on assembly and disassembly of VLPs indicate that the stability of such particles often depends on the ionic or reducing conditions in which the assembly occurs. Formation of disulfide bonds between interacting domains of viral structural proteins or binding of divalent ions that may link corresponding elements often leads to conformational alterations of the VLP structure and can influence functions such as intracellular trafficking or disassembly.

The icosahedral polyomaviruses, Murine polyomavirus, Simian virus 40 (SV 40), Human BK polyomavirus, and JC polyomavirus (JCV) possess a capsid consisting of three proteins VP1, VP2, and VP3, of which VP1 is the major structural protein. When overexpressed in bacteria, VP1 forms pentameric capsomers and assembles into VLPs stabilized at low ionic strengths (108). In contrast, when expressed in the cytoplasm of insect cells, VP1 is transported to the nucleus and forms VLPs (109, 110) that may be disrupted into pentameric capsomers by EDTA or DTT (110). This indicates that the minor capsid

proteins are not essential for VLP formation and implies a role for disulfide bonds and/or metal ions.

When the assembled VP1 capsids of JCV were treated with chelating and reducing agents, they dissociated into capsomers that retained the ability to reassemble (110). Substitutions of the calcium ion binding residues of SV40 VP1 capsid protein resulted in the formation of tubular particles as well as VLPs with increased stability in the absence of calcium ions, suggesting that appropriate affinities of calcium ion binding are important for assembly of the capsid. Cysteine mutations revealed that the disulfide linkage stabilizes the calcium ions and, hence, the stability of the particles of SV40 (111). Recombinant capsids assembled upon expression of the major structural protein VP1 of JCV form disulfide linkages that stabilize dimeric and trimeric interactions. Such VLPs remained intact even after treatment with DTT, and disassembly occurred only after treatment with EGTA. When the capsids were treated with EGTA without reducing the disulfide bonds, they failed to disassemble, suggesting that the disulfide bonds are important to maintain the capsid structure by protecting the calcium ions from chelation (112). In the icosahedral SeMV, calcium-mediated interactions are proposed to regulate the assembly/disassembly of the virus. The calcium ion is octahedrally coordinated with six ligands, two carboxylates from one subunit, and three other ligands from the neighboring subunits. The sixth ligand is a water molecule. The calcium-binding site is conserved in all the Sobemoviruses. Mutation of the residues involved in the calcium coordination does not affect the assembly of $T = 1$ VLPs. Further, the crystal structure of mutant $T = 1$ particles revealed that they indeed lack calcium and are slightly expanded (113). In contrast, mutation of aspartates involved in calcium coordination severely affected the $T = 3$ VLP assembly (114).

The role of calcium ions in the formation of SV40 VLPs was demonstrated in a series of *in vitro* assembly experiments (115). The VP1 protein obtained as pentamers was exposed to various reconstitution buffers and the results clearly showed that, in absence of CaCl_2 , only tiny particles were formed. Enrichment of the assembly buffer with calcium, cadmium, or manganese ions whose ionic radii are similar, resulted in VLP formation. This supports the notion that appropriate divalent ions must bind to calcium-binding pockets of VP1 before the protein can assemble into larger particles. Interestingly, VP1 pentamers could also form long tubular particles similar to those of rod-shaped viruses.

Expression of the major late 1 (L1) structural protein using the prokaryotic, baculovirus, yeast, or mammalian systems results in the self-assembly of Human papillomavirus (HPV; family *Papillomaviridae*) VLPs. Coexpression of the minor late 2 (L2) structural protein increases the efficiency of particle formation but is not strictly required. Reduction of intermolecular disulfide

bonds by DTT led to disassembly of VLPs into L1 capsomers to which L2 remained associated, indicating that disulfide bond formation could be essential for capsid assembly (116). Further experiments demonstrated that mutation of only two highly conserved cysteine residues in L1 leads to the formation of monomers. It has also been suggested that trimerization of L1 is indispensable for the stabilization of intercapsomeric contacts in papillomavirus VLPs (117). Expression of L1 from two different serotypes resulted in VLPs with covalent disulfide bonds between these two types of proteins. This may provide a new tool to induce antibodies against multiple HPV types (118).

G. Role of Proteolytic Maturation in VLP Formation

The final step of a viral infection event is the development of mature virus particles. To reach this point, viruses adopt a series of gene expression strategies allowing them to control their multiplication. Expression of immature precursor structural components of viral envelopes followed by their processing seems to be an effective strategy in controlling the completion of the viral particles.

When the FHV CP precursor CP-alpha was expressed in insect cells, precursor VLPs were formed. Maturation by autocatalytic cleavage led to polypeptide chains beta and gamma that form particles indistinguishable from authentic FHV. Alteration of proteolytic cleavage by mutations resulted in defective VLPs, some of which possessed unusual structural features. Particles with the N363D mutation were fragile and broke into half-shells. Those with the N363A mutation displayed a distinct hole (77).

The pre-CP of *Thosea asigna* virus (TaV; family *Tetraviridae*) is cleaved at two positions to produce the L and S CPs as well as a predicted third nonstructural protein (119). In insect cells, VLPs were only formed when the L and S fragments were expressed from the same promoter, indicating that cleavage between the L and S CPs is an essential step in TaV capsid assembly (120).

The rotavirus (family *Reoviridae*) VP6 expressed as a free protein in plants using the Potato potexvirus X- (PVX) based vector forms only paracrystalline sheets and tubes. Expression of VP6 fused to the PVX CP yields flexuous rods containing a heterologous surface overcoat. Interestingly, in plants, such presentation of VP6 on the surface of the flexuous rod, followed by proteolytic cleavage, resulted in the formation of some icosahedral VP6 rotavirus-like particles, indicating a possible role of maturation in VLP assembly (121). The engineering of a morphogenesis switch to control a particular type of capsid protein assembly was reported for Infectious bursal disease virus (family *Birnaviridae*) (122). The expression of the pVP2-VP4-VP3 segment in insect cells resulted mainly in the formation of tubular structures composed of pVP2, the precursor of VP2. Expression of pVP2 alone resulted in the formation of

isometric particles smaller than the VLPs. When the entire GFP was fused to the C terminus of VP3 of the pVP2-VP4-VP3 segment, a large number of VLPs could be visualized by electron microscopy. This is the first report in which addition of a foreign protein to the inner VP3 capsid protein triggered VLP assembly.

H. Host Factors Supporting VLP Formation

In most cases, the formation of VLPs depends on viral structural or functional elements that are expressed during the infection stage. Yet, co-evolution of viruses with their hosts has resulted in cellular factors enhancing or controlling the assembly of VLPs. As a consequence, the host cell is frequently an important player in defining the efficiency of virus and VLP production.

It was found that expression of the RSV Gag protein in insect cells failed to produce VLPs even after artificial introduction of a myristoylation signal that was lacking from the N terminus of the protein, whereas deletion of the proteinase domain from the Gag protein restored VLP formation. The same Gag protein expressed in mammalian and avian cells was fully competent for assembly into VLPs without any modifications. It has been speculated that a chaperone activity in vertebrate cells may cause proper folding of the proteinase-containing Gag protein and that this activity is absent or quantitatively insufficient in insect cells (123).

Similarly, Human T-cell leukemia virus type I (HTLV-I) Gag and Gag-pro polyproteins showed differential budding efficiencies, depending on the cell system used for their expression. The Pr53^{Gag} polyprotein expressed from a recombinant baculovirus accumulated in insect cells at the plasma membrane but was unable to drive budding of immature VLPs. In contrast, in human 293 cells Gag particles were released into the medium as enveloped VLPs. Such cell type-dependent assembly strongly suggests involvement of an unknown mammalian host factor in the final stages of VLP assembly (124).

As mentioned previously, expression of the HIV-1 Gag-Pol protein resulted in VLP assembly and release from human cells and yeast spheroplasts, suggesting that no other viral factor is required. Using insect cells and baculovirus vectors for the expression of the Gag protein, only VLPs representing the immature form of the virus were obtained. Expression of Gag-Pol constructs in insect cells abolished VLP formation. Even intermediate levels of proteinase activity obtained by mutations did not result in mature VLPs (125). This indicates that specific cell factors are required for the maturation of the Gag protein and for VLP synthesis of HIV-1.

It was demonstrated that retroviral Gag proteins contain sequences termed late (L) domains that facilitate the final stages of VLP budding from the plasma membranes. The L domains of HIV-1 and Ebola virus encode small sequence

motifs that recruit two human cell factors: Tsg101, a tumor susceptibility gene and a component of the vesicular sorting machinery, and Nedd4, an ubiquitin ligase (126–128). These interactions are crucial for the budding process during VLP assembly. It was shown that Nedd4 and Tsg101 might play complementary roles by recruiting cellular factors of two independent pathways to the budding site at the plasma membrane.

III. VLPs as Epitope Carriers and Foreign Antigen Presentation Systems

Although it is known that chemically synthesized peptides are capable of eliciting the production of protective antibodies against the antigens from which they derive, the peptides by themselves present a low immunogenicity level. Antigenicity can be increased by conjugating/fusing the peptides to carrier proteins, thereby extending their lifetime and presenting them in a manner in which they will be better recognized by the immune system. Hence, the attractive prospect of presenting antigenic epitopes on the surface of viruses or VLPs and of using these chimeric constructs for vaccination and gene therapy.

The potential use of viruses as epitope presentation systems has had some success, but this strategy has largely been supplemented by the use of VLPs in place of viruses. This new approach has been facilitated by the development of various systems such as bacterial, yeast, insect, and mammalian cells, in which VLPs can be produced and assembled (129). VLPs are generally easy to produce on a large scale, and are non-infectious. Two classes of VLPs can be considered: (1) non-chimeric VLPs and (2) chimeric VLPs harboring a given peptide in an appropriate VLP context. Crucially, the use of VLPs for epitope presentation depends on precise knowledge of the detailed structure of the VLPs, so as to evaluate the region of the viral protein into which the foreign sequence is to be introduced. Moreover, the size of the insert (130, 131), the site of insertion, the mode of presentation together with the presence of short flanking sequences, and the expression system employed can dramatically affect the immunological response (132, 133).

Antigenic epitopes presented on the surface of VLPs can be taken up by antigen-presenting cells. The peptides presented on the surface of VLPs are exposed on MHC class I molecules at the cell surface. This primes the T cell response, either against the viral particle-forming protein itself or against the additional peptide sequence fused to the VLP. Recent achievements in the development of VLPs as epitope carriers are summarized here and in Table II.

The full-sized capsid of parvoviruses is composed of three proteins whose major structural protein VP2, by itself, is able to form stable VLPs (134, 135).

TABLE II
VIRUSLIKE PARTICLES AS FOREIGN EPITOPE CARRIERS^a

Family/Genus	Virus	VLP modification	Foreign sequence	Function	References
<i>Parvoviridae</i>	CPV, PPV	N-terminus and loop 2 of VP2	Fluorescent epitope (eGFP)	Particle entry, intracellular trafficking	(136–140)
		N-terminus of VP2	CD8+ CTL epitope from LCMV	CD8+ class I-restricted CTL response	(136)
<i>Retroviridae</i>	SIV	SIV Gag and Env proteins	Conjugation with CTB	Dose-dependent antigen response	(141)
	SIV, HIV	SIV Gag and HIV Env proteins	HA	Increased humoral and cellular response	(142)
<i>Hepadnaviridae</i>	Hepatitis B	Surface and core antigens	HPV16-E7 oncoprotein	Antibody response	(143)
		C-terminus of the core protein	17 kDa nuclease from <i>S. aureus</i>	Encapsulation of nuclease	(144)
		c/e 1 epitope	GFP (238 aminoacids)	Fluorescence and humoral response	(145)
		c/e 1 epitope	Influenza virus, malaria, Hantavirus epitopes	Antibody response	(146–148)
<i>Reoviridae</i>	Bluetongue	N-terminus of VP7	Rabies virus glycoprotein, HBV preS2	Epitope presentation	(149, 150)
	Rotavirus	N-terminus of VP2	Encapsulation of GFP and DsRed protein	Delivery system into living cells	(156)
<i>Caliciviridae</i>	Hepatitis E	C-terminus of ORF2 product	B-cell epitope	IgG and IgA to VLP and epitope	(152)
<i>Orthomyxoviridae</i>	Influenza	Replacement of HA	G protein or their ectodomain of VSV	Epitope presentation	(153)

(Continues)

TABLE II (Continued)

Family/Genus	Virus	VLP modification	Foreign sequence	Function	References
<i>Potyviridae</i>	Johnsongrass mosaic	N- or C-termini replacement	Merozoite surface antigen of malaria and/or luteinizing hormone-releasing hormone	Highly immunogenic in mice and rabbits	(154)
<i>Papovaviridae</i>	Polyoma	VP1 fusion	<i>E. coli</i> dihydrofolate reductase	Epitope presentation	(157)
			Tumor-specific antibody Fv fragment	Cell specific attachment and entry	(158, 159)
			Protein Z (antibody binding domain)	Antibody directed cell targeting	(160)

^aNote: CPV, Canine parvovirus; PPV, Porcine parvovirus; eGFP, enhanced green fluorescent protein; LCMV, Lymphocytic choriomenengitis virus; CTL, cytotoxic T lymphocyte; CTB, cholera toxin B subunit; SIV, Simian immunodeficiency virus; HIV, Human immunodeficiency virus; SHIV, Simian/Human immunodeficiency virus; HA, hemagglutinin; HBV, Hepatitis B virus; VSV, Vesicular stomatitis virus.

The specific three-dimensional structure of the Canine or Porcine parvovirus (CPV and PPV, respectively) VP2, with its four loops between eight-stranded antiparallel β -barrel motifs, appears to be a particularly suitable site for the insertion of epitopes to produce VLPs as carriers of molecules for antigen delivery. Two regions of VP2 dispensable for capsid formation, the N terminus which is directed toward the inside of the VLP, and loop 2, which is partially presented on the surface of the capsid, proved suitable sites for foreign epitope insertion in antigen presentation when expressed in insect cells (136–139). To elucidate events related to CPV infection, fluorescent VLPs were developed. The insertion of the enhanced GFP at the N terminus of VP2 did not alter assembly of the VLPs. Using these chimeric particles, their entry process into mammalian cells was followed and intracellular trafficking was observed (140). Inserting into the N terminus of the PPV VP2, a CD8+ cytotoxic T lymphocyte (CTL) epitope from Lymphocytic choriomeningitis virus (LCMV; family *Arenaviridae*) produced a readily self-assembling PPV:VLP-LCMV when expressed in insect cells, with a morphology closely related to that of PPV:VLP. Such chimeric particles elicited a strong CD8+ class I-restricted CTL response and totally protected mice against lethal chorimeningitis (136).

Purified SIV-derived VLPs, to which cholera toxin (CT), a well-known mucosal adjuvant, had been conjugated that increases antigen uptake at the mucosal surfaces, were produced and used for intranasal immunization of mice. CT could efficiently be replaced by its nontoxic B subunit (CTB) either free or conjugated to the VLP, the conjugated CTB being more efficient than the free CTB (141). The antigen response was dose-dependent and high levels of serum IgG antibody production was achieved. Antibodies were detected at the mucosal surface and increased numbers of MHC I-restricted peptide-specific T cells were obtained. Other experiments have been directed toward developing the use of CTB to enhance the immunogenicity of SIV or Simian/human immunodeficiency virus (SHIV) VLPs (142). Since Influenza virus induces strong immune responses by systemic as well as mucosal administration, it was investigated whether its surface glycoprotein hemagglutinin (HA) would enhance immunogenicity of SHIV. Chimeric HA/SHIV VLPs, indeed, increased the humoral and cellular immune responses in the systemic and mucosal compartments.

To develop an experimental vaccine against HPV-induced tumors, fragments of the HPV16-E7 oncoprotein were expressed as fusions with three proteins capable of VLP formation: the Hepatitis B virus (HBV; family *Hepadnaviridae*) surface (HBs) antigens, the HBV core (HBc) antigens, and the RNA phage fr coat protein. Only the HBV chimeras carrying the HBs and HBc assembled into VLPs. Nevertheless, analysis of the IgG isotype profile from immunized mice showed that the epitopes carried by all three viral proteins induced efficient antibody response to the antigens delivered (143).

The icosahedral nucleocapsid of HBV is composed of a single core protein that can form VLPs in the absence of its genome. The C terminus of the protein can be replaced by the 17 kDa nuclease of *Staphylococcus aureus*, also leading to VLPs. The foreign epitope is internally located in the chimeric VLPs, yet retains its nuclease activity (144). A highly appropriate central location in the HBV VLP was chosen to introduce GFP (238 amino acids). The hybrid protein formed fluorescent particles, the GFP domains being exposed to the surface, and humoral response was elicited against GFP (145).

Advantage has also been taken of the availability of the HBV core to improve immunogenicity of protein fragments derived from Influenza virus (146) and human malaria parasite, *Plasmodium falciparum* (147). In 2004 (148), HBV core fusions were produced in *E. coli* by introducing the 120 N-terminal amino acids of the N protein of one of three hantaviruses into an internal major immunodominant region of the HBV core. This region is preferred over the N- or the C-terminal region as insertion site for epitope presentation in HBV because of the surface exposure of the inserts that result in high immunogenicity. In all cases, chimeric core particles were formed. Cryo-electron microscopy revealed that the chimeric cores present a fuzzier outline with spikes emerging from the shell than the original HBV cores. The chimeric core particles elicited a strong N protein-specific antibody response in two mouse strains.

BTV forms icosahedral particles composed of two or three concentric shells (see Section III.C. Role of Viral Scaffolding Proteins). The outer and inner cores are composed of VP7 and VP3, respectively, as well as three minor proteins. The outer shell is composed of VP2 and VP5. CLPs are assembled in insect cells using the recombinant baculovirus system. Such particles have been used as foreign epitope carriers of peptide sequences from the Rabies virus glycoprotein (149) or the HBV preS2 region (150) that were introduced at the N terminus of the VP7 protein. Moreover, the BTV nonstructural protein NS1 of unknown function encoded by the RNA segment M6, when expressed in insect cells, produces tubular structures with a helical conformation. These chimeric structures have been successfully used for epitope presentation. Introducing sequences (ranging from amino acids 44 to 116) from the *Clostridium difficile* toxin A, the HBV preS2 region, or the entire bovine leukemia virus p15 protein at the C terminus of NS1 produced highly immunogenic tubules, in which the foreign epitope was exposed on the surface (131). Similar results were obtained when a peptide derived from VP1 of Foot and mouth disease virus or a peptide derived from the Influenza virus HA protein was used as epitope (151). The results demonstrate that the purified tubules elicit both humoral and cell-mediated responses.

Overexpressing part of open reading frame (ORF) 2 of Hepatitis E virus (HEV; family *Calicivirus*) in a baculovirus expression system produces VLPs

similar to mature HEV, although slightly smaller. Chimeric constructs in which a B-cell epitope was introduced into various insertion sites in the protein derived from ORF2 were used as oral vaccines. The C terminus of ORF2 was the only appropriate site for insertion of the foreign epitope; it induced specific IgG and IgA to the epitope and to the VLP in intestinal fluids (152).

To study the formation and release of nonchimeric Influenza virus VLPs from the surface of insect cells, a quadruple baculovirus recombinant expressing simultaneously the viral neuramidase (NA), the HA protein, and the matrix M1 and M2 proteins was constructed. These four proteins were sufficient to produce VLPs that were secreted into the medium. Immunogold labeling and electron microscopy examination indicated that HA and NA were exposed on the surface of these wild-type VLPs. Furthermore, using the same expression system, replacing HA by the G protein of Vesicular stomatitis virus or by a hybrid containing the cytoplasmic tail and transmembrane domain of HA and the ectodomain of the G protein resulted in the formation of chimeric VLPs (153). Such a system may serve as a convenient tool for the development of novel vaccines.

VLPs of the flexible filamentous JGMV also yield highly ordered aggregates forming particles in which vaccine subunits are presented. Short peptides or large antigens of foreign epitopes, such as the merozoite surface antigen of malaria and/or the luteinizing hormone releasing hormone fused to, or replacing, the N- or C-termini of the JGMV CP, were highly immunogenic in mice and rabbits without adjuvant (154). Moreover, the JGMV CP expressed in a recombinant Vaccinia virus to sufficiently high levels led to the formation of potyviral VLPs in mammalian cells (155). Synthesis of such self-adjuvanting molecules carrying foreign epitopes without the need of protein purification before vaccination would be of immense value.

As has been discussed (Section III.C) in Rotavirus VLP assembly, the N terminus of VP2 of double-layered VLPs composed of VP2 and VP6 is dispensable for the formation of such particles. The GFP protein was introduced at the N terminus of VP2 as model insert (156). VP2 thus modified assembled correctly into VLPs when coexpressed with VP6, and the GFP molecules were present inside the core at the 5-fold vertices. Such chimeric particles enhanced by the presence of the outer capsid layer VP4 and VP7 were able to penetrate mammalian cells, demonstrating their usefulness in macromolecule delivery into living cells.

The major coat protein VP1 of polyomavirus was shown to be competent in forming VLPs when it presented a foreign protein such as the *E. coli*-derived dihydrofolate reductase on its surface (157). Further engineering of the surface of polyomavirus VLPs by modifying the natural binding site of VP1 to mammalian cells resulted in particles that could serve as epitope-presenting systems, e.g., for tumor-specific antibody fragments (158). Evaluation of this

system showed that it resulted in VLPs with the ability of cell type specific gene transfer whose main role was played by a tumor specific antibody, the Fv fragment, which allows highly specific attachment and cell entry of the chimeric VLPs (159). Such cell entry was demonstrated for a variant of VP1 fused to protein Z, which is an engineered antibody-binding domain of protein A from *Staphylococcus aureus*; in this case, antibodies directed against the receptor tyrosine kinase ErbB2 allowed specific transduction of mammalian ErbB2-positive cells with plasmids encoding eGFP or beta-galactosidase (160).

IV. Concluding Remarks

VLP technology is a rapidly advancing domain of molecular and structural biology. Extensive progress in VLP studies was achieved since the insect cell-based protein production system was developed. This baculovirus expression system has many advantages for the synthesis of viral structural proteins resulting in the formation of VLPs. It allows production of large amounts of correctly folded proteins while also providing cell membranes that can serve as structural elements for enveloped viruses. These features give us the opportunity to gain insights into the interactions and requirements accompanying VLP formation that are similar to the assembly events occurring in mammalian cells. Other encouraging elements are the ability to easily scale-up the system and the simplicity of purification of the assembled VLPs.

To date, aspects of VLP technology are mostly concentrated on three areas. The first focuses on the characterization and extension of our knowledge of viral structural elements participating in the assembly process of particular VLPs. The main efforts are directed toward solving the structure of VLPs and defining the rules managing their formation. The second concentrates mostly on the development of new VLP-based vaccines. Special attention is given to chimeric VLPs, in particular, those that can expose foreign epitopes on their surface. The third area of interest focuses basically on engineering effective carry/delivery systems, new vehicles useful in gene therapy (161), and virus trafficking studies.

Structural characterization of VLPs is mainly performed by X ray crystallography and advanced EM techniques with the help of computational analyses, and focuses on the comparison of VLPs composed of different numbers and combinations of structural proteins (162). The use of biochemical methods provides not only details on individual viral structural components, but also answers questions regarding the structural basis of assembly, packaging, and the interactions of VLPs with host components. Intensive studies are aimed at characterizing the minimal requirements for VLP formation and on prospects of modifying the original proteins without hampering the natural ability of

these proteins to assemble into highly organized macromolecules. As a consequence, modified structural proteins appropriate for assembly of multipurpose chimeric VLPs can be designed. Our growing knowledge of the rules directing VLP assembly makes it possible to engineer particles composed of fusion proteins carrying viral structural domains and foreign epitopes. It has been shown that such modifications can lead to the formation of VLPs which are highly effective as subunit vaccines and which can mimic the overall structure of virus particles without containing the viral genetic material. The use of VLPs as novel vaccines and immunogens has been widely reviewed (*129, 163*).

The growing number of VLPs carrying foreign protein fragments on their surface and studies on the successful assembly of these chimeric molecules is a promising avenue towards the development of a new technology in which the newly designed VLPs will be directed to particular mammalian cell types by exposing specific binding domains. The progress made in modeling the surface of VLPs makes them to date the best candidates for the design of delivery systems that can efficiently reach their targets.

ACKNOWLEDGMENTS

This work was supported in part by the Center of Excellence of Molecular Biotechnology (Poland), by the Indo-French Center for the Promotion of Advanced Research, by the French-Polish Center of Plant Biotechnology, the CNRS (France), and COLCIENCIAS (Colombia).

REFERENCES

1. Roy, P. (1996). Genetically engineered particulate virus-like structures and their use as vaccine delivery systems. *Intervirology* **39**, 62–71.
2. Casal, J. I. (2001). Use of the baculovirus expression system for the generation of virus-like particles. *Biotechnol. Genetic Engineering Rev.* **18**, 73–87.
3. Ou, W. C., Chen, L. H., Wang, M., Hseu, T. H., and Chang, D. (2001). Analysis of minimal sequences on JC virus VP1 required for capsid assembly. *J. Neurovirol.* **7**, 298–301.
4. Pumpens, P., and Grens, E. (2001). HBV core particles as a carrier for B cell/T cell epitopes. *Intervirology* **44**, 98–114.
5. Schiller, J. T., and Lowy, D. R. (2001). Papillomavirus-like particle based vaccines: Cervical cancer and beyond. *Expert Opin. Biol. Therapy* **1**, 571–581.
6. Liljas, L. (1999). Virus assembly. *Curr. Opin. Struct. Biol.* **9**, 129–134.
7. Zlotnick, A., Aldrich, R., Johnson, J. M., Ceres, P., and Young, M. J. (2000). Mechanism of capsid assembly for an icosahedral plant virus. *Virology* **277**, 450–456.
8. Jiang, X., Wang, M., Graham, D. Y., and Estes, M. K. (1992). Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* **66**, 6527–6532.
9. Green, K. Y., Lew, J. F., Jiang, X., Kapikian, A. Z., and Estes, M. K. (1993). Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of

- the native Norwalk virus antigen in serologic assays and some epidemiologic observations. *J. Clin. Microbiol.* **31**, 2185–2191.
10. Venkataram Prasad, B. V., Hardy, M. E., and Estes, M. K. (2000). Structural studies of recombinant Norwalk capsids. *J. Infect. Diseases* **181**, 317–321.
 11. Bertolotti-Ciarlet, A., White, L. J., Chen, R., Prasad, B. V., and Estes, M. K. (2002). Structural requirements for the assembly of Norwalk virus-like particles. *J. Virol.* **76**, 4044–4055.
 12. Baric, R. S., Yount, B., Lindesmith, L., Harrington, P. R., Greene, S. R., Tseng, F. C., Davis, N., Johnston, R. E., Klapper, D. G., and Moe, C. L. (2002). Expression and self-assembly of Norwalk virus capsid protein from Venezuelan equine encephalitis virus replicons. *J. Virol.* **76**, 3023–3030.
 13. Glass, P. J., White, J. M., Ball, J. M., Leparco-Goffart, I., Hardy, M. E., and Estes, M. K. (2000). Norwalk virus open reading frame 3 encodes a minor structural protein. *J. Virol.* **74**, 6581–6591.
 14. Glass, P. J., Zeng, C. Q., and Estes, M. K. (2003). Two nonoverlapping domains on the Norwalk virus open reading frame 3 (ORF3) protein are involved in the formation of the phosphorylated 35K protein and in ORF3-capsid protein interactions. *J. Virol.* **77**, 3569–3577.
 15. Bertolotti-Ciarlet, A., Crawford, S. E., Hutson, A. M., and Estes, M. K. (2003). The 3' end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: A novel function for the VP2 protein. *J. Virol.* **77**, 11603–11615.
 16. Lochridge, V. P., and Hardy, M. E. (2003). Snow Mountain virus genome sequence and virus-like particle assembly. *Virus Genes* **26**, 71–82.
 17. Croizier, L., Jousset, F. X., Veyrunes, J. C., Lopez-Ferber, M., Bergoin, M., and Croizier, G. (2000). Protein requirements for assembly of virus-like particles of Junonia coenia densovirus in insect cells. *J. Gen. Virol.* **81**, 1605–1613.
 18. Hoque, M. N., Shimizu, K., Ishizu, H., Yajima, F., Arisaka, K., Suzuki, H., Watanabe, H., and Handa, H. (1999). Chimeric virus-like particle formation of adeno-associated virus. *Biochem. Biophys. Res. Commun.* **266**, 371–376.
 19. Wellink, J., Verver, J., Van, LentJ., and Van Kammen, A. (1996). Capsid proteins of cowpea mosaic virus transiently expressed in protoplasts form virus-like particles. *Virology* **224**, 352–355.
 20. Shanks, M., and Lomonosoff, G. P. (2000). Coexpression of the capsid proteins of Cowpea mosaic virus in insect cells leads to the formation of virus-like particles. *J. Gen. Virol.* **81**, 3093–3097.
 21. Zhang, F., Li, Y., Liu, Y., An, C., and Chen, Z. (1997). Molecular cloning, sequencing, functional analysis, and expression in *E. coli* of major core protein gene (S3) of rice dwarf virus Chinese isolate. *Acta Virologica* **41**, 161–168.
 22. Zheng, H., Yu, L., Wei, C., Hu, D., Shen, Y., Chen, Z., and Li, Y. (2000). Assembly of double-shelled, virus-like particles in transgenic rice plants expressing two major structural proteins of rice dwarf virus. *J. Virol.* **74**, 9808–9810.
 23. Hagiwara, K., Higashi, T., Namba, K., Uehara-Ichiki, T., and Omura, T. (2003). Assembly of single-shelled cores and double-shelled virus-like particles after baculovirus expression of major structural proteins P3, P7, and P8 of Rice dwarf virus. *J. Gen. Virol.* **84**, 981–984.
 24. Hagiwara, K., Higashi, T., Miyazaki, N., Naitow, H., Cheng, R. H., Nakagawa, A., Mizuno, H., Tsukihara, T., and Omura, T. (2004). The amino-terminal region of major capsid protein P3 is essential for self-assembly of single-shelled core-like particles of Rice dwarf virus. *J. Virol.* **78**, 3145–3148.
 25. Krishna, S. S., Hiremath, C. N., Munshi, S. K., Prahadeeswaran, D., Sastri, M., Savithri, H. S., and Murthy, M. R. (1999). Three-dimensional structure of physalis mottle virus: Implications for the viral assembly. *J. Mol. Biol.* **289**, 919–934.

26. Krishna, S. S., Sastri, M., Savithri, H. S., and Murthy, M. R. (2001). Structural studies on the empty capsids of Physalis mottle virus. *J. Mol. Biol.* **307**, 1035–1047.
27. Sastri, M., Kekuda, R., Gopinath, K., Kumar, C. T., Jagath, J. R., and Savithri, H. S. (1997). Assembly of physalis mottle virus capsid protein in *Escherichia coli* and the role of amino and carboxy termini in the formation of the icosahedral particles. *J. Mol. Biol.* **272**, 541–552.
28. Sastri, M., Reddy, D. S., Krishna, S. S., Murthy, M. R., and Savithri, H. S. (1999). Identification of a discrete intermediate in the assembly/disassembly of physalis mottle tymovirus through mutational analysis. *J. Mol. Biol.* **289**, 905–918.
29. Umashankar, M., Murthy, M. R., and Savithri, H. S. (2003). Mutation of interfacial residues disrupts subunit folding and particle assembly of Physalis mottle tymovirus. *J. Biol. Chem.* **278**, 6145–6152.
30. Garcea, R. L., Salunke, D. M., and Caspar, D. L. (1987). Site-directed mutation affecting polyomavirus capsid self-assembly *in vitro*. *Nature* **329**, 86–87.
31. Yusibov, V., Kumar, A., North, A., Johnson, J. E., and Loesch-Fries, L. S. (1996). Purification, characterization, assembly, and crystallization of assembled alfalfa mosaic virus coat protein expressed in *Escherichia coli*. *J. Gen. Virol.* **77**, 567–573.
32. Choi, J., and Loesch-Fries, L. S. (1999). Effect of C-terminal mutations of alfalfa mosaic virus coat protein on dimer formation and assembly *in vitro*. *Virology* **260**, 182–189.
33. Lamb, J. W., Duncan, G. H., Reavy, B., Gildow, F. E., Mayo, M. A., and Hay, R. T. (1996). Assembly of virus-like particles in insect cells infected with a baculovirus containing a modified coat protein gene of potato leafroll luteovirus. *J. Gen. Virol.* **77**, 1349–1358.
34. Perera, R., Owen, K. E., Tellinghuisen, T. L., Gorbalenya, A. E., and Kuhn, R. J. (2001). Alphavirus nucleocapsid protein contains a putative coiled coil alpha-helix important for core assembly. *J. Virol.* **75**, 1–10.
35. Perera, R., Navaratnarajah, C., and Kuhn, R. J. (2003). A heterologous coiled coil can substitute for helix I of the Sindbis virus capsid protein. *J. Virol.* **77**, 8345–8353.
36. Butler, P. J. (1999). Self-assembly of tobacco mosaic virus: The role of an intermediate aggregate in generating both specificity and speed. *Philosophical Transactions: Biological Sciences. The Royal Society London* **354**, 537–550.
37. Hwang, D. J., Roberts, I. M., and Wilson, T. M. (1994). Expression of tobacco mosaic virus coat protein and assembly of pseudovirus particles in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **91**, 9067–9071.
38. Bragard, C., Duncan, G. H., Wesley, S. V., Naidu, R. A., and Mayo, M. A. (2000). Virus-like particles assemble in plants and bacteria expressing the coat protein gene of Indian peanut clump virus. *J. Gen. Virol.* **81**, 267–272.
39. Jagadish, M. N., Ward, C. W., Gough, K. H., Tulloch, P. A., Whittaker, L. A., and Shukla, D. D. (1991). Expression of potyvirus coat protein in *Escherichia coli* and yeast and its assembly into virus-like particles. *J. Gen. Virol.* **72**, 1543–1550.
40. Jagadish, M. N., Huang, D., and Ward, C. W. (1993). Site-directed mutagenesis of a potyvirus coat protein and its assembly in *Escherichia coli*. *J. Gen. Virol.* **74**, 893–896.
41. Voloudakis, A. E., Malpica, C. A., Aleman-Verdagner, M. E., Stark, D. M., Fauquet, C. M., and Beachy, R. N. (2004). Structural characterization of Tobacco etch virus coat protein mutants. *Arch. Virol.* **149**, 699–712.
42. Joseph, J., and Savithri, H. S. (1999). Determination of 3'-terminal nucleotide sequence of pepper vein banding virus RNA and expression of its coat protein in *Escherichia coli*. *Arch. Virol.* **144**, 1679–1687.
43. Anindya, R., and Savithri, H. S. (2003). Surface-exposed amino- and carboxy-terminal residues are crucial for the initiation of assembly in Pepper vein banding virus: A flexuous rod-shaped virus. *Virology* **316**, 325–336.

44. Dokland, T. (2000). Freedom and restraint: Themes in virus capsid assembly. *Structure Folding Design* **8**, R157–R162.
45. Thomsen, D. R., Newcomb, W. W., Brown, J. C., and Homa, F. L. (1995). Assembly of the herpes simplex virus capsid: Requirement for the carboxyl-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes. *J. Virol.* **69**, 3690–3703.
46. Tatman, J. D., Preston, V. G., Nicholson, P., Elliott, R. M., and Rixon, F. J. (1994). Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *J. Gen. Virol.* **75**, 1101–1113.
47. Kennard, J., Rixon, F. J., McDougall, I. M., Tatman, J. D., and Preston, V. G. (1995). The 25 amino acid residues at the carboxy terminus of the herpes simplex virus type 1 UL26.5 protein are required for the formation of the capsid shell around the scaffold. *J. Gen. Virol.* **76**, 1611–1621.
48. Matusick-Kumar, L., Newcomb, W. W., Brown, J. C., McCann, P. J., Hurlburt, W., Weinheimer, S. P., and Gao, M. (1995). The C-terminal 25 amino acids of the protease and its substrate ICP35 of herpes simplex virus type 1 are involved in the formation of sealed capsids. *J. Virol.* **69**, 4347–4356.
49. Newcomb, W. W., Juhas, R. N., Thomsen, D. R., Homa, F. L., Burch, A. D., Weller, S. K., and Brown, J. C. (2001). The UL6 gene product forms the portal for the entry of DNA into the herpes simplex virus capsid. *J. Virol.* **75**, 10923–10932.
50. Crawford, S. E., Labbe, M., Cohen, J., Burroughs, M. H., Zhou, Y. J., and Estes, M. K. (1994). Characterization of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. *J. Virol.* **68**, 5945–5952.
51. Lawton, J. A., Zeng, C. Q.-Y., Mukherjee, S. K., Cohen, J., Estes, M. K., and Venkataram Prasad, B. V. (1997). Three-dimensional structural analysis of recombinant rotavirus-like particles with intact and amino-terminal-deleted VP2: Implications for the architecture of the VP2 capsid layer. *J. Virol.* **71**, 7353–7360.
52. Zeng, C. Q., Estes, M. K., Charpilienne, A., and Cohen, J. (1998). The N terminus of rotavirus VP2 is necessary for encapsidation of VP1 and VP3. *J. Virol.* **72**, 201–208.
53. Crawford, S. E., Estes, M. K., Ciarlet, M., Barone, C., O'Neal, C. M., Cohen, J., and Conner, M. E. (1999). Heterotypic protection and induction of a broad heterotypic neutralization response by rotavirus-like particles. *J. Virol.* **73**, 4813–4822.
54. Kim, Y., Chang, K. O., Kim, W. Y., and Saif, L. J. (2002). Production of hybrid double- or triple-layered virus-like particles of group A and C rotaviruses using a baculovirus expression system. *Virology* **302**, 1–8.
55. Tosser, G., Labbe, M., Bremont, M., and Cohen, J. (1992). Expression of the major capsid protein VP6 of group C rotavirus and synthesis of chimeric single-shelled particles by using recombinant baculovirus. *J. Virol.* **66**, 5825–5831.
56. French, T. J., and Roy, P. (1990). Synthesis of bluetongue virus (BTV) core-like particles by a baculovirus expressing the two major structural proteins of BTV. *J. Virol.* **64**, 1530–1536.
57. Roy, P., Mikhailov, M., and Bishop, D. H. (1997). Baculovirus multigene expression vectors and their use for understanding the assembly process of architecturally complex virus particles. *Gene* **190**, 119–129.
58. French, T. J., Marshall, J. J., and Roy, P. (1990). Assembly of double-shelled, virus-like particles of bluetongue virus by the simultaneous expression of four structural proteins. *Virology* **64**, 5695–5700.
59. Limn, C. K., Staeuber, N., Monastyrskaya, K., Gouet, P., and Roy, P. (2000). Functional dissection of the major structural protein of bluetongue virus: Identification of key residues within VP7 essential for capsid assembly. *J. Virol.* **74**, 8658–8669.
60. Tanaka, S., and Roy, P. (1994). Identification of domain in bluetongue virus VP3 molecules essential for the assembly of virus cores. *J. Virol.* **68**, 2795–2802.

61. Limn, C. K., and Roy, P. (2003). Intermolecular interactions in a two-layered viral capsid that requires a complex symmetry mismatch. *J. Virol.* **77**, 11114–11124.
62. Ona, A., Luque, D., Abaitua, F., Maraver, A., Caston, J. R., and Rodriguez, J. F. (2004). The C-terminal domain of the pVP2 precursor is essential for the interaction between VP2 and VP3, the capsid polypeptides of infectious bursal disease virus. *Virology* **322**, 135–142.
63. Maraver, A., Ona, A., Abaitua, F., Gonzalez, D., Clemente, R., Ruiz-Diaz, J. A., Caston, J. R., Pazos, F., and Rodriguez, J. F. (2003). The oligomerization domain of VP3, the scaffolding protein of infectious bursal disease virus, plays a critical role in capsid assembly. *J. Virol.* **77**, 6438–6449.
64. Chevalier, C., Lepault, J., Da Costa, B., and Delmas, B. (2004). The last C-terminal residue of VP3, glutamic acid 257, controls capsid assembly of infectious bursal disease virus. *J. Virol.* **78**, 3296–3303.
65. Kaito, M. K., Watanabe, S., Tsukiyama-Kohara, K., Yamaguchi, K., Kobayashi, Y., Konishi, M., Yokoi, M., Ishida, S., Suzuki, S., and Kohara, M. (1994). Hepatitis C virus particle detected by immunoelectron microscopic study. *J. Gen. Virol.* **75**, 1755–1760.
66. Shimizu, Y. K., Feinstone, S. M., Kohara, M., Purcell, R. H., and Yoshikura, H. (1996). Hepatitis C virus: Detection of intracellular virus particles by electron microscopy. *Hepatology* **23**, 205–209.
67. Falcon, V., Garcia, C., de la Rosa, M. C., Menendez, I., Seoane, J., and Grillo, J. M. (1999). Ultrastructural and immunocytochemical evidences of core-particle formation in the methylotrophic *Pichia pastoris* yeast when expressing HCV structural proteins (core-E1). *Tissue Cell* **31**, 117–125.
68. Blanchard, E., Brand, D., Trassard, S., Goudeau, A., and Roingard, P. (2002). Hepatitis C virus-like particle morphogenesis. *J. Virol.* **76**, 4073–4079.
69. Baumert, T. F., Ito, S., Wong, D. T., and Liang, T. J. (1998). Hepatitis C virus structural proteins assemble into virus-like particles in insect cells. *J. Virol.* **72**, 3827–3836.
70. Lorenzo, L. J., Duenas-Carrera, S., Falcon, V., Acosta-Rivero, N., Gonzales, E., de la Rosa, M. C., Menendez, I., and Morales, J. (2001). Assembly of truncated HCV core antigen into virus-like particles in *Escherichia coli*. *Biochem. Biophys. Res. Commun* **281**, 962–965.
71. Kunkel, M., Lorneci, M., Rijnbrand, R., Lemon, S. M., and Watowich, S. J. (2001). Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. *J. Virol.* **75**, 2119–2129.
72. Lokesh, G. L., Gowri, T. D., Satheshkumar, P. S., Murthy, M. R., and Savithri, H. S. (2002). A molecular switch in the capsid protein controls the particle polymorphism in an icosahedral virus. *Virology* **292**, 211–223.
73. Bhuvaneshwari, M., Subramanya, H. S., Gopinath, K., Savithri, H. S., Nayudu, M. V., and Murthy, M. R. (1995). Structure of sesbania mosaic virus at 3 Å resolution. *Structure* **3**, 1021–1030.
74. Sangita, V., Parthasarathy, S., Toma, S., Lokesh, G. L., Gowri, T. D., Satheshkumar, P. S., Savithri, H. S., and Murthy, M. R. (2002). Determination of the structure of the recombinant T = 1 capsid of sesbania mosaic virus. *Curr. Sci.* **82**, 1123–1131.
75. Choi, Y. G., Dreher, T. W., and Rao, A. L. (2002). tRNA elements mediate the assembly of an icosahedral RNA virus. *Proc. Natl. Acad. Sci. USA* **99**, 655–660.
76. Fisher, J., McKinney, B. R., Schneemann, A., Rueckert, R. R., and Johnson, J. E. (1993). Crystallization of virus-like particles assembled from flock house virus coat protein expressed in a baculovirus. *J. Virol.* **67**, 2950–2953.
77. Schneemann, A., Dasgupta, R., Johnson, J. E., and Rueckert, R. R. (1993). Use of recombinant baculoviruses in synthesis of morphologically distinct virus-like particles of flock house virus, a nodavirus. *J. Virol.* **67**, 2756–2763.

78. Dong, X. F., Natarajan, P., Tihova, M., Johnson, J. E., and Schneemann, A. (1998). Particle polymorphism caused by deletion of a peptide molecular switch in a quasiaequivalent icosahedral virus. *J. Virol.* **72**, 6024–6033.
79. Marshall, D., and Schneemann, A. (2001). Specific packaging of nodaviral RNA2 requires the N-terminus of the capsid protein. *Virology* **285**, 165–175.
80. Lin, C. S., Lu, M. W., Tang, L., Liu, W., Chao, C. B., Lin, C. J., Krishna, N. K., Johnson, J. E., and Schneemann, A. (2001). Characterization of virus-like particles assembled in a recombinant baculovirus system expressing the capsid protein of a fish nodavirus. *Virology* **290**, 50–58.
81. Tellinghuisen, T. L., Hamburger, A. E., Fisher, B. R., Ostendorp, R., and Kuhn, R. J. (1999). *In vitro* assembly of alphavirus cores by using nucleocapsid protein expressed in *Escherichia coli*. *J. Virol.* **73**, 5309–5319.
82. Tellinghuisen, T. L., and Kuhn, R. J. (2000). Nucleic acid-dependent cross-linking of the nucleocapsid protein of Sindbis virus. *J. Virol.* **74**, 4302–4309.
83. Tellinghuisen, T. L., Perera, R., and Kuhn, R. J. (2001). *In vitro* assembly of Sindbis virus core-like particles from cross-linked dimers of truncated and mutant capsid proteins. *J. Virol.* **75**, 2810–2817.
84. Yu, F., Joshi, S. M., Ma, Y. M., Kingston, R. L., Simon, M. N., and Vogt, V. M. (2001). Characterization of Rous sarcoma virus Gag particles assembled *in vitro*. *J. Virol.* **75**, 2753–2764.
85. Johnson, M. C., Scobie, H. M., Ma, Y. M., and Vogt, V. M. (2002). Nucleic acid-independent retrovirus assembly can be driven by dimerization. *J. Virol.* **76**, 11177–11185.
86. Ma, Y. M., and Vogt, V. M. (2002). Rous sarcoma virus Gag protein–oligonucleotide interaction suggests a critical role for protein dimer formation in assembly. *J. Virol.* **76**, 5452–5462.
87. Ma, Y. M., and Vogt, V. M. (2004). Nucleic acid binding-induced Gag dimerization in the assembly of Rous sarcoma virus particles *in vitro*. *J. Virol.* **78**, 52–60.
88. Delchambre, M., Gheysen, D., Thines, D., Thiriart, C., Jacobs, E., Verdin, E., Horth, M., Burny, A., and Bex, F. (1989). The GAG precursor of simian immunodeficiency virus assembles into virus-like particles. *EMBO J.* **8**, 2653–2660.
89. Gheysen, D., Jacobs, E., de Foresta, F., Thiriart, C., Francotte, M., Thines, D., and De Wilde, M. (1989). Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**, 103–112.
90. Karacostas, V., Nagashima, K., Gonda, M. A., and Moss, B. (1989). Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. *Proc. Natl. Acad. Sci. USA* **86**, 8964–8967.
91. Sakuragi, S., Goto, T., Sano, K., and Morikawa, Y. (2002). HIV type 1 Gag virus-like particle budding from spheroplasts of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **99**, 7956–7961.
92. Shaw, K. L., Lindemann, D., Mulligan, M. J., and Goepfert, P. A. (2003). Foamy virus envelope glycoprotein is sufficient for particle budding and release. *J. Virol.* **77**, 2338–2348.
93. Hobman, T. C., Lundstrom, M. L., Mauracher, C. A., Woodward, L., Gillam, S., and Farquhar, M. G. (1994). Assembly of rubella virus structural proteins into virus-like particles in transfected cells. *Virology* **202**, 574–585.
94. Garbutt, M., Chan, H., and Hobman, T. C. (1999). Secretion of rubella virions and virus-like particles in cultured epithelial cells. *Virology* **261**, 340–346.
95. Qiu, Z., Ou, D., Hobman, T. C., and Gillam, S. (1994). Expression and characterization of virus-like particles containing rubella virus structural proteins. *J. Virol.* **68**, 4086–4091.
96. Garbutt, M., Law, L. M., Chan, H., and Hobman, T. C. (1999). Role of rubella virus glycoprotein domains in assembly of virus-like particles. *J. Virol.* **73**, 3524–3533.

97. Vennema, H., Godeke, G. J., Rossen, J. W., Voorhout, W. F., Horzinek, M. C., Opstelten, D. J., and Rottier, P. J. (1996). Nucleocapsid-independent assembly of coronavirus-like particles by coexpression of viral envelope protein genes. *EMBO J.* **15**, 2020–2028.
98. de Haan, C. A., Kuo, L., Masters, P. S., Vennema, H., and Rottier, P. J. (1998). Coronavirus particle assembly: Primary structure requirements of the membrane protein. *J. Virol.* **72**, 6838–6850.
99. de Haan, C. A., Vennema, H., and Rottier, P. J. (2000). Assembly of the coronavirus envelope: Homotypic interactions between the M proteins. *J. Virol.* **74**, 4967–4978.
100. Corse, E., and Machamer, C. E. (2003). The cytoplasmic tails of infectious bronchitis virus E and M proteins mediate their interaction. *Virology* **312**, 25–34.
101. Gomez-Puertas, P., Albo, C., Perez-Pastrana, E., Vivo, A., and Portela, A. (2000). Influenza virus matrix protein is the major driving force in virus budding. *J. Virol.* **74**, 11538–11547.
102. Coronel, C., Murti, K. G., Takimoto, T., and Portner, A. (1999). Human parainfluenza virus type 1 matrix and nucleoprotein genes transiently expressed in mammalian cells induce the release of virus-like particles containing nucleocapsid like structures. *J. Virol.* **73**, 7035–7038.
103. Timmins, J., Scianimanico, S., Schoehn, G., and Weissenhorn, W. (2001). Vesicular release of ebola virus matrix protein VP40. *Virology* **283**, 1–6.
104. Jasenosky, L. D., Neumann, G., Lukashevich, I., and Kawaoka, Y. (2001). Ebola virus VP40-induced particle formation and association with the lipid bilayer. *J. Virol.* **75**, 5205–5214.
105. Noda, T., Sagara, H., Suzuki, E., Takada, A., Kida, H., and Kawaoka, Y. (2002). Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP. *J. Virol.* **76**, 4855–4865.
106. Swenson, D. L., Warfield, K. L., Kuehl, K., Larsen, T., Hevey, M. C., Schmaljohn, A., Bavari, S., and Aman, M. J. (2004). Generation of Marburg virus-like particles by coexpression of glycoprotein and matrix protein. *FEMS Immunol. Med. Microbiol.* **40**, 27–31.
107. Schmitt, A. P., Leser, G. P., Waning, D. L., and Lamb, R. A. (2002). Requirements for budding of paramyxovirus simian virus 5 virus-like particles. *J. Virol.* **76**, 3952–3964.
108. Salunke, D. M., Caspar, D. L., and Garcea, R. L. (1986). Self-assembly of purified polyomavirus capsid protein VP1. *Cell* **46**, 895–904.
109. Montross, L., Watkins, S., Moreland, R. B., Mamon, H., Caspar, D. L., and Garcea, R. L. (1991). Nuclear assembly of polyomavirus capsids in insect cells expressing the major capsid protein VP1. *J. Virol.* **65**, 4991–4998.
110. Chang, C. Y., Fung, W. C., Ou, P. C., Chao, S. Y., Li, M., Wang, Y. L., Huang, T. Y., Tzeng, and Tsai, R. T. (1997). Self-assembly of the JC virus major capsid protein, VP1, expressed in insect cells. *J. Gen. Virol.* **78**, 1435–1439.
111. Ishizu, K. I., Watanabe, H., Han, S. I., Kanesashi, S. N., Hoque, M., Yajima, H., Kataoka, K., and Handa, H. (2001). Roles of disulfide linkage and calcium ion-mediated interactions in assembly and disassembly of virus-like particles composed of simian virus 40 VP1 capsid protein. *J. Virol.* **75**, 61–72.
112. Chen, P.-L., Wang, M., Ou, W.-C., Lii, C.-K., Chen, L.-S., and Chang, D. (2001). Disulfide bonds stabilize JC virus capsid-like structure by protecting calcium ions from chelation. *FEBS Lett.* **500**, 109–113.
113. Sangita, V., Lokesh, G. L., Satheshkumar, P. S., Vijay, C. S., Saravanan, V., Savithri, H. S., and Murthy, M. R. (2004). T = 1 capsid structures of Sesbania mosaic virus coat protein mutants: Determinants of T = 3 and T = 1 capsid assembly. *J. Mol. Biol.* **342**, 987–999.
114. Satheshkumar, P. S., Lokesh, G. L., Sangita, V., Saravanan, V., Vijay, C. S., Murthy, M. R., and Savithri, H. S. (2004). Role of metal ion-mediated interactions in the assembly and stability of Sesbania mosaic virus T = 3 and T = 1 capsids. *J. Mol. Biol.* **342**, 1001–1014.

115. Kanesashi, S. N., Ishizu, K., Kawano, M. A., Han, S. I., Tomita, S., Watanabe, H., Kataoka, K., and Handa, H. (2003). Simian virus 40 VP1 capsid protein forms polymorphic assemblies *in vitro*. *J. Gen. Virol.* **84**, 1899–1905.
116. Sapp, M., Volpers, C., Muller, M., and Streeck, R. E. (1995). Organization of the major and minor capsid proteins in human papillomavirus type 33 virus-like particles. *J. Gen. Virol.* **76**, 2407–2412.
117. Sapp, M., Fligge, C., Petzak, I., Harris, J. R., and Streeck, R. E. (1998). Papillomavirus assembly requires trimerization of the major capsid protein by disulfides between two highly conserved cysteines. *J. Virol.* **72**, 6186–6189.
118. Buonamassa, T., Greer, C. E., Capo, S., Yen, T. S., Galeotti, C. L., and Bensi, G. (2002). Yeast coexpression of human papillomavirus types 6 and 16 capsid proteins. *Virology* **293**, 335–344.
119. Pringle, F. M., Gordon, K. H., Hanzlik, T. N., Kalkmakoff, J., Scotti, P. D., and Ward, V. K. (1999). A novel capsid expression strategy for *Thosea asigna* virus (Tetraviridae). *J. Gen. Virol.* **80**, 1855–1863.
120. Pringle, F. M., Kalkmakoff, J., and Ward, V. K. (2001). Analysis of the capsid processing strategy of *Thosea asigna* virus using baculovirus expression of virus-like particles. *J. Gen. Virol.* **82**, 259–266.
121. O'Brien, G. J., Bryant, C. J., Voogd, C., Greenberg, H. B., Gardner, R. C., and Bellamy, A. R. (2000). Rotavirus VP6 expressed by PVX vectors in *Nicotiana benthamiana* coats PVX rods and also assembles into virus-like particles. *Virology* **270**, 444–453.
122. Chevalier, J., Lepault, J., Erk, I., Da Costa, B., and Delmas, B. (2002). The maturation process of pVP2 requires assembly of infectious bursal disease virus capsids. *J. Virol.* **76**, 2384–2392.
123. Johnson, M. C., Scobie, H. M., and Vogt, V. M. (2000). PR domain of rous sarcoma virus Gag causes an assembly/budding defect in insect cells. *J. Virol.* **75**, 4407–4412.
124. Bouamr, F., Garnier, L., Rayne, F., Verna, A., Rebeyrotte, N., Cerutti, M., and Mamoun, R. Z. (2000). Differential budding efficiencies of human T-cell leukemia virus type I (HTLV-I) Gag and Gag-Pro polyproteins from insect and mammalian cells. *Virology* **278**, 597–609.
125. Adamson, C. S., Nermut, M., and Jones, I. M. (2003). Control of human immunodeficiency virus type-1 protease activity in insect cells expressing Gag-Pol rescues assembly of immature but not mature virus-like particles. *Virology* **308**, 157–165.
126. Martin-Serrano, J., Zang, T., and Bieniasz, P. D. (2001). HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. *Nat. Med.* **7**, 1313–1319.
127. Martin-Serrano, J., Perez-Caballero, D., and Bieniasz, P. D. (2004). Context-dependent effects of L domains and ubiquitination on viral budding. *J. Virol.* **78**, 5554–5563.
128. Timmins, J., Schoehn, G., Ricard-Blum, S., Scianimanico, S., Vernet, T., Ruigrok, R. W., and Weissenhorn, W. (2003). Ebola virus matrix protein VP40 interaction with human cellular factors Tsg101 and Nedd4. *J. Mol. Biol.* **326**, 493–502.
129. Noad, R., and Roy, P. (2003). Virus-like particles as immunogens. *Trends Microbiol.* **11**, 438–444.
130. Miyamura, K., Kajigaya, S., Momoeda, M., Smith-Gill, S. J., and Young, N. S. (1994). Parvovirus particles as platforms for protein presentation. *Proc. Natl. Acad. Sci. USA* **91**, 8507–8511.
131. Mikhailov, M., Monastyrskaya, K., Bakker, T., and Roy, P. (1996). A new form of particulate single and multiple immunogen delivery system based on recombinant bluetongue virus-derived tubules. *Virology* **217**, 323–331.
132. Taylor, K. M., Lin, T., Porta, C., Mosser, A. G., Giesing, H. A., Lomonosoff, G. P., and Johnson, J. E. (2000). Influence of three-dimensional structure on the immunogenicity of a peptide expressed on the surface of a plant virus. *J. Mol. Recognit.* **13**, 71–82.

133. Rueda, P., Moron, G., Sarraseca, J., Leclerc, C., and Casal, J. I. (2004). Influence of flanking sequences on presentation efficiency of a CD8⁺ cytotoxic T-cell epitope delivered by parvovirus-like particles. *J. Gen. Virol.* **85**, 563–572.
134. Lopez de Turiso, J. A., Cortes, E., Martinez, C., Ruiz de Ybanez, R., Simarro, I., Vela, C., and Casal, I. (1992). Recombinant vaccine for parvovirus in dogs. *J. Virol.* **66**, 2748–2753.
135. Saliki, J. T., Mizak, B., Flore, H. P., Gettig, R. R., Burand, J. P., Carmichael, L. E., Wood, H. A., and Parrish, C. R. (1992). Canine parvovirus empty capsids produced by expression in a baculovirus vector: Use in analysis of viral properties and immunization of dogs. *J. Gen. Virol.* **73**, 369–374.
136. Sedlik, C., Saron, M.-F., Sarraseca, J., Casal, I., and Leclerc, C. (1997). Recombinant parvovirus-like particles as an antigen carrier: Novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T-cells. *Proc. Natl. Acad. Sci. USA* **94**, 7503–7508.
137. Sedlik, C., Sarraseca, J., Rueda, P., Leclerc, C., and Casal, I. (1995). Immunogenicity of poliovirus B and T cell epitopes presented by hybrid porcine parvovirus particles. *J. Gen. Virol.* **76**, 2361–2368.
138. Rueda, P., Hurtado, A., del Barrio, M., Martinez-Torrecuadrada, J. L., Kamstrup, S., Leclerc, C., and Casal, J. I. (1999). Minor displacements in the insertion site provoke major differences in the induction of antibody responses by chimeric parvovirus-like particles. *Virology* **263**, 89–99.
139. Hurtado, A., Rueda, P., Nowicky, J., Sarraseca, J., and Casal, J. I. (1996). Identification of domains in canine parvovirus VP2 essential for the assembly of virus-like particles. *J. Virol.* **70**, 5422–5429.
140. Gilbert, L., Toivola, J., Lehtomaki, E., Donaldson, L., Käpylä, P., Vuento, M., and Oker-Blom, C. (2004). Assembly of fluorescent chimeric virus-like particles of canine parvovirus in insect cells. *Biochem. Biophys. Res. Commun.* **313**, 878–887.
141. Kang, S.-M., Yao, Q., Guo, L., and Compans, R. W. (2003). Mucosal immunization with virus-like particles of Simian immunodeficiency virus conjugated with cholera toxin subunit B. *J. Virol.* **77**, 9823–9830.
142. Guo, L., Lu, X., Kang, S.-M., Chen, C., Compans, R. W., and Yao, Q. (2003). Enhancement of mucosal immune response by chimeric influenza HA/SHIV virus-like particles. *Virology* **313**, 502–513.
143. Pumpens, P., Razanskas, R., Pushko, P., Renhof, R., Gusars, I., Skrastina, D., Ose, V., Borisova, G., Sominskaya, I., Petrovskis, I., Jansons, J., and Sasnauskas, K. (2002). Evaluation of HBs, HBc, and rCP virus-like particles for expression of human Papillomavirus 16 E7 oncoprotein epitopes. *Intervirology* **45**, 24–32.
144. Beterams, G., Bottecher, B., and Nassal, M. (2000). Packaging of up to 240 subunits of a 17 kDa nuclease into the interior of recombinant hepatitis B virus capsids. *FEBS Lett.* **481**, 169–176.
145. Kratz, P. A., Bottecher, B., and Nassal, M. (1999). Native display of complete foreign protein domains on the surface of hepatitis B virus capsids. *Proc. Natl. Acad. Sci. USA* **96**, 1915–1920.
146. Neiryneck, S., Deroo, T., Saelens, X., Vanlandschoot, P., Jou, W. M., and Fiers, W. (1999). A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat. Med.* **5**, 1157–1163.
147. Sällberg, M., Hughes, J., Jones, J., Phillips, T. R., and Milich, D. R. (2002). A malaria vaccine candidate based on an HBV core platform. *Intervirology* **45**, 350–361.
148. Geldmacher, A., Skrastina, D., Petrovskis, I., Borisova, G., Berriman, J. A., Roseman, A. M., Crowther, R. A., Fischer, J., Musema, S., Gelderblom, H. R., Lundkvist, A., Renhofa, R., Ose, V., Kruger, D. H., Pumpens, P., and Ulrich, R. (2004). An amino-terminal segment of hantavirus nucleocapsid protein presented on hepatitis B virus core particles induces a strong and highly cross-reactive antibody response in mice. *Virology* **323**, 108–119.

149. Le Blois, H., and Roy, P. (1993). A single point mutation in the VP7 major core protein of bluetongue virus prevents the formation of core-like particles. *J. Virol.* **67**, 353–359.
150. Belyaev, A. S., and Roy, P. (1992). Presentation of hepatitis B virus preS2 epitope on bluetongue core-like particles. *Virology* **190**, 840–844.
151. Ghosh, M. K., Borca, M. V., and Roy, P. (2002). Virus-derived tubular structure displaying foreign sequences on the surface elicit CD4+ Th cell and protective humoral responses. *Virology* **302**, 383–392.
152. Nikura, M., Takamura, S., Kim, G., Kawa, G., Saijo, M., Morikawa, S., Kurane, I., Li, T.-C., Takeda, S., and Yasutomi, Y. (2002). Chimeric recombinant Hepatitis E virus-like particles as an oral vaccine vehicle presenting foreign epitopes. *Virology* **293**, 273–280.
153. Latham, T., and Galarza, J. M. (2001). Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. *J. Virol.* **75**, 6154–6165.
154. Jagadish, M. N., Edwards, S. J., Hayden, M. B., Grusovin, J., Vandenberg, K., Schoofs, P., Hamilton, R. C., Shukla, D. D., Kalnins, H., McNamara, M., Haynes, J., Nisbet, I. T., Ward, C. W., and Pye, D. (1996). Chimeric potyvirus-like particles as vaccine carriers. *Intervirology* **39**, 85–92.
155. Hammond, J. M., Sproat, K. W., Wise, T. G., Hyatt, A. D., Jagadish, M. N., and Coupar, B. E. (1998). Expression of the potyvirus coat protein mediated by recombinant vaccinia virus and assembly of potyvirus-like particles in mammalian cells. *Arch. Virol.* **143**, 1433–1439.
156. Charpilienne, A., Nejmeddine, M., Berois, M., Parez, N., Neumann, E., Hewat, E., Trugnan, G., and Cohen, J. (2001). Individual rotavirus-like particles containing 120 molecules of fluorescent protein are visible in living cells. *J. Biol. Chem.* **276**, 29361–29367.
157. Gleiter, S., Stubenrauch, K., and Lilie, H. (1999). Changing the surface of a virus shell fusion of an enzyme to polyoma VP1. *Protein Sci.* **8**, 2562–2569.
158. Stubenrauch, K., Gleiter, S., Brinkmann, U., Rudolph, R., and Lilie, H. (2001). Conjugation of an antibody Fv fragment to a virus coat protein: Cell-specific targeting of recombinant polyoma-virus-like particles. *Biochem. J.* **356**, 867–873.
159. May, T., Gleiter, S., and Lilie, H. (2002). Assessment of cell type specific gene transfer of polyoma virus-like particles presenting a tumor-specific antibody Fv fragment. *J. Virol. Methods* **105**, 147–157.
160. Gleiter, S., and Lilie, H. (2003). Cell-type specific targeting and gene expression using a variant of polyoma VP1 virus-like particles. *Biol. Chem.* **384**, 247–255.
161. Petry, H., Goldmann, C., Ast, O., and Luke, W. (2003). The use of virus-like particles for gene transfer. *Curr. Opin. Mol. Therapeutics* **5**, 524–528.
162. Johnson, J. E., and Chiu, W. (2000). Structures of virus and virus-like particles. *Curr. Opin. Struct. Biol.* **10**, 229–235.
163. Lechner, F., Jegerlehner, A., Tissot, A. C., Maurer, P., Sebbel, P., Renner, W. A., Jennings, G. T., and Bachmann, M. F. (2002). Virus-like particles as a modular system for novel vaccines. *Intervirology* **45**, 212–217.