

Chimeric Virus-like Particle Formation of Adeno-Associated Virus

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Adeno-associated virus (AAV) capsids are composed of three proteins, VP1, VP2, and VP3. These capsid proteins have a common amino acid sequence, being expressed from different initiation codons on the same open reading frame. Although VP1 is necessary for viral infection, it is not essential for capsid formation. The other capsid proteins, VP2 and VP3, are sufficient for capsid formation, but their functions are poorly understood. To investigate the role(s) of the capsid proteins in capsid formation, we used a baculovirus protein expression system to produce virus-like particles (VLPs). We found that varying the ratios of VP2 and VP3 did not affect VLP formation. Further, their physical properties were equivalent to those of empty wild-type particles. The function of VP3 was studied further by fusing a peptide tag, FLAG, to its N-terminus. This chimeric viral protein, in combination with VP2, could assemble into VLPs, indicating that the chimerism of VP3 did not affect VLP formation. Although the monomeric native form of the FLAG-VP3 chimera could react with anti-FLAG antibody, VLP containing the chimeric VP3 could not, suggesting that the N-terminal region of VP3 is located inside the VLP. These observations indicate that it may be possible to utilize AAV VLP as vectors of a broad range of drugs since fusion of the VP3 N-terminus with defined molecules could impose distinct physical properties onto the internal environment of the VLP. © 1999 Academic Press

Adeno-associated virus (AAV) type 2, a nonpathogenic human parvovirus, contains a 4.7 kb single-stranded DNA (ssDNA) genome packaged into an 20–25 nm diameter icosahedral capsid (1, 2). The ge-

nome consists of two open reading frames (ORFs) which encode nonstructural (Rep) and structural (Cap) proteins, and the entire genome is flanked by two identical 145-base inverted terminal repeats (ITRs) (3). The capsid is composed of three proteins, VP1, VP2 and VP3, which have molecular masses of 87, 72 and 58 kDa, respectively. They are expressed from the same open reading frame by means of alternative initiation codons (4, 5). A 2.6 kb precursor RNA is spliced into two 2.3 kb mRNAs using the same donor but different acceptor sites (6). While VP2 and VP3 can be expressed from both mRNAs, VP1 is expressed from a minor mRNA. VP3 is efficiently expressed from an AUG initiation codon at position 2809, while VP2 is expressed with approximately 10-fold lower efficiency from an unusual initiation codon (ACG) at position 2614. The ratio of the three proteins in wild type AAV-2 is 1:1:10 (7) and this probably reflects the amounts expressed in the cell (8). Studies of mutant viruses have indicated that VP1 is necessary for infection but not for capsid formation (9–11). VP2 and VP3, however, are considered to be necessary for both capsid formation and the accumulation and sequestration of DNA, but the functions of each protein are as yet poorly defined.

In recent years, baculovirus and insect cells have been used to analyze the capsid formation of many viruses (12–16). This system is suitable for the study of virus-like particle (VLP) formation because only the capsid proteins can be expressed and the effects of other viral genes can be eliminated. Using this system, we were able to express the VP2 and VP3 proteins in various ratios in insect cells. We found that VLPs could be generated regardless of the VP2/VP3 ratio and that the ratios correlated with the ratios observed in cell extracts. Further, we found that when peptide-tagged VP3 was expressed with VP2, it was incorporated into the VLP, indicating that the N-terminal region of VP3 is quite flexible. Using this chimeric VP3, we could also show that the N-terminal region of VP3 is located

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inside the VLP. This suggests that it may be possible to alter the environment of the AAV protein cage by combining exogenous peptide to the N-terminal of VP3 and this in turn indicates the potential of AAV VLP as a broad range virus vector.

MATERIALS AND METHODS

Construction of recombinant baculoviruses. The genes encoding the capsid proteins were subcloned from pAV2, which contains the complete genome of AAV-2 (17). For the subcloning of the VP2 and VP3 genes, either *Bam*HI sites or *Bam*HI and *Eco*RI sites were introduced at both ends of the genes by PCR with *rth* polymerase. The PCR products were digested and subcloned into a transfer vector, pFastbacl and recombinant baculoviruses were generated according to the manufacturer's instructions (Invitrogen). For construction of the VP2 expression vector, the unusual translation initiation codon, ACG, was mutated to ATG. The DNA sequences of the recombined DNAs were determined by the dye terminator cycle sequencing method. For construction of the FLAG-VP3 chimera, the FLAG peptide sequence was included in the PCR primer used to obtain the VP3 gene. The oligonucleotide sequences of the primers are as follows: VP2 5' primer, ATGCGGATCCATGGCTCCGG-GAAAAAGAGG; VP3 5' primer, ATGCGGATCCATGGCTACAG-GCAGTGGCGC; VP2/VP3 3' primer, ATGCGAATTCATTAACG-GTTTATTGATTAAC; FLAG-VP3 5' primer, ATGCGGATCCATG-GACTACAAGGATGACGACAAGATGGCTACAGGCAGTGGCGC.

Insect cell culture and viral infection. *Spodoptera frugiperda* Sf9 cells were cultured at 27°C as either a suspension or a monolayer in Sf900 II medium (Gibco BRL) supplemented with 0.3% fetal calf serum (FCS) and 180 µg/ml kanamycin sulfate. Cells were grown to the logarithmic phase at a density of 1.5×10^6 cells/ml and were then infected with recombinant baculovirus which had been purified and amplified. During infection, cells were maintained in TC-100 medium supplemented with 10% FCS, bactotryptose phosphate broth (2.6 mg/ml) and kanamycin sulfate (100 µg/ml).

Determination of VLP production. 5×10^8 infected Sf9 cells were harvested on day 4 and suspended in 20 ml of sonication buffer A (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and sonicated for 10 min at 50% duty cycle (TITEC Ultra Sonicator). The extract was centrifuged at 15,000 g for 10 min at 4°C and the supernatant was transferred to a fresh tube. The pellet was resuspended in 5 ml of sonication buffer B (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, and 1 mM PMSF), sonicated for 2 min at 50% duty cycle and centrifuged as before. The supernatant was pooled with the first and centrifuged at 100,000 g for 1 h to precipitate the VLP. The pellet was then resuspended in sonication buffer A.

The VLP-containing extract was layered onto a CsCl cushion consisting of four densities ranging from 1.15 to 1.44 mg/ml. After centrifugation at 34,000 rpm for 2.5 h at 15°C in an SW50.1 rotor, the VLP-containing fractions were collected, pooled and dialyzed against 20 mM Tris/HCl, pH 8.0, and 300 mM NaCl. The dialyzed sample was then treated with DNase and RNase in the presence of 5 mM MgCl₂ and again purified by CsCl step gradient ultracentrifugation. The VLP-containing portion was collected and dialyzed to remove CsCl as described above. Purified VLPs were stored at 4°C until use.

Detection of capsid proteins by Western blotting. The expression of the capsid proteins was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Cell lysates or fractionated proteins were loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to an Immobilon transfer membrane (Millipore) and the VP3 protein was detected using an anti-VP3 antibody which was raised against a recombinant VP3 (courtesy of M. Ikeda and I. Tamai, MBL, Nagoya, Japan). This

antibody could also detect VP1 and VP2 because their amino acid sequences are equivalent to that of VP3.

Electron microscopy. One drop of protein sample was absorbed onto a carbon-coated collodion film on a copper grid for 30 s. Excess fluid was removed and the preparation was briefly washed. The grid was then soaked in 1% uranyl acetate for 30 s and dried in air at room temperature. The samples were examined with an H-7000 (Hitachi) electron microscope.

Purification of wild type AAV-2. HeLa cells in which the complete AAV-2 genome was stably integrated were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS and penicillin streptomycin at 37°C in 5% CO₂. For adenovirus infection, cells were grown to 80% confluency and the medium was changed to DMEM containing 2% FCS. The cells were infected with Adenovirus type 5 in a multiplicity of infection (MOI) of 1.0 and were incubated until the cytopathic effect of the infection was complete. The infected cells were then harvested, suspended in five volumes of 20 mM Tris/HCl, pH 8.0 and disrupted by sonication at maximum output (6–7 bursts of 30 s each using a handy sonicator (Tomy Seiko). The sonicated extract was centrifuged at 3000 rpm for 10 min at 4°C and the supernatant was collected. The supernatant was then heated to 56°C for 30 min to inactivate the adenovirus, loaded onto a CsCl step gradient and centrifuged as above. After two rounds of ultracentrifugation, empty and DNA-containing particles were collected and dialyzed.

Sedimentation velocity analysis. Sedimentation velocity analysis was performed in the Optima XLA analytical ultracentrifuge using an An-60 Ti rotor (Beckman) at 20,000 rpm at 20°C. 0.4 ml of each sample with an OD between 0.5 and 1.0 at 280 nm was used. Sedimentation patterns were recorded every 10 min with a UV scanner at 280 nm. The sedimentation coefficients were calculated using XLABEL software (Beckman) and corrected for the viscosity and density of the solvent to yield $S_{20,w}$ according to the equation

$$S_{20,w} = S_{T,b} \frac{(1 - \nu\rho)_{20,w} \cdot \eta_{T,b} \eta_{T,w}}{(1 - \nu\rho)_{20,w} \cdot \eta_{T,w} \eta_{20,w}}$$

where ν , ρ , η , and T represent the partial specific volume of the solute, the solvent density, the viscosity, and the absolute temperature, respectively. The suffixes 20, b, and w represent 20°C, buffer, and water, respectively.

Immunoreactivity of VLP containing the chimeric FLAG-VP3 protein. Purified VLP consisting of VP2 and FLAG-VP3 were mixed with either anti-FLAG monoclonal antibody (BABC0) or anti-VP3 antibody (see above) in a reaction mixture containing 20 mM Tris/HCl (pH 7.9), 150 mM NaCl and incubated at 4°C for 90 min. After the incubation, the mixtures were loaded onto a sucrose density gradient (5–20% sucrose in 20 mM Tris/HCl pH 8.0 and 150 mM NaCl) and centrifuged at 35,000 rpm at 4°C for 2 h using an SW55Ti rotor (Beckman). Each fraction (0.4 ml) was collected from top to bottom and subjected to 10% SDS-PAGE followed by Western blot analysis. This procedure was also used to detect the native monomeric form of the chimeric FLAG-VP3 protein (obtained by infecting Sf9 cells with recombinant baculovirus expressing FLAG-VP3). In this case, however, centrifugation was performed for 18 h.

RESULTS

Expression of Capsid Proteins and Formation of VLPs

We used a baculovirus protein expression system to study the formation of AAV VLP. Two recombinant baculoviruses expressing large amounts of the AAV capsid proteins VP2 and VP3 were constructed. After

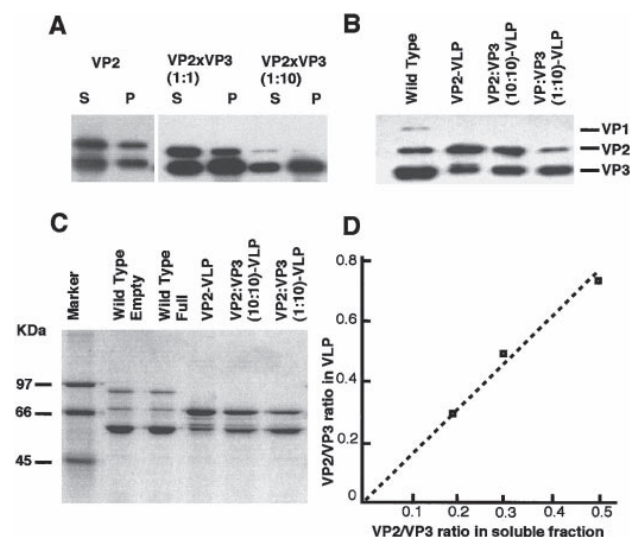


FIG. 1. Analysis of purified VLPs formed in insect cells from recombinant baculoviruses expressing VP2 and VP3. (A) Sf9 cells were infected with two recombinant baculoviruses expressing VP2 and VP3 and cell extracts were analysed by SDS-PAGE followed by Western blot analysis with anti-VP3 antibody. S: supernatant of the cell extracts; P: insoluble pellet of the cell extract. (B) VLPs were purified from the cell extracts by CsCl density gradient ultracentrifugation and analyzed by SDS-PAGE followed by Western blotting with anti-VP3 antibody. Wild type AAV (see Materials and Methods) was also analyzed as a control. (C) Purified VLPs were also analyzed by SDS-PAGE followed by Coomassie brilliant blue staining. As a control, both empty and DNA-containing wild type AAVs were prepared (see Materials and Methods) and analyzed by SDS-PAGE. As a control, both empty and DNA-containing wild type AAVs were prepared from AAV-integrated HeLa cells infected with adenovirus and analyzed by SDS-PAGE. The MOIs of recombinant baculovirus infection are indicated in parentheses. (D) Molar ratios of VP2 and VP3 in soluble fractions in infected cells and purified VLPs. Each protein was quantified by comparing known quantity of VPs by Western blot analysis and their molar ratios were calculated and plotted.

purification and amplification of the recombinant viruses, Sf9 cells were infected with the recombinant baculoviruses and harvested. Expression of each protein was determined by Western blot analysis (Fig. 1A). Both recombinant proteins were highly expressed in soluble fractions.

Western blot analysis indicated that cells expressing VP2 also produced substantial amounts of two smaller proteins similar in size to VP3 which were recognized by the anti-VP3 antibody. Thus, VP3-like polypeptides were generated when VP2 was expressed in insect cells. To determine whether VP2 alone could form VLPs, we attempted to abrogate the production of the VP3-like polypeptides by introducing some mutations. Mutations at G52A (the number indicates the amino acid position of VP2 protein) and Q53N were designed to eliminate N-terminal proteolysis, and mutations at M66I (the true VP3 initiation codon) and M74I were

designed to prevent VP3 initiation. However, these mutations failed to completely eliminate the VP3-like products and further, the mutated VP2 lost its ability to form VLP even when the VP3-like polypeptides were present (data not shown). Thus it was not possible to determine whether VP2 alone could form VLP. As VP3 on its own cannot form VLPs (8), we infected recombinant baculoviruses expressing VP2 alone or VP2 and VP3 to produce VLPs in following experiments.

Alteration of the VP2/VP3 Ratio and Effect on VLP Formation

Sf9 cells were co-infected at various MOIs with the recombinant viruses expressing VP2 and VP3 to assess the relationship between expressed protein and VLP composition. The quantities of each VP protein in both the cell extract and the purified VLPs were determined by SDS-PAGE followed by Western blotting. The ratios of VP2/VP3 in purified VLPs correlated well with those in the cell extracts (Fig. 1D), suggesting that the VLP composition is determined by the natural expression level of each protein.

Electron microscopic images of the recombinant VLPs were compared with those of the wild type empty and DNA-containing particles of AAV-2 (Fig. 2). The recombinant VLPs were indistinguishable in size and shape from the wild type AAV-2 regardless of the VP2/VP3 ratio.

To elucidate the physical properties of the VLPs, we purified the VLPs on a large scale and measured their sedimentation coefficients by analytical ultracentrifugation. The VLPs isolated from cells infected with higher ratios of VP2/VP3 were found to have higher sedimentation coefficients (Fig. 3), indicating that VLP containing more VP2 was heavier. As the molecular weight of VP2 is heavier than VP3, this result agrees well with the expectation. These results indicate that VLP can be formed with a wide range of VP2/VP3 ratios.

VLP Can Form If VP3 Is Tagged at the N-Terminus with an Exogenous Peptide

The fact that VLP can be formed in proportion to VP2/VP3 ratios in soluble fractions suggests that VP2 and VP3 function as equivalent subunits, which indicates that the VP2-specific region is not responsible for VLP formation. If this is true, it should be possible to place an exogenous peptide at the VP3 N-terminus without it affecting VLP formation. To test this, we fused a FLAG peptide as a tag to the VP3 N-terminus and examined if it could be incorporated into VLP. The recombinant baculovirus encoding the FLAG-VP3 chimera was generated and then co-infected with the VP2-expressing recombinant baculovirus into Sf9 cells. The ensuing VLP was purified from the cell extract and

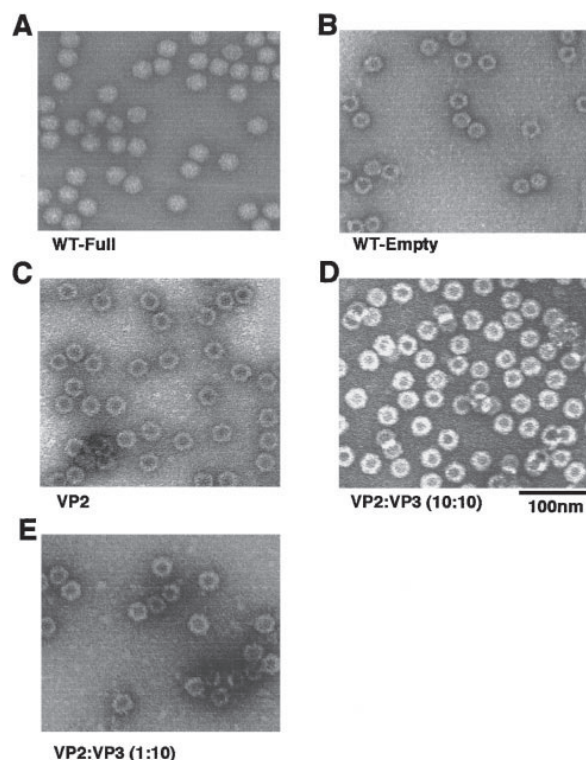


FIG. 2. Electron micrographs of purified VLPs from recombinant baculovirus-infected insect cells and controls. (A) AAV wild type full particle. (B) AAV wild type empty particle. (C) VLP purified from recombinant baculovirus-infected cells expressing VP2. (D, E) VLP purified from recombinant baculovirus-infected cells expressing VP2 and VP3. The MOIs of recombinant baculovirus infection are indicated in parentheses.

examined by SDS-PAGE followed by Western blotting with anti-VP3 or anti-FLAG antibody. VP3 derived from purified VLP was recognized by both anti-VP3 and anti-FLAG, whereas VP2 was recognized only by anti-VP3. This result indicates that the FLAG-VP3 chimera could be incorporated in the VLP (Fig. 4A).

The N-Terminus of VP3 Is Located Inside the VLP

The location of the N-terminus of VP3 in the VLP was determined by assessing ability of the anti-FLAG antibody to recognize the VLP containing the FLAG-VP3. First, however, we checked that the native monomeric FLAG-VP3 protein could be recognized by this antibody. When Sf9 cells were infected only with FLAG-VP3, VLP formation did not occur and the monomeric form of FLAG-VP3 could be obtained. The monomeric FLAG-VP3 protein was purified and incubated with anti-FLAG antibody. Sucrose density gradient centrifugation indicated that the FLAG-VP3 and anti-FLAG antibody formed a complex that was heavier than either protein (Fig. 4B). Thus, when fused

to the N-terminus of VP3, the FLAG peptide was exposed at the surface of the native protein and could be recognized by the anti-FLAG antibody.

Using this antibody, we examined if it could recognize the FLAG when FLAG-VP3 was incorporated into the VLP. The anti-FLAG antibody was incubated with VLP composed of VP2 and FLAG-VP3 and then subjected to sucrose density gradient centrifugation. Fractions of the sample were then subjected to SDS-PAGE followed by Western blotting analysis to determine the distribution of VLP and anti-FLAG. In this experiment, anti-FLAG was used as a first antibody and anti-mouse IgG was used as a second antibody. The distribution of the VLP was detected by anti-FLAG antibody and the distribution of the anti-FLAG antibody incubated with the VLP was detected by anti-mouse IgG antibody that was used as a second antibody. If the FLAG peptide was exposed outside of the VLP, the incubated anti-FLAG antibody could bind to the VLP and co-migrated with VLP. Our result showed that the anti-FLAG antibody incubated with the VLP remained at the top of the gradient and it was not detected in the fraction containing the VLP (Fig. 4C). Such co-migration of antibody was observed only when anti-VP3 was incubated with the VLP as a control (Fig. 4C). This result indicates that the FLAG peptide, when fused to the N terminus of VP3, is located inside the VLP where it cannot be accessed by the anti-FLAG antibody.

DISCUSSION

Two proteins, VP2 and VP3, are required for the formation of the AAV virus-like particle (VLP). Their

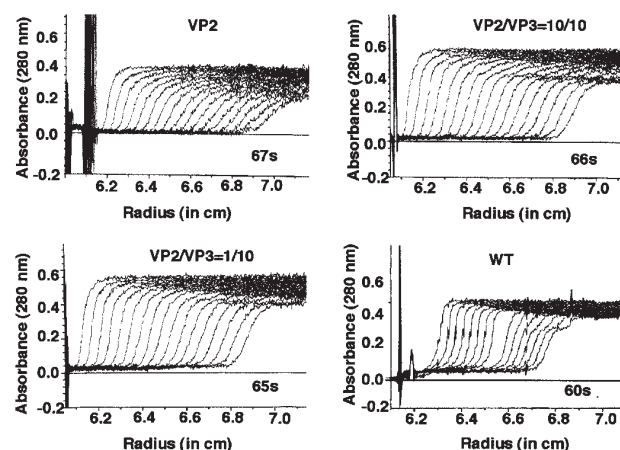


FIG. 3. Sedimentation velocity analysis of VLP. Sedimentation velocity was analyzed by centrifugation in a XLA analytical ultracentrifuge. Centrifugation was carried out at 20,000 rpm and samples were scanned every 10 min. Calculated *S* values are also shown. Arrows indicate the meniscus.

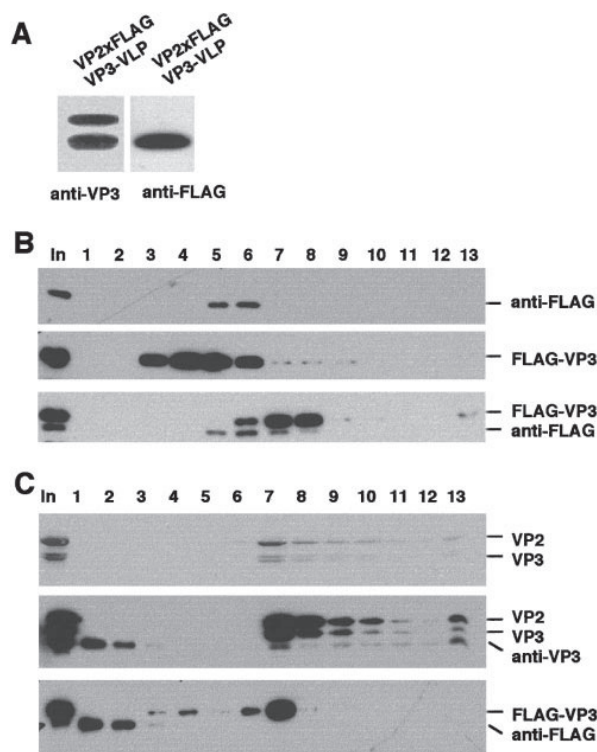


FIG. 4. Recognition of FLAG fused to the N-terminus of VP3 by anti-FLAG antibody. (A) The recombinant baculovirus encoding the FLAG-VP3 chimera was co-infected with the VP2-expressing recombinant baculovirus into Sf9 cells. The ensuing VLP was purified from the cell extract by CsCl density gradient centrifugation and examined by SDS-PAGE followed by Western blotting with anti-VP3 (left) and anti-FLAG (right) antibody. (B) The recombinant baculovirus encoding the FLAG-VP3 chimera was infected into Sf9 cells and the FLAG-VP3 was purified. The purified monomeric FLAG-VP3 (top) or anti-FLAG (middle) antibody or both (bottom) were subjected to sucrose density gradient centrifugation. Then each fraction was further fractionated by SDS-PAGE followed by Western blotting with anti-FLAG antibody. Note that anti-FLAG antibody fractionated by SDS-PAGE could be detected by anti-mouse IgG used as a second antibody. (C) The recombinant baculovirus encoding the FLAG-VP3 chimera was co-infected with the recombinant baculovirus encoding the VP2 into Sf9 cells and the VLPs were purified. Sedimentation pattern of sucrose density gradient centrifugation was indicated (top). The purified VLP composed of VP2 and FLAG-VP3 was mixed with anti-VP3 (middle) or anti-FLAG (bottom) antibody and then subjected to sucrose density gradient centrifugation. Fractions of the sample were then subjected to SDS-PAGE followed by Western blotting analysis with an anti-VP3 and anti-FLAG, respectively. Note that the anti-FLAG antibody incubated with VLP could be detected by anti-mouse IgG antibody in Western blot analysis.

functions in VLP assembly are, however, as yet poorly understood. To assess the contributions of each protein in VLP assembly, we have used the baculovirus expression system with insect cells. This is a system which has previously been used for the study of VLPs in many other viral systems (12–16). We produced various VLPs by infecting insect cells with recombinant bacu-

loviruses at different MOI ratios. This resulted in the expression of VP2 and VP3 in different ratios in the insect cells. The ratios of the VP proteins in the purified VLPs reflected those of the soluble fraction of the cell extracts, and there was no effect on the ability of the VLP to assemble.

Further, electron microscopy work and physical characterization indicated that the structure of these VLPs resembled that of empty wild-type particles. This suggests that VP2 and VP3 are equivalent in VLP formation and that the VP2-specific region is not responsible for VLP formation. This is in contrast to polyomavirus. In polyomavirus, the stoichiometry of viral proteins is determined by its structure and the ratio of VP1 to VP2/VP3 (5:1) is not affected by the expression level of each protein (18).

This finding led us to further examine the role of VP3 in VLP formation by fusing the FLAG peptide to its N-terminus. The fact that the FLAG peptide fused to VP3 did not affect VLP formation suggests that this portion of the protein is flexible. Supporting our observation is work with canine parvovirus (CPV), which is homologous to AAV. X-ray analysis showed that the CPV VP3 N-terminal region up to residue 37 is not visible, suggesting that this region is not rigid (19). We could also show that although the anti-FLAG antibody recognized the native monomeric form of the FLAG-VP3 chimeric protein, it could not bind to the VLP containing this protein. This suggests that the N-terminus of VP3 may be located inside the VLP. In autonomous parvoviruses such as B19 and CPV, it is thought that N-terminus of VP1 is located outside the VLP (20). Despite the similarity of the amino acid sequences of CPV and AAV (25%), CPV has a glycine stretch at the N-terminus of VP3 (19). It is believed that this region is needed to allow VP1 and/or VP2 to position their N-terminal specific regions outside the VLP. Since the N-terminal region of the AAV VP3 does not have the glycine stretch, presumably it cannot pass through the 5-fold axis pore, suggesting that the VP3 N-terminal region might remain inside the VLP. It is possible that the N-terminal region of the AAV VP3 may contribute to the terminal environment of the VLP.

That the N-terminal region of VP3 is located internally indicates that this region might be useful in the development of AAV as a virus vector. Further, since attaching an exogenous peptide to the VP3 N-terminus had no effect on VLP assembly, this suggests that the internal environment of the VLP could be altered to permit a wide range of substrates to be encapsulated. Generally, virus capsids contain polyanionic DNA and they can be used for drug delivery only when target molecule is anionic. In the case of CCMV, the negatively charged molecules paratungstate and decavanadate could be successively encapsulated (21). This is partly because the inside of the virus is an ideal cage for negatively charged DNA. In AAV VLPs, however, the addition of a peptide with particular polarity or

hydrophobicity characteristics might permit the packaging of different types of chemical substrates without modification of the core structure of VP3. This system could thus be used as a drug delivery tool for a wide range of chemicals.

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