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Modest Truncation of the Major Capsid Protein Abrogates B19 Parvovirus Capsid Formation

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In vitro studies have suggested an important role for the minor capsid protein (VP1) unique region and the junction between VP1 and the major capsid protein (VP2) in the neutralizing immune response to B19 parvovirus. We investigated the role of the NH_2-terminal region of the major structural protein in capsid structure by expressing progressively more truncated versions of the VP2 gene followed by analysis using immunoblotting and electron microscopy of density gradient-purified particles. Deletion of the first 25 amino acids (aa) of VP2 did not affect capsid assembly. Altered VP2 with truncations to aa 26 to 30, including a single amino acid deletion at position 25, failed to self-assemble but did participate with normal VP2 in the capsid structure. The altered region corresponds to the beginning of the βA antiparallel strand. Truncations beyond aa 30 were incompatible with either self-assembly or coassembly, probably because of deletion of the βB strand, which helps to form the core structure of the virus.

B19 human parvovirus causes a variety of diseases in humans, including fifth disease, transient aplastic crisis, congenital and acquired pure erythrocyte aplasia, and hydrops fetalis (27); viral infection has also been associated with childhood thrombocytopenia (12), neutropenia (17), and vasculitis (8, 14). B19 virus is a member of the family *Parvoviridae*, which consists of small, nonenveloped, linear single-stranded DNA viruses; this virus and its close relative simian parvovirus (15) constitute the genus *Erythrovirus* (18). The native B19 virion consists of a 5.6-kb genome encased in an icosahedral capsid of approximately 260-Å (26-nm) diameter. The two structural proteins, VP1 (83 kDa), and VP2 (58 kDa), self-assemble naturally into the capsid, which is composed of 60 copies of ≤5% minor capsid protein (VP1) and 95% major capsid protein (VP2) (7, 16). VP1 and VP2 are encoded in overlapping reading frame, but VP1 contains an additional 227 amino acids (aa) at the NH_2 terminus (23).

Formation of recombinant B19 virus capsids in a baculovirus system does not require the presence of VP1 (9). VP1 alone forms capsids with low efficiency and distorted morphology (26). However, VP1 is crucial in the immune response in animals and probably also in humans. VP2-only capsids do not elicit a neutralizing antibody response in animals (4, 9). Linear neutralizing epitopes cluster in the VP1 unique and VP1-VP2 junction regions (21). The major specificity in convalescent-phase human sera is directed to the VP1 unique region (11, 22). Antibody binding and neutralization studies have shown that the unique region is located on the surface of the virion (3, 10, 19) and can be substituted in chimeric empty capsids to display foreign protein sequences, including a complete lysozyme protein (13).

The fine structure of canine parvovirus has been solved by X-ray crystallography (25), but similar studies in B19 virus have achieved resolution to only 8 Å (0.8 nm) (1). In neither case has either the unique region of VP1 or the most NH_2-terminal portion of VP2 been visualized. Because of the importance of this latter region in both vaccine development and production of chimeric capsids, we have investigated the effects of NH_2-terminal truncation of B19 virus VP2 on capsid formation.

Capsid formation by NH_2-terminally truncated VP2. To examine whether NH_2-terminal truncations of B19 virus VP2 would affect capsid formation, progressively shortened major capsid proteins were generated in the baculovirus expression system. First, 11 plasmids termed pVP2/T1 to pVP2/T11 were constructed to produce 11 truncated VP2 proteins in which the NH_2 termini were sequentially deleted by 5 or 10 aa from the terminus of VP2 (Fig. 1A). In brief, 11 fragments encoding truncated NH_2-termini of VP2 protein were created by using PCR in which pYT103c (a nearly full length clone of B19 virus; a kind gift from P. Tattersall) served as the template for appropriate primers (7, 23) (Fig. 1B). Eleven different 5′ primers containing *Bam*HI linker sequences and an ATG start codon were designed in order to generate the different truncated VP2 segments. One 3′ primer was synthesized to encode nucleotides 3962 to 3982, located approximately 60 bp downstream of the *Bam*HI site in VP2. PCR was performed with a combination of one of the 5′ primers and the 3′ primer, resulting in 11 PCR fragments containing various truncated VP2 sequences. Plasmid pFCP1, in which a full-length B19 virus VP1 capsid protein is fused to the Flag peptide (10), was cleaved with *Bam*HI to eliminate a region between two *Bam*HI sites, after which each PCR-amplified fragment that had been digested with *Bam*HI was inserted; thus, the two VP2 segments were recombined at the *Bam*HI site to produce the final truncated VP2 sequence. The resulting plasmids, pVP2/T1 to pVP2/T11, were predicted to encode truncated proteins, termed VP2/T1 to VP2/T11 (Fig. 1A). Using these plasmids, we generated 11 recombinant baculoviruses as instructed by the manufacturer (Invitrogen, San Diego, Calif.). Briefly, each recombinant plasmid and linear wild-type *Autographa californica* nuclear polyhedrosis virus was cotransfected into *Spodoptera frugiperda* Sf9 cells (American Type Culture Collection, Rockville, Md.) and cultured as described previously (9). Recombinant baculoviruses were purified by two cycles of plaque assays; baculoviruses expressing VP2/T1 to VP2/T11 (VP2/T1 to VP2/T11 baculoviruses) were derived from plasmids pVP2/T1 to pVP2/T11. To examine expression of truncated VP2 proteins, Sf9 cells were infected with each of the 11 different recombinant baculoviruses and harvested on day 3. Aliquots of the Sf9 cell

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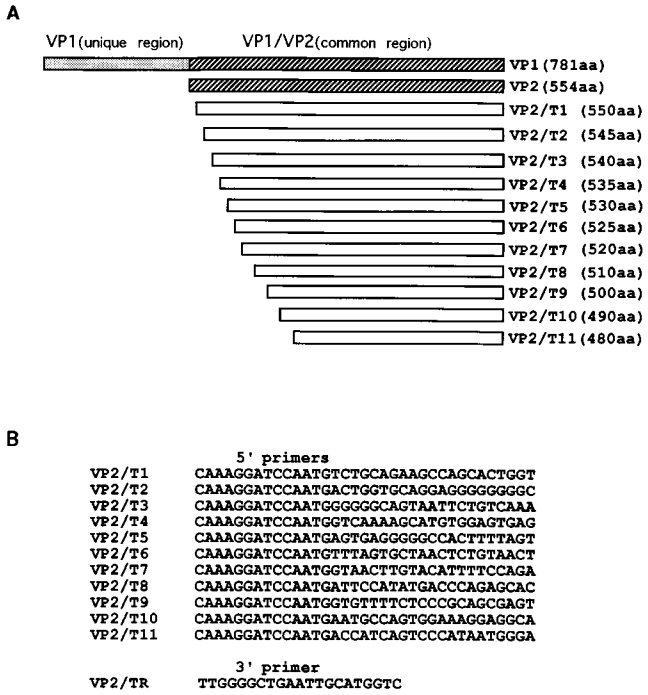


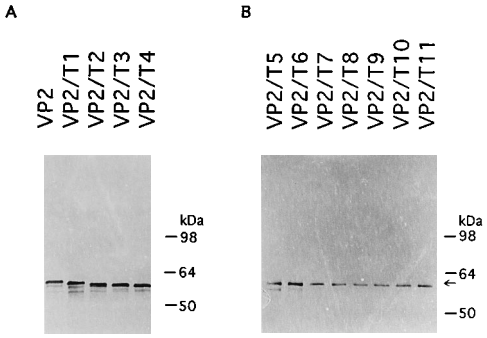
FIG. 1. Construction of plasmids. (A) Schematic representation of truncated VP2 genes. Eleven plasmids (pVP2/T1 to pVP2/T11) were constructed to generate recombinant baculoviruses for the expression of VP2/T1 to VP2/T11 proteins. The amino acid numbers of the proteins are shown in parentheses. Normal VP1 and VP2 genes are illustrated at the top. (B) Primers for PCR amplification. Shown are the 11 5′ primers and the single 3′ primer that were used to generate progressively truncated versions of VP2.

lysates were subjected to sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis followed by immunoblot analysis as described previously (10).

VP2/T1 to VP2/T11 capsid proteins were successfully produced in Sf9 cells infected with the 11 recombinant baculoviruses (data not shown). The protein products (each contained an additional methionine as a consequence of creation of a translation initiation codon, AUG, at the 5′ ends of the genes) were of the relative molecular masses predicted by the amino acid sequences, and each was produced at high levels comparable to that of normal VP2, as judged from reactivity on immunoblots (data not shown).

To examine the ability of truncated versions of VP2 protein to self-assemble (or to maintain a stable capsid structure), each of 11 different baculoviruses, which contained VP2/T1 to VP2/T11 genes, were separately infected into Sf9 cells, and the cells were harvested 3 days after inoculation. To determine formation of empty capsids, cell homogenates were subjected to centrifugation over a 40% (wt/vol) sucrose cushion in phosphate-buffered saline (PBS) and then subjected to CsCl gradient sedimentation as described previously (9). The CsCl fractions were dialyzed against PBS and used in further analyses. After a single passage through the CsCl gradient, preparations containing VP2/T1 to VP2/T4 gave rise to visible bands of the appropriate densities for empty capsids. However, no visible bands were observed in preparations from VP2/T5 to VP2/T11. The protein concentration of each fraction was determined by spectrophotometry. Sharp peaks were detected in fractions obtained from VP2/T1 to VP2/T4 samples, but for VP2/T5 to VP2/T11 preparations, there were no detectable peaks present in fractions corresponding to the density of empty capsids (multiple attempts to detect proteins were made after a repeated experiment using different truncated VP2 baculovirus clones). The CsCl fractions containing empty capsids from VP2/T1 to VP2/T4 specimens and the fractions showing the proper densities for empty capsids which were collected from the VP2/T5 to VP2/T11 samples were subjected to a second CsCl gradient density procedure. After two cycles, capsids were still visualized as light-scattering bands from VP2/T1 to VP2/T4 preparations. Fractions from the second CsCl gradient showed strong bands of protein of the expected size for VP2/T1 to VP2/T4 on immunoblots but no detectable bands for VP2/T5 to VP2/T11 (Fig. 2A).

Each of the 11 recombinant baculoviruses was coinfected into Sf9 cells with normal VP2 baculovirus in order to examine whether truncated VP2 would coassemble with normal VP2. After two cycles of CsCl gradient sedimentation, visible bands were observed in all preparations for truncated VP2 proteins plus normal VP2 at the predicted densities. Immunoblot analyses were performed with the CsCl fractions derived from the individual preparations. VP2/T1 to VP2/T6 proteins were detected in fractions containing empty capsids, indicating that these proteins were capable of coassembly with normal VP2 into capsids. However, no VP2/T7 to VP2/T11 bands were detected on immunoblots derived from the CsCl fractions containing empty capsids, indicating that VP2/T7 to VP2/T11 proteins failed to coassemble with VP2. Figure 2B shows immunoblots of the peak fractions of CsCl gradient sedimentation of preparations from VP2/T5 to VP2/T11 plus normal VP2 preparations. Capsid formation with truncated VP2 alone and truncated VP2 plus normal VP2 is summarized in Fig. 2C. (In these



C

	Capsid Formation alone	with VP2
VP2	+	
VP2/T1	+	+
VP2/T2	+	+
VP2/T3	+	+
VP2/T4	+	+
VP2/T5	–	+
VP2/T6	–	+
VP2/T7	–	–
VP2/T8	–	–
VP2/T9	–	–
VP2/T10	–	–
VP2/T11	–	–

FIG. 2. Formation of empty capsids after infection with VP2/T1 to VP2/T11 baculoviruses. Eleven different recombinant baculoviruses were infected into Sf9 cells with or without normal VP2 baculoviruses. Formation of empty capsids was assessed by immunoblotting with anti-B19 virus rabbit antiserum and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. Illustrated are immunoblot analyses of empty capsids containing truncated VP2 alone (A) or truncated VP2 plus normal VP2 (B) after purification by two cycles of CsCl gradient sedimentation. (C) Summary of empty capsid formation. The arrow in panel B indicates the position of normal VP2 protein.

A

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M V K S M W S E G A T F S A N . . . . . .VP2/T4 (535aa)
  M K S M W S E G A T F S A N . . . . . .VP2/T41(534aa)
    M S M W S E G A T F S A N . . . . . .VP2/T42(533aa)
      M M W S E G A T F S A N . . . . . .VP2/T43(532aa)
        M W S E G A T F S A N . . . . . .VP2/T44(531aa)
M V K S L W S E G A T F S A N . . . . . .VP2/T45(535aa)
  M K S L W S E G A T F S A N . . . . . .VP2/T46(534aa)
    M S L W S E G A T F S A N . . . . . .VP2/T47(533aa)
      M L W S E G A T F S A N . . . . . .VP2/T48(532aa)
          M S E G A T F S A N . . . . . .VP2/T5 (530aa)
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B

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                5' primers
VP2/T41  CAAAGGATCCAATGAAAAGCATGTGGAGTGAGGGG
VP2/T42  CAAAGGATCCAATGAGCATGTGGAGTGAGGGGCC
VP2/T43  CAAAGGATCCAATGATGTGGAGTGAGGGGCCACT
VP2/T44  CAAAGGATCCAATGTGGAGTGAGGGGCCACTTTT
VP2/T45  CAAAGGATCCAATGGTCAAAAGCCTGTGGAGTGAG
VP2/T46  CAAAGGATCCAATGAAAAGCCTGTGGAGTGAGGGG
VP2/T47  CAAAGGATCCAATGAGCCTGTGGAGTGAGGGGCC
VP2/T48  CAAAGGATCCAATGCTGTGGAGTGAGGGGCCACT

                3' primer
VP2/TR     TTGGGGCTGAATTGCATGGTC
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FIG. 3. Formation of empty capsids by infection with VP2/T41 to VP2/T48 baculoviruses. (A) NH_2-terminal sequences of eight different truncated VP2 proteins. Amino acid numbers are shown in parentheses. (B) Primers for PCR amplification. Eight different 5′ primers and one 3′ primer were used to create the progressively truncated VP2 by 1 aa at the NH_2 terminus.

experiments, three clones of individual VP2/T recombinant baculoviruses were tested with similar results.)

Identification of a crucial NH_2-terminal site affecting capsid formation. To determine the site in the VP2 NH_2 terminus which affects capsid formation, we produced baculovirus constructs containing progressive single-amino-acid deletions in the region defined between VP2/T4 and VP2/T5 proteins. For this purpose, eight plasmids designated pVP2/T41 to pVP2/T48 generated sequentially truncated and/or amino acid-substituted VP2 proteins specifically between aa 21 and 25 of VP2 (VP2/T41 to VP2/T48) (Fig. 3A). Eight fragments encoding truncated VP2 proteins with or without mutation were produced by PCR using one of eight different 5′ primers, designed to produce single-amino-acid truncations, and the 3′ primer (Fig. 3B). The 5′ primers for pVP2/T45 to pVP2/T48 were synthesized so as to replace the downstream AUG with CUG, but the lengths of the truncated VP2 sequences were the same as those of the corresponding pVP2/T4, pVP2/T41, pVP2/T42, and pVP2/T43 constructs. All 5′ primers were flanked with *Bam*HI linkers and start codons, except that in pVP2/T44, the native methionine 24 was used as a start codon. The eight PCR-amplified fragments were inserted between two *Bam*HI sites of plasmid pFCP1. Constructs pVP2/T41 to pVP2/T44 were used to generate VP2/T41 to VP2/T44 proteins (Fig. 3A). VP2/T41 to VP2/T43 contained an additional methionine residue at the NH_2 terminus. In VP2/T44, the AUG codon at aa 24 of normal VP2 was used for initiation of translation. (In the baculovirus system, translation initiation frequently occurs at AUG codons adjacent to the original translation initiation site. Therefore, it was likely for the VP2/T41 to VP2/T43 genes that translation would also be initiated at the position 24 AUG downstream of the engineered site. To avoid translation initiation at two different AUG codons, the downstream AUG was altered to CUG by PCR-mediated site-directed mutagenesis, resulting in plasmids pVP2/T45 to pVP2/T48, in which the methionine 24 was substituted by lysine.) Using the pVP2/T41 to pVP2/T48 constructs, VP2/T41 to VP2/T48 recombinant baculoviruses were generated and used to infect Sf9 cells. After two cycles of CsCl gradient sedimentation, visible bands were detected in all preparations containing VP2/T41 to VP2/T48, with protein yields similar to that of normal VP2. Immunoblot analysis demonstrated that VP2/T41 to VP2/T48 proteins self-assembled into empty capsids (data not shown).

Peak fractions of two cycles of CsCl gradient sedimentation were subjected to transmission electron microscopy after negative staining as described previously (26). Typical parvovirus particles were observed in all VP2/T1 to VP2/T4 specimens, but no empty capsids were observed for the VP2/T5 preparation (Fig. 4).

These results demonstrate that the most NH_2-terminal 25 aa of VP2 are not required for B19 parvovirus capsid assembly. Deletion or alteration of aa 26 abolished self-assembly by truncated VP2, although truncated versions of VP2 to aa 30 did coassemble with full-length VP2. Truncation of VP2 beyond aa 30 prevented both self-assembly and coassembly. These results

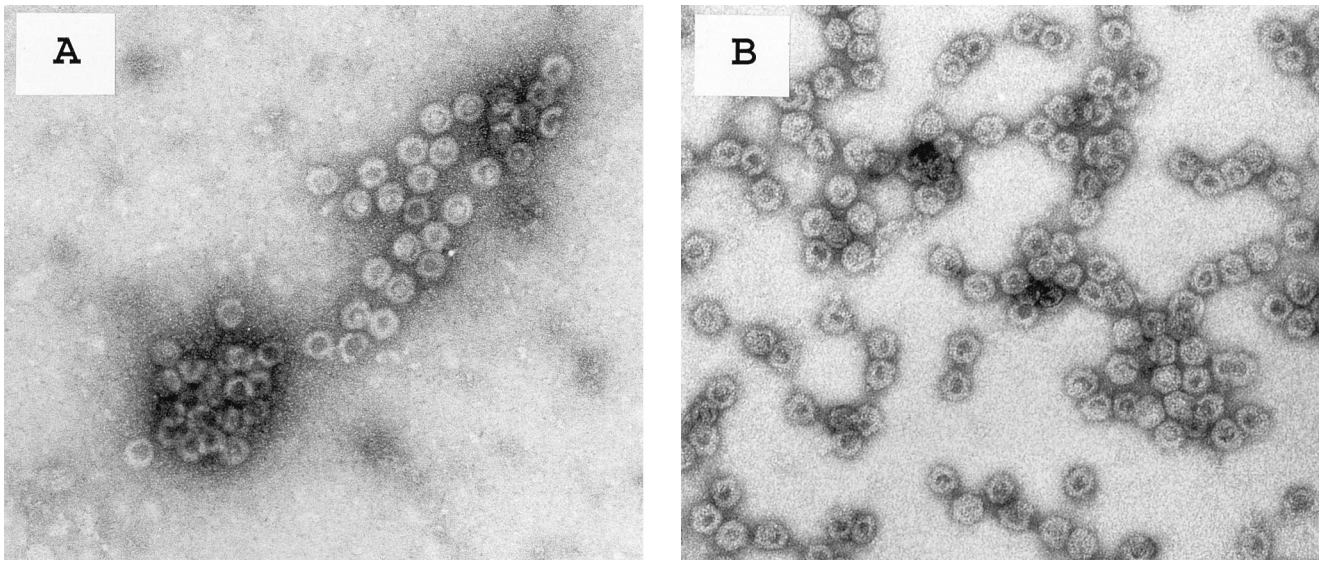

FIG. 4. Electron microscopy. Purified VP2 alone (A) and VP2/T4 alone (B) empty capsids were negatively stained and examined by transmission electron microscopy.

can be interpreted in the context of known structural features of parvoviruses and B19 virus empty capsids in particular, and they have implications for the design of chimeric B19 virus capsids for antigen or protein delivery.

When the capsid protein sequences of the *Parvoviridae* are aligned, a glycine-rich region adjacent to the NH_2 terminus is found to be well conserved in all the autonomous parvoviruses (5, 6). The polyglycine sequence that includes aa 22 to 39 in canine parvovirus is similar in length to sequences of the other autonomous parvoviruses, with the exception of B19 virus. For the human virus, the glycine-rich region is shorter, aa 12 to 17, and also localized within the initial 25 amino acid residues. The function of the polyglycine region is unknown. Although the fine structure of canine parvovirus has been resolved by X-ray crystallography, neither the first 38 aa of canine parvovirus nor the first 38 aa of empty capsids of the closely related feline panleukopenia virus were visualized (2, 25). (B19 parvovirus empty capsids have also been subjected to X-ray crystallography, but the structure has been resolved only at low resolution [1].) Residue 38 of canine parvovirus was situated near the interior of the capsid, near the fivefold axis of symmetry. By analogy with other icosahedral viruses, disorder of the NH_2 termini of the major capsid proteins was assumed to be due to association of this region with internal nucleic acids. Alternatively, this region may serve to allow exposure of part of VP2 and of the unique region of VP1 on the capsid surface. Examination of minute virus of mice particles exposed to trypsin showed that the NH_2-terminal regions of VP2 were internal to the capsid in empty particles but external on full particles (24). These results are consistent with our own findings that the B19 parvovirus VP1 unique region and probably also the VP1-VP2 junction sequence are external to the B19 parvovirus capsid (see the introduction). The flexibility and narrow diameter of the conserved polyglycine sequence could serve to allow the protein to tunnel through cylindrical structures located about the fivefold axis of symmetry. One function apparently not conferred by the NH_2-terminal domain is participation in the capsid's structural conformation, as indicated also by normal capsid formation by truncated versions of VP2 lacking the first 25 NH_2-terminal amino acids.

Canine parvovirus, feline panleukopenia virus, and B19 parvovirus share the central eight-stranded antiparallel β-barrel motif that is the common core structure of all icosahedral viruses (1, 5, 20). An additional β strand (βA) is situated immediately after the glycine-rich region of VP2 (1). For B19 virus, the βA strand extends between aa 26 to 32. VP2/T5, in which the NH_2-terminal tryptophan (residue 25) was replaced with methionine, was unable to self-assemble into capsids; the methionine residue which was located just before the βA strand likely altered the conformation of VP2. Similarly, in other constructs, alteration of the first residue to the βA strand, a serine, consistently inhibited capsid self-assembly. Shortening of VP2 alone appears an unlikely explanation for this effect, as in other experiments, in which truncated VP2 capsids fused to an 8-aa Flag peptide were generated, chimeric capsids joined at aa 26 also failed to assemble (our unpublished data).

When VP2/T5 and VP2/T6 baculoviruses were coinfected with normal VP2 baculoviruses, empty capsids containing both VP2/T5 or VP/T6 plus normal VP2 were produced, indicating that truncated VP2 missing up to 30 amino acid residues could participate in forming the capsid structure. However, the ratio of VP2/T5 or VP2/T6 to normal VP2 in capsids was $<5\%$, indicating that these deletions severely affected the efficiency of coassembly with normal VP2. NH_2-terminal deletion of >31 amino acid residues completely abolished the coassembly ability of truncated VP2. In B19 virus, the second β strand (βB) of VP2 begins at aa 35. βB is an essential element of the eight-stranded antiparallel β barrel topological motif. Its loss appears to be incompatible with both self-assembly and participation in the capsid structure.

We have suggested that the unique region of VP1 and the NH_2 terminus of VP2 may be relatively unconstrained, accounting for the structural features described above as well as their high degree of immunogenicity. The VP1 unique region can be entirely substituted by a foreign protein, with retention of the heterologous peptide's structural conformation and functional activity (13). The current data suggest that for production of chimeric capsids, only limited deletions or substitutions within VP2 will be feasible.

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