

Poliovirus Capsid Proteins Derived from P1 Precursors with Glutamine-Valine Cleavage Sites Have Defects in Assembly and RNA Encapsidation

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Assembly of poliovirus virions requires proteolytic cleavage of the P1 capsid precursor polyprotein between two separate glutamine-glycine (QG) amino acid pairs by the viral protease 3CD. In this study, we have investigated the effects on P1 polyprotein processing and subsequent assembly of processed capsid proteins caused by substitution of the glycine residue at the individual QG cleavage sites with valine (QG→QV). P1 cDNAs encoding the valine substitutions were created by site-directed mutagenesis and were recombined into wild-type vaccinia virus to generate recombinant vaccinia viruses which expressed the mutant P1 precursors. The recombinant vaccinia virus-expressed mutant P1 polyproteins were analyzed for proteolytic processing defects in cells coinfecting with a recombinant vaccinia virus (VVP3) that expresses the poliovirus 3CD protease and for processing and assembly defects by using a *trans* complementation system in which P1-expressing recombinant vaccinia viruses provide capsid precursor to a defective poliovirus genome that does not express functional capsid proteins (D. C. Ansardi, D. C. Porter, and C. D. Morrow, *J. Virol.* 67:3684-3690, 1993). The QV-substituted precursors were proteolytically processed at the altered sites both in cells coinfecting with VVP3 and in cells coinfecting with defective poliovirus, although the kinetics of cleavage at the altered sites were slower than those of cleavage at the wild-type QG site in the precursor. Completely processed capsid proteins VP0, VP3, and VP1 derived from the mutant precursor containing a valine at the amino terminus of VP3 (VP3-G001V) were unstable and failed to assemble stable subviral structures in cells coinfecting with defective poliovirus. In contrast, capsid proteins derived from the P1 precursor with a valine substitution at the amino terminus of VP1 (VP1-G001V) assembled empty capsid particles but were deficient in assembling RNA-containing virions. The assembly characteristics of the VP1-G001V mutant were compared with those of a previously described VP3-VP1 cleavage site mutant (K. Kirkegaard and B. Nelsen, *J. Virol.* 64:185-194, 1990) which contained a deletion of the first four amino-terminal residues of VP1 (VP1-Δ1-4) and which was reconstructed for our studies into the recombinant vaccinia virus system. Complete proteolytic processing of the VP1-Δ1-4 precursor also occurred more slowly than complete cleavage of the wild-type precursor, and formation of virions was delayed; however, capsid proteins derived from the VP1-G001V mutant assembled RNA-containing virions less efficiently than those derived from the VP1-Δ1-4 precursor. These results demonstrate that maintenance of the glycine residues at the QG cleavage sites of the poliovirus P1 precursor is required for assembly and RNA encapsidation events in addition to proteolytic processing and provide further evidence that the amino-terminal portion of VP1 plays a role in the RNA encapsidation process of poliovirus.

Poliovirus is the prototype member of the enterovirus group of the *Picornaviridae*, a family of icosahedral, nonenveloped plus-strand RNA viruses. The messenger sense poliovirus RNA genome is approximately 7.4 kb in length and contains a single open reading frame which is translated to generate a long polyprotein (25). Virus-encoded proteases cleave the genomic polyprotein to release the individual viral proteins required for replication of the viral genome and assembly of progeny virions and therefore have an obligatory role in poliovirus morphogenesis (12, 40). The viral protease 2A releases the structural proteins from the genomic polyprotein as a single 97-kDa precursor, designated P1, through an intramolecular cleavage event (40). The mature virion proteins, VP1, VP2, VP3, and VP4, are released by further cleavages of the P1 precursor. Viral protease 3CD cleaves the peptide bonds between two glutamine-glycine (QG) amino acid pairs in the P1 polyprotein, releasing proteins VP0, VP3, and VP1 (12, 20, 42). Upon encapsidation of the RNA genome, VP0 is cleaved at a site on the interior of the virus

capsid between an asparagine-serine (NS) amino acid pair to generate VP2 and a small capsid protein, VP4 (4, 18, 19, 38). The cleavage of VP0 is termed the maturation cleavage and is believed to be an intramolecular event (4). The mature virion consists of 60 copies of VP3 and VP1 and nearly 60 copies of VP2 and VP4; a few residual copies of uncleaved VP0 are believed to be present in the mature virion (38). The genome-linked protein, VPg, is covalently linked to the viral RNA genome at its 5' end via a phosphodiester linkage with a tyrosine residue and is the only other viral protein in the mature poliovirion (41).

Proteolytic processing of picornaviral P1 precursors is required for virus assembly and may be important for temporally controlling the different stages of the assembly process (14, 15, 32, 34). The enzymatic activity of poliovirus protein 3C^{pro} is responsible for cleavage of the poliovirus P1 capsid precursor to generate protomers consisting of single copies of VP0, VP3, and VP1 (VP0-3-1)₁ (12). The 3CD polyprotein, which consists of uncleaved 3C^{pro} and 3D^{pol} proteins, is the form of 3C^{pro} responsible for P1 cleavage (20, 42). Although the determinants for cleavage site recognition by picornaviral 3C^{pro} enzymes have not entirely been identified, the primary sequence

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of the dipeptide cleavage site is clearly important (9, 13, 22, 33, 34). 3C^{Pro}-mediated cleavages of poliovirus polyproteins occur exclusively at QG amino acid pairs (12, 25). The 3C^{Pro} enzymes of other picornaviruses, however, exhibit more flexibility in dipeptide recognition, although cleavage sites are typically composed of either glutamine or glutamic acid at the P1 position of the bond to be cleaved and either glycine, serine, or alanine at the P1' position (33). The importance of the amino acid constituents of 3C^{Pro} cleavage sites has been extensively characterized for a member of the cardiovirus group of picornaviruses, encephalomyocarditis virus (EMCV). Studies of EMCV capsid precursor processing at the VP3-VP1 cleavage site by 3C^{Pro} enzymes in vitro demonstrated limited flexibility in the dipeptide recognition sequence, with only substitutions of the glycine residue of the wild-type QG cleavage site with amino acids containing small aliphatic side chains permitting cleavage of the peptide bond at the altered sites (34). In addition to the primary sequence, the location of the cleavage site within the poliovirus polyprotein is important for accessibility to the enzyme (29, 43), and the presence of an alanine residue at the -4 position relative to the cleavage site peptide bond has also been demonstrated to play an important role in site recognition for poliovirus (5, 31).

The precise relationship between proteolytic processing of P1 and the capsid assembly process has not been completely defined. The amino termini of adjacent VP3 molecules form a β -cylinder structure on the interior of the icosahedral capsid at the fivefold axes of symmetry (16). A concentric bundle formed from portions of the amino termini of VP0 and VP1 surrounds the β -cylinder structure. Because cleavage of P1 is required to free these termini, complete processing of P1 by the 3CD protease has been considered to be a prerequisite for capsid assembly, and this notion has been supported by studies of EMCV capsid protein processing and assembly with a cell-free in vitro system in which capsid precursors that were cleaved between VP0 and VP3 but not between VP3 and VP1 failed to assemble (34). Protomers consisting of single copies of the processed capsid proteins VP0, VP3, and VP1 assemble into subviral intermediate particles, including 14S pentamers (VP0-3-1)₅ and 75S empty capsids or procapsids (VP0-3-1)₆₀ (36, 38). Formation of these particles can occur in the absence of full-length poliovirus RNA (1a, 21, 36); however, it is not clear whether poliovirus RNA facilitates assembly of subviral intermediates. Both 14S pentamers and 75S empty capsids have been proposed to be the direct precursors to the mature virion, with the RNA encapsidation process proceeding by condensation of pentamers around the RNA genome or by threading of the RNA genome into an existing empty capsid (36, 38), but the determinants for recognition of poliovirus RNA by the capsid proteins have not been determined at either the protein or RNA level. A temperature-sensitive poliovirus capsid mutant with a deletion of the first four amino acids of VP1 was shown by Kirkegaard to encapsidate RNA with slower kinetics at the nonpermissive temperature, suggesting a role for the amino terminus of VP1 in the RNA encapsidation process (23).

Insight into the poliovirus assembly process has been gained both from knowledge of the three-dimensional structure of the virus and from analysis of poliovirus capsid mutants by a variety of methods (14, 16). Previous reports from our laboratory have described the use of recombinant vaccinia virus-based expression systems to study the poliovirus assembly process (1a-3). Recently, we have demonstrated that poliovirus assembly can be analyzed by complementation of a capsid gene-deficient poliovirus RNA genome (11) by using recombinant vaccinia viruses to provide the type 1 poliovirus P1

precursor in *trans* (3). In this report, we describe the analysis of poliovirus capsid protein mutants with valine substitutions for the glycine residues at the QG sites of the P1 precursor by using recombinant vaccinia viruses to express the mutant P1 precursors. We chose the valine substitutions because EMCV P1 precursors with the glycine-to-valine change at the QG cleavage site between VP3 and VP1 were not substrates for cleavage by 3C^{Pro} at the altered sites in vitro (34), and the valine substitutions represented changes less drastic than substitutions with other amino acids with longer aliphatic side chains. Furthermore, a previous paper reported that a valine substitution at the QG cleavage site between 3C^{Pro} and 3D^{Pol} of poliovirus was lethal for virus growth, suggesting that QV could not serve as a functional cleavage site at that location (22). Our initial aim was to use the recombinant vaccinia viruses expressing the QV-substituted P1 precursors to analyze the role, if any, of processing intermediates in assembly by using precursors that could be cleaved at only one site. Unexpectedly, we found that mutant P1 precursors were proteolytically processed at QV sites by 3CD protease provided in *trans* by a second recombinant vaccinia virus, although processing was hindered at the altered sites. The P1 precursors with QV cleavage sites were also completely proteolytically processed in cells coinfecting with the recombinant vaccinia viruses and defective polioviruses which provide the capsid gene-deficient RNA genome. The assembly phenotypes of the QV mutants were compared with that of the previously described poliovirus capsid mutant with an amino-terminal deletion in VP1 (23, 24), which was reconstructed for this study into the recombinant vaccinia virus expression system. The results of these studies demonstrate that mutations at the amino-terminal glycine residues of VP3 and VP1 affect virus assembly and RNA encapsidation in addition to proteolytic cleavage and provide further evidence that the amino-terminal portion of the VP1 protein plays an important role in encapsidation of the poliovirus RNA genome (23).

MATERIALS AND METHODS

Chemicals and enzymes. All chemicals used for these studies were purchased from Sigma, unless otherwise specified. Enzymes used for DNA manipulations were purchased from New England Biolabs. [³⁵S]Translabel (methionine-cysteine) was purchased from ICN Biomedical. ³⁵S-dATP used for DNA sequencing and [α -³²P]UTP used for generating radiolabeled ribonucleotide probes (riboprobes) were purchased from Amersham.

Cells and viruses. HeLa cells were purchased from American Type Culture Collection and were maintained in monolayer culture in Dulbecco's modified Eagle medium (DMEM) (Gibco/BRL) supplemented with 5% fetal bovine serum (Biocell Laboratories) and the growth supplement GMS-G (Gibco/BRL) (complete medium).

Vaccinia viruses used for these studies were cultured in either TK-143_B cells (American Type Culture Collection) or HeLa cells and were concentrated for experimental use by previously described methods (28). Titers of vaccinia viruses were determined by plaque assay on HeLa cell monolayers. Poliovirus type 1 Mahoney used for these experiments was maintained in, and titers were determined by plaque assay on HeLa cell monolayers.

Defective poliovirus preparations. The generation of defective poliovirus stocks by *trans* complementation of a poliovirus genome containing an in-frame capsid gene deletion has been described previously (3). Defective polioviruses (PVdefSM) were maintained in HeLa cells coinfecting with recombinant

vaccinia virus VVP1 and were concentrated as previously described to remove residual infectious VVP1 in the preparations (3). Preparations of defective poliovirus were plated on HeLa cell monolayers in serial dilutions to determine the minimum concentration required to infect every cell in a monolayer of 3×10^5 HeLa cells as determined by a poliovirus-like cytopathic effect after overnight infection. Because the defective polioviruses cannot initiate a second round of infection, a complete cytopathic effect after overnight incubation indicated initial infection of every cell in a monolayer. The minimum concentration of defective viruses resulting in a complete cytopathic effect was then used for experimental purposes.

Generation of recombinant vaccinia viruses expressing mutant P1 polyproteins. Recombinant vaccinia viruses expressing mutant P1 polyproteins were generated by previously described methods (1a, 28). All DNA-subcloning procedures were performed by standard methods (39). Site-directed mutagenesis of P1 cDNA was performed by hybridization of mutagenic oligonucleotides (purchased from Oligos, Etc.) to a single-stranded template DNA generated from the plasmid pUC119-P1. The plasmid pUC119-P1 contains a cDNA copy of the poliovirus P1 gene flanked by *SalI* restriction sites and was derived by subcloning of the *SalI* insert from the previously described plasmid pSC11-P1 (1a, 6) into the unique *SalI* site of the pUC119 vector. The single-stranded DNA template was generated in the CJ236 (*dut ung*) strain of *Escherichia coli* to create a uracil-rich template to facilitate isolation of mutant cDNA plasmids (26). Mutagenesis reactions were performed as previously described (44). Plasmids harboring mutant P1 sequences were identified by dideoxy sequencing of the double-stranded DNA by using the Sequenase enzyme (U.S. Biochemical). pUC119-P1 plasmids containing mutant P1 sequences were digested with *SalI* restriction enzyme to release the P1 gene insert, and the insert was subcloned into the unique *SalI* site of vaccinia virus recombination plasmid pSC11-Sal I (1a, 6). Mutants encoding (i) a glycine-to-valine change at the amino terminus of VP3 (VP3-G001V), (ii) a glycine-to-valine change at the amino terminus of VP1 (VP1-G001V), or (iii) a deletion of the first four amino acids of VP1 (VP1- Δ 1-4) were generated. The sequences of the oligonucleotides used for site-directed mutagenesis were as follows, with mismatched bases underlined: VP3-G001V, 5'-GC-TTA-CAG-GTG-CTG-CCG-GTC-3'; VP1-G001V, 5'-CTA-GCA-CAG-GTC-TTA-GGT-CAG-3'; and VP1- Δ 1-4, 5'-GCG-CTA-GCA-CAG-ATG-CTT-GAA-GC-3'.

Recombinant vaccinia viruses were generated by transfection of pSC11-P1 plasmids encoding mutant P1 precursors into wild-type vaccinia virus-infected BSC-40 cells and selection for recombinants by resistance to the drug 5-bromo-2'-deoxyuridine and by a blue plaque phenotype as previously described (6). Putative recombinant viruses were plaque purified in TK-143_B cells and then analyzed for P1 expression by metabolic radiolabeling and immunoprecipitation with antipoliovirus antiserum.

Metabolic radiolabelings and immunoprecipitations. Metabolic protein radiolabelings of infected HeLa cell monolayers were conducted by incubation in DMEM without methionine-cysteine (ICN Biomedical) to which [³⁵S]Translabel had been added at a concentration of 100 μ Ci/ml. Following radiolabeling, monolayers were harvested in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate), and the cell lysates were clarified by microcentrifugation. The supernatants were adjusted to 0.2% sodium dodecyl sulfate (SDS) and incubated at 4°C overnight on a rotator with the appropriate

antiserum, either antiserum to poliovirus to detect poliovirus capsid proteins or an antiserum which recognizes a linear stretch of amino acids in the 3D^{pol} protein (anti-3D^{pol}) to detect the poliovirus protein 3CD (17). Antibody-bound proteins were collected on protein A-Sepharose for 1 h at room temperature. The Sepharose beads were washed twice in RIPA buffer containing 0.2% SDS and once in 25 mM Tris-HCl (pH 6.8), and the bound proteins were eluted by boiling in gel sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% glycerol, 0.7 M 2-mercaptoethanol, 0.025% bromophenol blue). The immunoprecipitated proteins were separated by electrophoresis on SDS-10% polyacrylamide gels (SDS-PAGE). The gels were fixed in methanol-acetic acid and then treated with the water-soluble fluor sodium salicylate to enhance visualization of the labeled proteins (7). The gels were dried and exposed to X-ray film at -70°C.

Pulse-chase analysis of P1 polyproteins expressed by recombinant vaccinia viruses. The recombinant vaccinia viruses (20 PFU per cell) were adsorbed to two separate HeLa cell monolayers for 1 h in serum-free DMEM. DMEM containing medium supplements (complete medium) was then added and left until 3.5 h postinfection. The infected cells were starved for methionine-cysteine in DMEM without methionine and cysteine (ICN Biomedical) from 3.5 to 4 h postinfection and were then metabolically radiolabeled with [³⁵S]Translabel for 1 h. After the labeling period, the monolayers were rinsed twice in DMEM containing a 20-fold excess of methionine and cysteine. One sample per virus infection was then harvested in RIPA buffer, while the other sample was incubated for an additional 4 h in complete medium with a 20-fold excess of methionine and cysteine. The cell lysates were analyzed for presence of the P1 polyprotein by immunoprecipitation using antipoliovirus antiserum.

Pulse-chase analysis of proteolytic processing of mutant P1 precursors. Proteolytic processing of mutant P1 precursors expressed by recombinant vaccinia viruses was analyzed in cells coinfecting with a recombinant vaccinia virus which expresses the poliovirus 3CD protein or in cells coinfecting with defective polioviruses which provide 3CD protease by expression from a defective poliovirus genome. For analysis by coinfection with two recombinant vaccinia viruses, HeLa cell monolayers were infected with a P1-expressing recombinant vaccinia virus alone (20 PFU per cell) or with a P1-expressing recombinant vaccinia virus and VVP3, a recombinant vaccinia virus which expresses the poliovirus 3CD protease (1a, 35) (20 PFU per cell each). After adsorption in DMEM, complete medium was added and left until 3.5 h postinfection. The monolayers were starved for methionine and cysteine for 30 min and then incubated with [³⁵S]methionine-cysteine for 30 min (pulse). The monolayers were rinsed twice in complete DMEM containing a 20-fold excess of methionine and cysteine. Pulse samples were then harvested in RIPA buffer, whereas chase samples were incubated for an additional designated period of time in complete DMEM containing a 20-fold excess of methionine and cysteine prior to harvest. The cell lysates were analyzed for presence of poliovirus capsid-specific proteins by immunoprecipitation using antipoliovirus antiserum.

For analysis of proteolytic processing of P1 precursors by protease provided by the defective poliovirus genome, several parallel HeLa cell monolayers were infected with 20 PFU of the designated recombinant vaccinia virus per cell and 2 h later were superinfected with a preparation of PVdefSM. After 2.5 h of infection with PVdefSM, the monolayers were rinsed with methionine-cysteine-free DMEM and then incubated with [³⁵S]Translabel for 1 h. At the end of the labeling period, the cells were rinsed twice with complete DMEM containing a

20-fold excess of methionine and cysteine, and the pulse-chase analysis was completed as described above. The overnight (14-h) chase samples, however, were harvested by addition of concentrated RIPA buffer to the medium on the monolayer to a final concentration of $1 \times$, allowing recovery of extracellular and intracellular proteins. When required, immunoprecipitated proteins were quantitated by phosphorimager (Molecular Dynamics).

trans complementation of defective poliovirus genome by recombinant vaccinia viruses. To determine whether recombinant vaccinia viruses would support passage of a defective poliovirus genome by supplying functional capsid proteins in *trans*, HeLa cell monolayers were infected with 20 PFU of the designated recombinant vaccinia virus per cell and 2 h later were superinfected with a preparation of PVdefSM. The cell monolayers were washed with fresh medium at 2 h postinfection with PVdefSM and then again at 4 h postinfection to remove any unattached or eluted PVdefSM particles. The coinfecting cell monolayers were allowed to incubate overnight and then were lysed by four rounds of freezing and thawing. The lysates were clarified by microcentrifugation and incubated with RNase A (40 $\mu\text{g/ml}$; 37°C for 15 min) to digest any unencapsidated RNA in the cell extracts. One-half of each lysate was used to infect a fresh monolayer of HeLa cells. The infected HeLa cells were incubated with [³⁵S]Translabel from 5 to 7 h postinfection, and cell lysates were analyzed for the presence of 3CD protein by immunoprecipitation with anti-3D^{pol}.

Sucrose density gradient analysis of infected-cell extracts. Sucrose density gradient analysis of infected-cell extracts was carried out essentially as previously described (3). HeLa cell monolayers were coinfecting with recombinant vaccinia viruses and PVdefSM and then incubated with [³⁵S]Translabel for 2 h in methionine-cysteine-free DMEM, beginning 1.5 h postinfection with PVdefSM. Complete medium was then added to the monolayers with a further incubation of either 4 h or overnight. The infected monolayers were lysed by addition of Triton X-100 to the medium on the cells at a final concentration of 1%. The lysates were clarified by microcentrifugation and were layered over linear 15 to 30% sucrose density gradients prepared in a buffer of 10 mM Tris-HCl (pH 7.0), 10 mM NaCl, 1.5 mM MgCl₂, and 0.01% bovine serum albumin. The sucrose gradients were centrifuged at 27,500 rpm for 4.25 h in a TH-641 rotor (Sorvall) at 4°C. Twenty fractions of 0.5 ml each were collected from the bottom of each gradient, and either even- or odd-numbered fractions were diluted with concentrated RIPA buffer and analyzed by immunoprecipitation for poliovirus capsid-specific proteins. Gradients were standardized for sedimentation of mature poliovirions (155S) and empty capsids (75S) by conducting a parallel gradient analysis of a lysate from poliovirus-infected cells which had been continuously incubated with [³⁵S]Translabel from 3.5 to 8 h postinfection. Aliquots of each of the fractions from the poliovirus gradient were analyzed directly for radioactivity by liquid scintillation. The locations of mature virions and empty capsids in the poliovirus gradient were determined by the corresponding peaks of radioactivity.

Northern (RNA) blot analysis of encapsidated viral RNA. Poliovirus-specific RNA was detected by Northern blot analysis essentially as previously described (8). Fractions 6 and 8 from sucrose density gradients were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and RNA was precipitated by addition of LiCl and ethanol. Poliovirus virion RNA was extracted from a culture supernatant from poliovirus-infected cells to serve as a marker. The recovered RNA was separated on a 6.6% formaldehyde-0.8%

agarose gel and transferred to a nitrocellulose membrane by capillary motion. The RNA was then hybridized to an in vitro-transcribed α -³²P-labeled riboprobe complementary to poliovirus plus-strand RNA between nucleotides 671 and 1174 (8). Following hybridization, the membrane was exposed to X-ray film at -70°C with an intensifying screen.

Quantitation of capsid proteins recovered from gradient fractions. For quantitative comparisons of the capsid protein distribution between virion and empty capsid fractions, sucrose density gradient fractionation was carried out on extracts of cells which had been coinfecting with PVdefSM and VVP1 mutants and incubated with [³⁵S]Translabel overnight as described in the previous section. A parallel fractionation of a lysate from ³⁵S-labeled poliovirus-infected cells allowed identification of fractions containing virions (fractions 5 to 8) and empty capsids (fractions 12 to 14). Capsid proteins were immunoprecipitated from fractions 5 to 8 and 12 to 14 from each gradient, and the virion samples and empty capsid samples from each gradient were pooled and analyzed by SDS-PAGE. The levels of protein VP3 recovered from the pooled virion and empty capsid samples were quantitated by phosphorimager (Molecular Dynamics).

RESULTS

Construction of recombinant vaccinia viruses which express mutant P1 polyproteins. The construction and characterization of a recombinant vaccinia virus which expresses the poliovirus type 1 Mahoney capsid precursor polyprotein, P1, has been described previously (1a). For the current studies, three new recombinant vaccinia viruses were generated, each of which expresses a mutant P1 precursor with an alteration at a site of proteolytic cleavage by the enzyme 3CD. Site-directed mutations encoding amino acid changes at sites of proteolytic cleavages were introduced into P1 capsid region cDNA (Fig. 1A). The mutant P1 cDNAs were subsequently introduced into the wild-type vaccinia virus (WTVV) genome by homologous recombination to generate recombinant vaccinia viruses (1a, 28). The mutations encoded a glycine-to-valine change at the amino terminus of VP3 (VP3-G001V) or at the amino terminus of VP1 (VP1-G001V). The third cleavage site mutation encoded a deletion of the first four amino-terminal residues of VP1 (VP1- Δ 1-4) and has been described previously by Kirkegaard and Nelsen in the context of the full-length poliovirus genome (24). This mutant was reconstructed for our studies by site-directed mutagenesis of P1 region cDNA and used to generate a recombinant vaccinia virus which expresses a P1 precursor containing the four-amino-acid deletion. The deletion results in the creation of a glutamine-methionine (QM) dipeptide which the previous study suggested could serve as the cleavage site between VP3 and VP1 (24). All four mutant vaccinia viruses were shown to express the P1 precursor by metabolic radiolabeling and immunoprecipitation with poliovirus capsid-specific antiserum (Fig. 1B). Each P1 protein showed a similar degree of stability upon further incubation for an additional 4 h after labeling with excess unlabeled methionine and cysteine, indicating that the mutations introduced into the P1 polyprotein did not significantly affect stability of the unprocessed P1 precursor proteins. A slight decrease in stability of the VP1- Δ 1-4 precursor relative to that of the other P1 precursors was observed in this experiment; however, in repeat experiments this has not been a consistent observation with the VP1- Δ 1-4 mutant. Two additional proteins were detected by immunoprecipitation with antipoliovirus antiserum. One protein migrated slightly faster than P1 and was also immunoprecipitated from wild-type vaccinia virus-in-

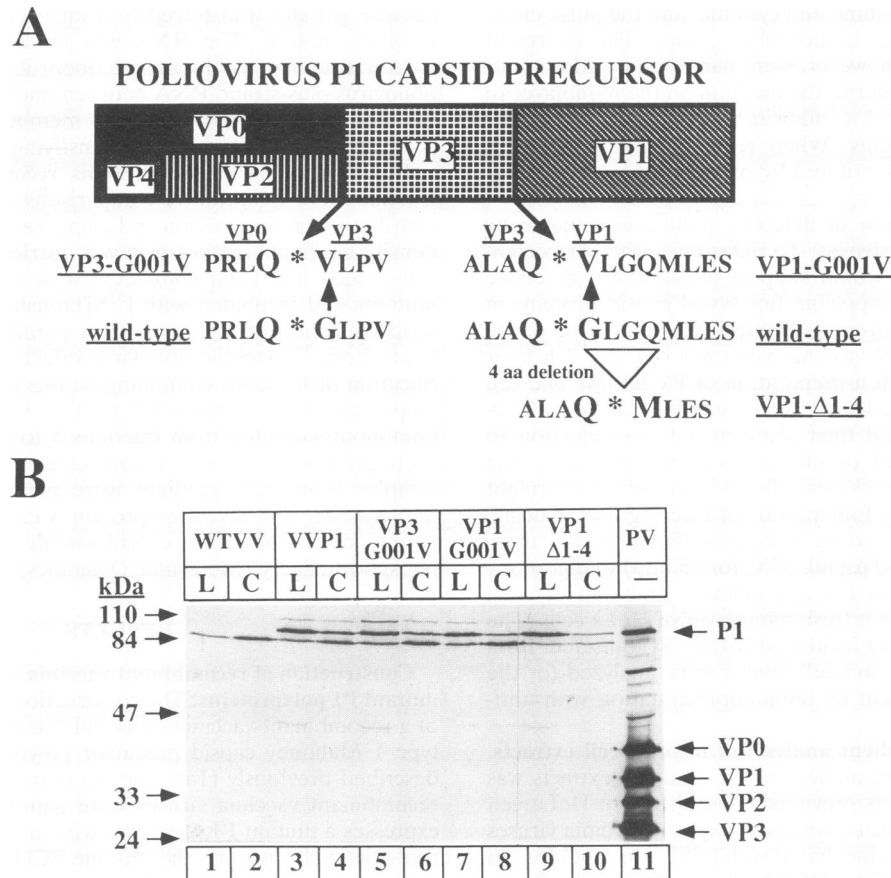


FIG. 1. Expression of poliovirus P1 capsid precursors with cleavage site mutations by using recombinant vaccinia viruses (A). Schematic diagram depicting the organization of individual poliovirus capsid proteins within the P1 capsid precursor. The glutamine-glycine cleavage sites of the wild-type P1 precursor and the altered cleavage sites encoded by the site-directed mutations constructed for this study are shown. (B) Stability of P1 precursors expressed by recombinant vaccinia viruses. The relative stabilities of the poliovirus P1 precursors containing QG cleavage site mutations were compared by immunoprecipitation analysis of P1 expression at the end of a 1-h metabolic radiolabeling period (lanes L) and after a 4-h chase period in medium containing a 20-fold excess of unlabeled methionine and cysteine (lanes C). The immunoprecipitated proteins were analyzed by SDS-PAGE, and an autoradiogram of the gel is shown. Samples were derived from cells infected with the following viruses: lanes 1 and 2, WTVV; lanes 3 and 4, VVP1; lanes 5 and 6, VVP1-VP3-G001V; lanes 7 and 8, VVP1-VP1-G001V; lanes 9 and 10, VVP1-VP1-Δ1-4. Lane 11 contains ^{35}S -radiolabeled proteins immunoprecipitated by antipoliovirus antiserum from cells infected with type 1 poliovirus to serve as marker proteins. The migrations of poliovirus P1 capsid precursor and the individual proteins VP0, VP1, VP2, and VP3 are noted. aa, amino acid.

infected cells. A second protein migrating more slowly than P1 was not immunoprecipitated from wild-type vaccinia virus-infected cells by antipoliovirus antiserum but has also been detected by immunoprecipitation from cells infected with other recombinant vaccinia viruses generated by using plasmid pSC11 and is most likely the β -galactosidase protein expressed as a selectable marker by these recombinants (data not shown).

Cleavage of mutant P1 polyproteins by 3CD protease provided by a separate recombinant vaccinia virus. Our initial aim in constructing the QV mutants was to generate P1 precursors that would be processed at only a single cleavage site. For our initial studies, the mutant P1 polyproteins VP3-G001V and VP1-G001V were analyzed for cleavage by the poliovirus protease 3CD by coinfecting cells with the recombinant vaccinia virus expressing the mutant P1 polyprotein and with a second recombinant vaccinia virus, VVP3, which expresses the poliovirus 3CD protease (1a, 35). Cleavage of the mutant polyproteins was compared with that of the wild-type P1 protein expressed in cells coinfecting with VVP1 and VVP3. Poliovirus capsid proteins generated in the recombinant vac-

cinia virus-coinfected cells were analyzed by immunoprecipitation after a pulse-chase radiolabeling experiment in which the coinfecting cells were incubated with [^{35}S]Translabel for 30 min and then subjected to a series of chase periods of up to 5 h in excess unlabeled methionine and cysteine (Fig. 2). In cells coinfecting with VVP1 and VVP3, complete proteolytic processing of the wild-type P1 precursor expressed by VVP1 was evident from the appearance of the cleavage products VP0, VP3, and VP1 (Fig. 2A). VP0, VP3, and VP1 were detected at the end of the 30-min labeling period (Fig. 2A, lane 1), and there was continued accumulation of these proteins as chase times were extended (Fig. 2A, lanes 2 to 7). Intermediates of P1 processing, uncleaved VP0-VP3 (1ABC) or uncleaved VP3-VP1 (1CD), were not detected at significant levels, indicating similar rates of processing at both QG cleavage sites in the P1 substrate. In contrast, formation of products of complete cleavage of both QV mutant P1 precursors was delayed in comparison with the formation of these products from wild-type P1 in cells coinfecting with VVP3 and either VVP1-VP3-G001V or VVP1-VP1-G001V (Fig. 2B and C). VP1 and

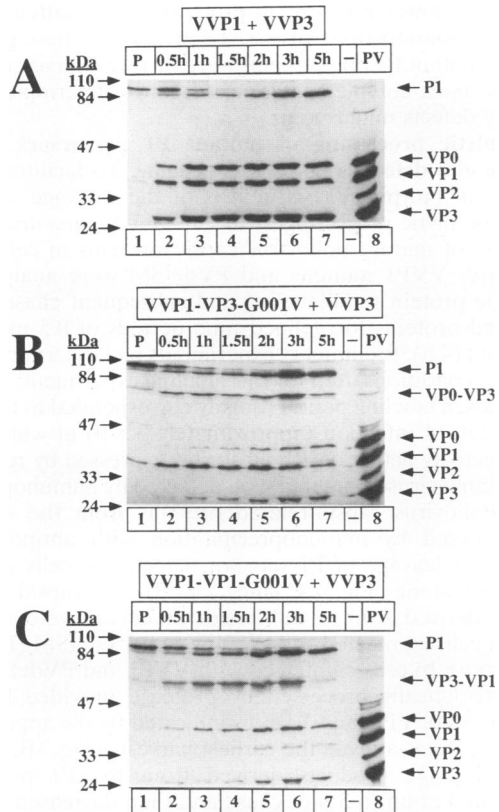


FIG. 2. Analysis of P1 precursor processing in cells coinfecting with P1-expressing recombinant vaccinia viruses and a second recombinant vaccinia virus which expresses 3CD protease. A pulse-chase analysis of P1 precursor processing was conducted by using cells coinfecting with VVP1 and VVP3 (A), VVP1-VP3-G001V and VVP3 (B), or VVP1-VP1-G001V and VVP3 (C). VVP3 is a recombinant vaccinia virus that expresses the poliovirus 3CD protease. Poliovirus capsid-specific proteins were immunoprecipitated from the coinfecting cells and analyzed by SDS-PAGE, and autoradiograms of the gels are shown. For each panel, the samples were derived after a 30-min pulse-radiolabeling (lane 1) or following subsequent chases in medium containing excess unlabeled methionine-cysteine for the indicated times (lanes 2 to 7). Lane 8 contains ^{35}S -radiolabeled poliovirus capsid-specific proteins immunoprecipitated from poliovirus-infected cells to serve as markers. The migrations of poliovirus capsid precursor P1, processing intermediates VP0-VP3 (1ABC) and VP3-VP1 (1CD), and individual capsid proteins VP0, VP1, VP2, and VP3 are indicated.

uncleaved VP0-VP3 (1ABC) were the major species of processed capsid proteins derived from the VP3-G001V mutant at the end of the pulse-labeling and at early chase harvests (Fig. 2B, lanes 1 to 3). VP0 and uncleaved VP3-VP1 (1CD) were the primary products detected at the end of the labeling period from cells coinfecting with VVP1-VP1-G001V and VVP3 and at early chase harvests (Fig. 2C, lanes 1 to 3). The apparent molecular masses of cleavage intermediates were consistent with those expected, approximately 63 kDa for VP0-VP3 and 60 kDa for VP3-VP1. The VP0-VP3 intermediate was also detected by immunoprecipitation from poliovirus-infected cells (Fig. 2B, lane 8). As chase times were extended, all three individual capsid proteins, VP0, VP3, and VP1, were gradually produced in cells coinfecting with VVP3 and either mutant P1-expressing recombinant vaccinia virus (Fig. 2B and 2C, lanes 4 to 7). These results demonstrated that the glycine-to-valine changes at the QG processing sites delayed, but did not

completely abolish, proteolytic processing at those sites by 3CD protease. However, a complete chase of the mutant P1 precursors to VP0, VP3, and VP1 was not observed, and the VP0, VP3, and VP1 products which did arise from complete cleavage of the mutant P1 precursors did not accumulate at levels comparable to those generated from wild-type P1, even at longer chase times. The apparent lack of a complete chase of radioactivity from precursors to products and the lack of accumulation of VP0, VP3, and VP1 derived from the mutant precursors suggest that the VP0-VP3 and VP3-VP1 intermediates and/or the VP0, VP3, and VP1 proteins generated from complete proteolytic processing of the mutant polyproteins were unstable.

trans complementation of a defective poliovirus genome by recombinant vaccinia viruses. The unexpected processing of the mutant P1 proteins containing QV cleavage sites led us to further characterize these mutants for possible assembly defects by comparison with wild-type P1 and the previously described VP1- Δ 1-4 mutant (23, 24). A report from our laboratory recently has described a new system for analysis of the poliovirus assembly and encapsidation processes in which recombinant vaccinia viruses are used to provide poliovirus capsid precursor in *trans* to a defective poliovirus genome which contains an in-frame deletion in the P1 capsid gene (3, 11). The defective poliovirus genomes do not synthesize functional capsid proteins and must be supplied with capsid proteins in *trans* in order to be encapsidated. The defective poliovirus genomes are delivered to cells by stock preparations of the defective poliovirus particles (PVdefSM) which are free of any contaminating helper wild-type virus since they have been maintained in serial passage with VVP1 to provide capsid precursor. The PVdefSM particles can be separated from residual VVP1 in the final preparations by using previously described techniques (3). By coinfecting cells with PVdefSM and recombinant vaccinia viruses which express mutant P1 precursors, the mutant precursors can be assayed for their abilities to complement the defective poliovirus genome and for assembly defects of cleavage products derived from the mutant precursors, as we have demonstrated previously for a recombinant vaccinia virus which expresses a nonmyristylated mutant P1 precursor (3). This system provides an advantage over methods which rely solely on characterization of mutant polioviruses or transfection of full-length RNA genomes containing capsid gene mutations which are subject to reversion during replication.

In the previous study by members of our laboratory, *trans* complementation of the defective poliovirus genome by VVP1 was shown to occur by demonstrating passage of the defective genome. Passage of the defective genome was indicated by expression of the poliovirus-specific protein 3CD upon infection of cells with particle preparations. In this study, we tested whether the recombinant vaccinia viruses expressing P1 polyproteins with cleavage site mutations were capable of *trans* complementing the defective poliovirus genome by providing functional capsid proteins required to generate infectious viral particles. HeLa cell monolayers were coinfecting with recombinant vaccinia viruses expressing mutant P1 polyproteins and with defective poliovirus (PVdefSM), and cell extracts from the primary coinfections were used to infect a new monolayer of HeLa cells following treatment with RNase A to remove unencapsidated RNA genomes in the preparations. The infected cells were analyzed for expression of poliovirus 3CD protein by immunoprecipitation using antiserum to 3D^{pol}. As expected from previous studies, 3CD expression was not detected upon infection of cells with extracts derived from cells infected with PVdefSM or VVP1 alone or from cells coin-

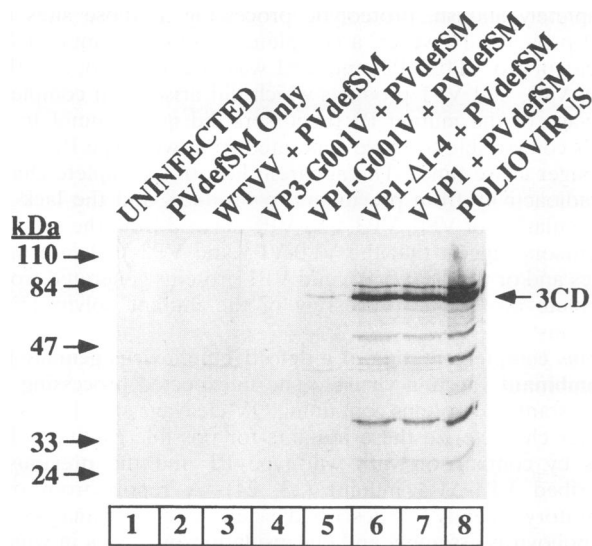


FIG. 3. Expression of poliovirus 3CD protein in cells infected with defective polioviruses generated by *trans* complementation of a defective poliovirus genome. Defective polioviruses were generated by coinfection of cells with recombinant vaccinia viruses expressing P1 precursors and preparations of defective polioviruses (PVdefSM) which contain RNA genomes with a capsid gene deletion and which do not encode functional capsid proteins. Defective polioviruses generated in the coinfecting cells were used to infect a new monolayer of cells, and the infected cells were analyzed for expression of poliovirus 3CD protein by metabolic radiolabeling and immunoprecipitation with anti-3D^{pol}. The immunoprecipitated proteins were analyzed by SDS-PAGE, and an autoradiogram of the gel is shown. Samples were derived from uninfected cells (lane 1) or from cells infected with extracts from cells initially infected with the indicated viruses (lanes 2 to 7). Lane 8 contains ³⁵S-radiolabeled proteins immunoprecipitated by anti-3D^{pol} from poliovirus-infected cells to serve as marker proteins. The migration of 3CD protein is indicated.

fecting with WTVV and PVdefSM (Fig. 3, lanes 1 to 3). 3CD expression also was not detected upon infection of cells with the extract from cells coinfecting with VVP1-VP3-G001V and PVdefSM (lane 4). In contrast, 3CD expression was detected upon infection of cells with extracts derived from cells coinfecting with VVP1-VP1G001V and PVdefSM (lane 5), VVP1-VP1Δ1-4 and PVdefSM (lane 6), or VVP1 and PVdefSM (lane 7). The level of 3CD expression resulting from infection of cells with the VVP1-VP1-G001V/PVdefSM extract was markedly lower than that from cells infected with either the VVP1-VP1-Δ1-4/PVdefSM or VVP1/PVdefSM extract. In serial passage experiments in which extracts from cells coinfecting with PVdefSM and recombinant vaccinia virus were passed two additional times with the same recombinant vaccinia virus, similar results were obtained: levels of 3CD expression from cells infected with the VVP1-VP1-Δ1-4/PVdefSM extract were similar to those from cells infected with the VVP1-VP1-Δ1-4/PVdefSM extract, whereas a lower level of expression of 3CD from cells infected with the VVP1-VP1-G001V/PVdefSM extract was detected (data not shown). Since formation of products of the complete cleavage of P1 precursors (VP0, VP3, and VP1) was observed in cells coinfecting with the QV mutants and VVP3, although at reduced levels in comparison with wild-type P1, the lack of complementation observed with the VP3-G001V mutant and the reduced level of complementation observed for the VP1-G001V mutant suggested that the glycine-to-valine changes resulted in virus assembly defects in

addition to slower polyprotein processing or resulted in production of noninfectious viruses. To investigate these possibilities, the mutant P1 polyproteins were further characterized by using the *trans* complementation system to determine where assembly defects might occur.

Proteolytic processing of mutant P1 precursors in the presence of a defective poliovirus genome. To facilitate identification of morphogenesis defects of the cleavage site mutants, proteolytic processing of the mutant P1 precursors and formation of mature poliovirus capsid proteins in cells coinfecting with VVP1 mutants and PVdefSM were analyzed by metabolic protein radiolabeling and subsequent chase of radiolabeled proteins for defined time periods of 0.5 to 4 h or overnight (14 h). Preliminary experiments had determined that under the conditions used for this labeling experiment, the end of the chosen labeling period roughly corresponded to the time post-PVdefSM infection (approximately 3.5 h) at which proteolytic activity acting on P1 substrates expressed by recombinant vaccinia viruses could first be detected by immunoprecipitation. Poliovirus capsid-related proteins from the samples were analyzed by immunoprecipitation with antipoliovirus antiserum. Cleavage of P1 was not detected in cells infected with VVP1 alone (Fig. 4A, lanes 1 to 3), and capsid-specific proteins derived from a full-length precursor were not detected in cells coinfecting with WTVV and PVdefSM (Fig. 4A, lanes 4 to 6). In cells coinfecting with VVP1 and PVdefSM, P1 was proteolytically processed by protease provided by PVdefSM to VP0, VP3, and VP1, as indicated by the appearance of these proteins at even the earliest harvests (Fig. 4B, lanes 1 to 3). Cleavage products derived from the P1 precursor accumulated at up to 3 h of chase and then decreased slightly after chase times of 4 h or overnight, as determined by quantitation of levels of immunoprecipitated VP3 by phosphorimager (Fig. 4B, lanes 1 to 8). After a 1-h chase and subsequent chases, the mature virion protein VP2 was detected, indicating that the maturation cleavage of VP0 associated with formation of mature virions had occurred (Fig. 4B, lanes 3 to 8).

Cleavage of mutant P1 precursors by protease provided by PVdefSM was analyzed in a similar manner and compared with the processing products derived from the wild-type precursor at different chase times. From cells coinfecting with VVP1-VP3-G001V and PVdefSM, P1, VP0-VP3 (1ABC), and VP1 were the major capsid species detected at the end of the labeling period and at chase times of up to 2 h (Fig. 4C, lanes 1 to 5); however, after further incubation, complete proteolytic processing of some of the mutant precursors was evident from the appearance of VP0, VP3, and VP1 (lanes 6 to 8). Levels of completely processed proteins were quantitated by phosphorimager for comparison with levels of proteins derived from wild-type P1. VP3 levels at each time were quantitated as an indicator of complete processing, since release of VP3 from the P1 precursor requires cleavage at both sites. VP3 derived from the VP3-G001V mutant precursor reached levels of 5% of that derived from wild-type precursor after 3 h of chase but dropped to approximately 1% of wild-type levels after an overnight chase. The decrease in levels of completely processed proteins detected after longer incubations indicated that the cleaved proteins derived from the VP3-G001V precursor were unstable.

Formation of cleavage products from complete cleavage of VP1-G001V precursors was reduced in comparison with that from the wild-type precursor, with less-frequent cleavage at the QV site between VP3 and VP1 apparent by the presence of an uncleaved VP3-VP1 (1CD) intermediate (Fig. 4D, lanes 1 to 5). A VP0-VP3 (1ABC) intermediate derived from this mutant

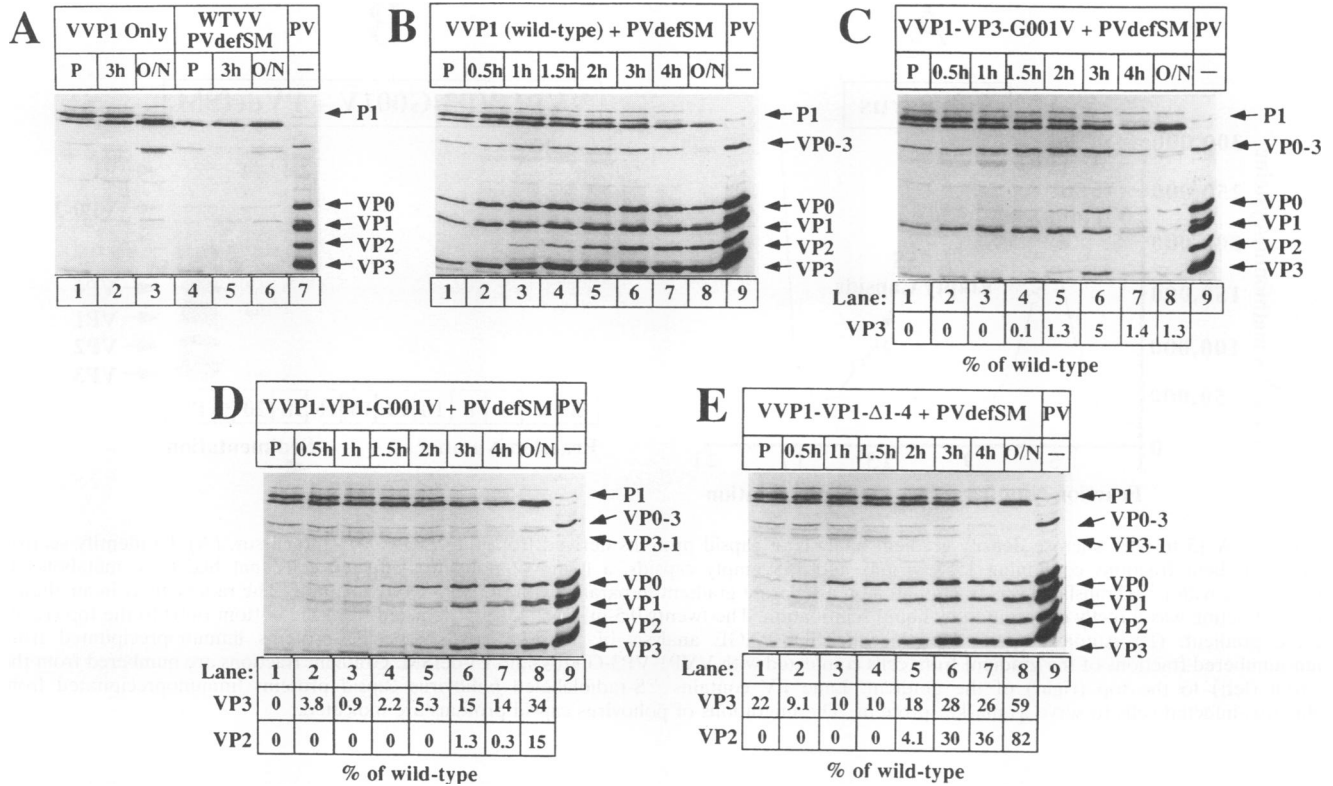


FIG. 4. Analysis of P1 precursor processing in cells coinfecting with P1-expressing recombinant vaccinia viruses and defective poliovirus. SDS-PAGE analysis of poliovirus capsid-specific proteins immunoprecipitated from lysates of cells coinfecting with recombinant vaccinia viruses and PVdefSM is shown. Infected cells were metabolically radiolabeled for 1 h (pulse) followed by subsequent chases of up to 14 h (overnight) in medium containing excess unlabeled methionine-cysteine. For each panel, the migrations of relevant poliovirus capsid proteins are indicated. (A) Samples were derived from cells infected with VVP1 alone (lanes 1 to 3) or coinfecting with WTVV and PVdefSM (lanes 4 to 6). The samples were collected at the end of the pulse (P) or after chases of 3 h or overnight (O/N). Lane 7 contains ³⁵S-radiolabeled proteins immunoprecipitated by antipoliovirus antiserum from a lysate of poliovirus-infected cells to serve as marker proteins. (B) Samples were derived from cells coinfecting with VVP1 and PVdefSM. The samples were collected at the end of the pulse (P) or following chases of the indicated times. Lane 9 contains poliovirus capsid proteins immunoprecipitated from poliovirus-infected cells. (C) Samples were derived from cells coinfecting with VVP1-VP3-G001V and PVdefSM and collected as described for panel B. For lanes 1 to 8, the VP3 band was quantitated by phosphorimagery and expressed as a percentage of the VP3 levels measured for that time point on the VVP1-PVdefSM gel. The percentages are indicated below lanes 1 to 8. Lane 9 contains poliovirus capsid proteins immunoprecipitated from poliovirus-infected cells. (D) Samples were derived from cells coinfecting with VVP1-VP1-G001V and PVdefSM and collected as described for panel B. The VP3 and VP2 bands in each lane were quantitated by phosphorimagery and expressed as a percentage of the levels measured in the corresponding lane on the VVP1-PVdefSM gel. The percentages are indicated below lanes 1 to 8. Lane 9 contains poliovirus capsid proteins immunoprecipitated from poliovirus-infected cells. (E) Samples were derived from cells coinfecting with VVP1-VP1Δ1-4 and collected as described for panel B. Levels of VP3 and VP2 in each lane were determined and are depicted as described for panel D.

was also detected, indicating that some of the VP1-G001V precursors were cleaved at the QV site first. Complete proteolytic processing was evident at 1.5 h of chase and thereafter by the appearance of VP3 (Fig. 4D, lanes 4 to 8), and VP3 levels derived from this mutant reached 34% of those derived from wild-type P1 after the overnight chase period.

The P1 protein expressed by VVP1-VP1Δ1-4 was proteolytically processed to individual capsid proteins VP0, VP3, and VP1, although, as with the VP1G001V mutant, an uncleaved VP3-VP1 (1CD) intermediate was detected, indicating less-frequent cleavage between VP3 and VP1 in comparison with that in the wild-type precursor. The four-amino-acid deletion resulted in a QM dipeptide at the cleavage site, and the generation of VP3 and VP1 from the VP1-Δ1-4 precursor lends support to the idea (24) that cleavage can occur between the glutamine and methionine residues (Fig. 4E, lanes 1 to 6). VP0, VP3, and VP1 derived from this mutant were increasingly evident as chase times were extended (lanes 2 to 8), and after

an overnight chase, VP3 levels reached 59% of those derived from wild-type P1 precursor. As with the VP1-G001V mutant, uncleaved VP0-VP3 was also detected, indicating that in some instances the VP1-G001V precursor was processed at the QM site prior to or in the absence of cleavage at the unaltered site between VP0 and VP3.

Levels of the mature virion protein VP2 produced in the recombinant vaccinia virus-PVdefSM-coinfecting cells were also compared quantitatively by phosphorimagery. Production of VP2 was observed from the wild-type precursor and from the VP1-G001V and VP1-Δ1-4 precursors but was not observed from the VP3-G001V precursor. The appearance of VP2 derived from the VP1-G001V and VP1-Δ1-4 precursors was delayed in comparison with that from the wild-type precursor. VP2 derived from the wild-type precursor was first observed after 1 h of chase (Fig. 4B, lane 3); that from the VP1-Δ1-4 precursor was first observed faintly after 2 h of chase (Fig. 4E, lane 5), and that from the VP1-G001V precursor was

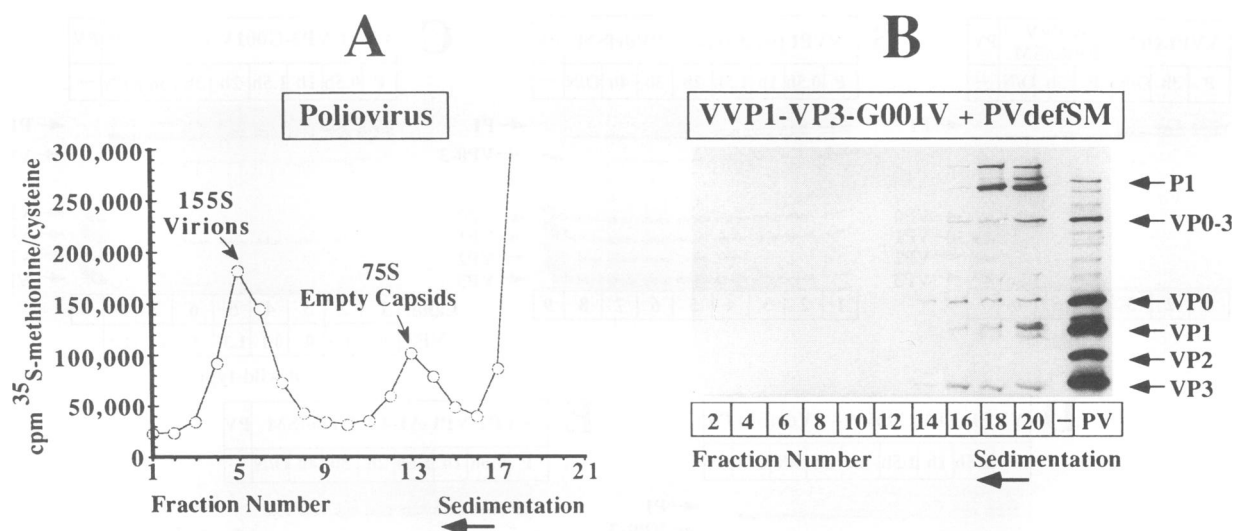


FIG. 5. A 15 to 30% sucrose density gradient analysis of capsid proteins derived from the VP3-G001V precursor. (A) To identify sucrose density gradient fractions containing 155S virions and 75S empty capsids, a lysate of poliovirus-infected cells that had been metabolically radiolabeled with [^{35}S]Translabel was fractionated on a separate gradient in parallel with the VP3-G001V sample. The radioactivity in an aliquot of each fraction was directly quantitated by liquid scintillation. The twenty 0.5-ml fractions are depicted from the bottom (left) to the top (right) of the gradient. (B) Autoradiogram depicting the SDS-PAGE analysis of poliovirus capsid-specific proteins immunoprecipitated from even-numbered fractions of the gradient from cells coinfecting with VVP1-VP3-G001V and PVdefSM. Gradient fractions are numbered from the bottom (left) to the top (right) of the gradient. Lane PV contains ^{35}S -radiolabeled poliovirus capsid proteins immunoprecipitated from poliovirus-infected cells to serve as marker proteins. The migrations of poliovirus capsid proteins are indicated.

first observed at low levels after 3 h of chase (Fig. 4D, lane 6). VP2 derived from the VP1- Δ 1-4 mutant achieved levels 82% of those of VP2 derived from the wild-type precursor after an overnight chase; however, VP2 derived from the VP1-G001V mutant reached only 15% of wild-type levels. The difference in amounts of completely processed proteins (VP0-VP3-VP1) derived from the VP1- Δ 1-4 mutant versus the VP1-G001V mutant (59 versus 34% of wild-type levels) was not as pronounced as the difference in amounts of VP2 derived from the two mutants (82 versus 15% of wild-type levels). The percent discrepancy in the levels of VP3 and VP2 derived from the VP1- Δ 1-4 precursor after the overnight chase might indicate a surplus of completely processed proteins derived from the wild-type precursor which were not virion associated. Since the presence of VP2 indicates that the maturation cleavage associated with mature virion formation has taken place, these results suggested that capsid subunits derived from the VP1-G001V mutant might have defects in a late stage of virion morphogenesis.

Assembly of capsid proteins derived from mutant P1 polyproteins. To better characterize assembly defects of processed capsid proteins derived from the mutant P1 precursors, the sedimentation properties of the cleavage products were analyzed on sucrose density gradients. A 15 to 30% sucrose density gradient fractionation was conducted with lysates of cells coinfecting with PVdefSM and recombinant vaccinia viruses expressing mutant P1 precursors. A sucrose gradient fractionation was conducted with a lysate of poliovirus-infected cells to identify fractions containing 155S virions and 75S empty capsids (Fig. 5A).

Capsid proteins derived from the VP1-G001V and VP1- Δ 1-4 mutants were analyzed after overnight incubation, whereas capsid proteins derived from the VP3-G001V mutant were analyzed 7.5 h postinfection with PVdefSM, since longer incubation times resulted in a decrease of radiolabeled processed proteins (Fig. 4C). Consistent with the pulse-chase

analysis of capsid proteins derived from the VP3-G001V precursor, we detected low levels of completely processed capsid proteins from cells coinfecting with VVP1-VP3-G001V and PVdefSM. The VP0, VP3, and VP1 proteins were detected only in the upper fractions of the sucrose gradient (fractions 14 to 20) (Fig. 5B). Quantitation of VP3 levels in each fraction by phosphorimager indicated that processed proteins were primarily localized from fractions 16 to 20 with lower amounts in fraction 14. Capsid proteins derived from this mutant were not detected in virion-corresponding fractions even upon overexposure of the gel depicted in Fig. 5B to X-ray film (data not shown). Although the presence of capsid proteins in fraction 14 suggested that some of the processed capsid proteins derived from this mutant might have assembled empty capsids, the instability of these proteins argues against the formation of stable assembly intermediates. In support of this idea, assembly of stable subviral intermediates derived from this mutant precursor was also not observed in cells coinfecting with VVP1-VP3-G001V and VVP3, the recombinant vaccinia virus that expresses 3CD protease (data not shown).

In contrast to those from the VP3-G001V mutant, capsid proteins derived from the VP1- Δ 1-4 and VP1-G001V precursors did assemble subviral intermediates and mature virions (Fig. 6A and B). For both of these mutants, VP0, VP3, and VP1 accumulated in fractions consistent with the empty capsid peak on the poliovirus gradient (fractions 13 to 15), and primarily capsid proteins VP1, VP2, and VP3 were immunoprecipitated from fractions corresponding to the virion peak on the poliovirus gradient (fractions 5 to 9). These results demonstrated that capsid proteins derived from the VP3-VP1 cleavage site mutant precursors were capable of forming mature virions. However, it was possible that fractions above the mature virion fractions might contain provirions that were not clearly resolved away from empty capsids in these gradients. To address this question, the sedimentation of capsid proteins derived from these mutants was compared with that of

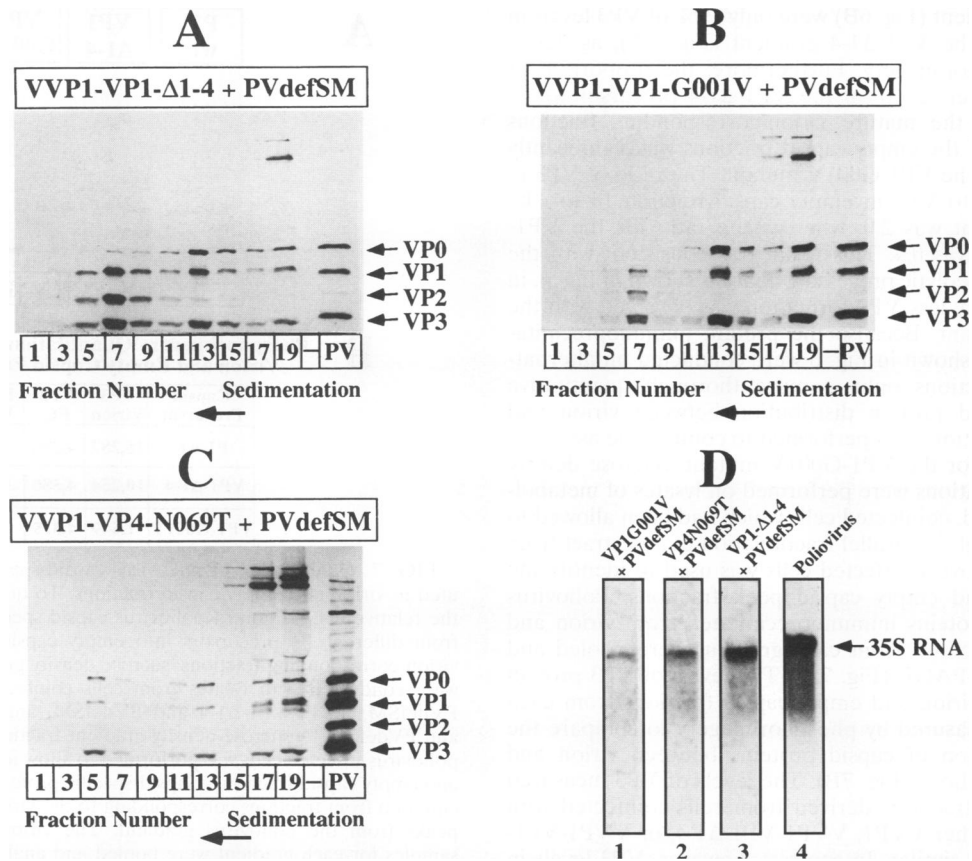


FIG. 6. Sucrose density gradient analysis of capsid proteins derived from precursors with mutations at the VP3-VP1 QG cleavage site. A 15 to 30% sucrose density gradient analysis of poliovirus capsid-specific proteins was performed as described for Fig. 5. Empty capsid and virion fractions were identified as described for Fig. 5. (A) Autoradiogram depicting an SDS-PAGE analysis of poliovirus capsid-specific proteins immunoprecipitated from the odd-numbered fractions of the gradient from cells coinfecting with VVP1-VP1- Δ 1-4 and PVdefSM. (B) Autoradiogram depicting an SDS-PAGE analysis of poliovirus capsid-specific proteins immunoprecipitated from the odd-numbered fractions of the gradient from cells coinfecting with VVP1-VP1-G001V and PVdefSM. (C) Autoradiogram depicting an SDS-PAGE analysis of poliovirus capsid-specific proteins immunoprecipitated from the odd-numbered fractions of the gradient from cells coinfecting with VVP1-VP4-N069T and PVdefSM. VVP1-VP4-N069T expresses a P1 precursor with a threonine substitution for asparagine at the asparagine-serine cleavage site between VP4 and VP2. The VP4-N069T mutant precursor is deficient in the maturation cleavage of VP0 and accumulates provirions or immature virions. Sucrose density gradient analysis of an extract from cells coinfecting with VVP1-VP4-N069T and PVdefSM was performed to identify fractions containing the provirion particles (fractions 5 to 7) (D) Northern blot analysis of poliovirus-specific RNA extracted from sucrose density gradient fractions. RNA was extracted from gradient fractions 6 and 8 from the VP1-G001V, VP4-N069T and VP1- Δ 1-4 gradients. The RNA was analyzed by Northern blot hybridization using a [32 P]UTP-labeled riboprobe complementary to poliovirus plus-strand RNA from nucleotides 671 to 1174. Samples were derived from the VP1-G001V gradient (lane 1), the VP4-N069T gradient (lane 2), and the VP1- Δ 1-4 gradient (lane 3). Lane 4 contains RNA extracted from type 1 poliovirus virions. Lanes 1 to 3 were exposed to X-ray film six times as long as lane 4.

capsid proteins derived from a fourth capsid mutant which has a threonine substitution for asparagine at residue 69 of VP4 (VP4-N069T). This substitution occurs at the asparagine-serine site of maturation cleavage of VP0; the corresponding mutation in human rhinovirus type 14 has been constructed and characterized by Lee et al. (27) and was demonstrated to result in an accumulation of provirions, or immature virions, in which RNA has been encapsidated but VP0 has not been cleaved to VP2 and VP4. The details of the construction and characterization of the VVP1-VP4-N069T recombinant vaccinia virus will be presented elsewhere (1). A sucrose density gradient analysis of a lysate from cells coinfecting with VVP1-VP4-N069T and PVdefSM resulted in detection of a peak of VP0, VP3, and VP1 in fractions corresponding to the virion peak on the poliovirus gradient (Fig. 6C, fractions 5 to 7). The use of this mutant, then, provided a marker for provirion particles for these gradient experiments and indicated that

capsid proteins derived from the VP1-G001V and VP1- Δ 1-4 mutants did not accumulate provirions. Finally, to confirm the identity of RNA-containing particle fractions, even-numbered fractions from the VP1-G001V, VP4-N069T, and VP1- Δ 1-4 gradients corresponding to the mature virion peak from the poliovirus gradient were shown to contain poliovirus-specific RNA upon phenol-chloroform extraction and Northern blot analysis using a riboprobe complementary to poliovirus plus-strand RNA (Fig. 6D). The RNA species detected from these fractions migrated slightly faster on the formaldehyde-agarose gel than full-length (35S) poliovirus RNA, consistent with the migration expected for the deletion-containing RNA genome (11).

Although capsid proteins derived from both the VP1-G001V and VP1- Δ 1-4 mutants assembled mature virions, significantly less material sedimented into virion fractions on the VP1-G001V gradient. For example, VP3 levels in fraction 7 on the

VP1-G001V gradient (Fig. 6B) were only 19% of VP3 levels in fraction 7 from the VP1- Δ 1-4 gradient (Fig. 6A), as determined by phosphorimagery. Furthermore, the proportion of capsid proteins derived from the VP1- Δ 1-4 precursor which sedimented into the mature virion-corresponding fractions relative to that in the empty capsid fractions was significantly higher than with the VP1-G001V mutant. The ratio of VP3 in virion fraction 7 to VP3 in empty capsid fraction 13 for the VP1- Δ 1-4 gradient was 2.0, whereas the ratio for the VP1-G001V gradient was 0.4. This result was consistent with the results of the proteolytic processing analysis shown in Fig. 4, in which considerably less VP2 formation was observed with the VP1-G001V mutant. Because the protein immunoprecipitation experiments shown in Fig. 6 were conducted on alternating gradient fractions only, a more thorough quantitative analysis of capsid protein distribution between virion and empty capsid fractions was performed to confirm the assembly defect observed for the VP1-G001V mutant. Sucrose density gradient fractionations were performed on lysates of metabolically radiolabeled, coinfecting cells which had been allowed to incubate overnight. A parallel fractionation of an extract from radiolabeled poliovirus-infected cells was used to identify the mature virion and empty capsid peak fractions. Poliovirus capsid-specific proteins immunoprecipitated from virion and empty capsid fractions from each gradient were pooled and analyzed by SDS-PAGE (Fig. 7A). The levels of VP3 protein recovered from virion and empty capsid fractions from each gradient were measured by phosphorimagery to compare the relative distribution of capsid proteins between virion and empty capsid fractions (Fig. 7B). The levels of VP3 measured in empty capsid fractions derived from cells coinfecting with PVdefSM and either VVP1, VVP1-VP1- Δ 1-4, or VVP1-VP1-G001V were very similar. In contrast, however, VP3 levels in virion fractions on the VP1-G001V gradient were markedly lower than those in the virion fractions from the VVP1 or VP1- Δ 1-4 gradient. Levels of VP3 derived from wild-type P1 expressed by VVP1 were found to be 3.3 times higher than levels in empty capsid fractions. In contrast, VP3 derived from the VP1-G001V mutant was detected in virion fractions at a level of 0.38 times that in the empty capsid fractions. Thus, the proportion of capsid proteins in the empty capsid fractions relative to those in the virion fractions was essentially inverted when derived from the VP1-G001V mutant. The ratio of virion VP3 to empty capsid VP3 was approximately 2.2 when derived from the VP1- Δ 1-4 mutant. These results demonstrated that the capsid proteins derived from the VP1-G001V mutant were inhibited in formation of virions, since empty capsids were found in abundance over mature virion particles.

DISCUSSION

In this report, we have described the construction and characterization of poliovirus P1 capsid mutants with altered QG cleavage sites. The mutant P1 polyproteins were expressed by using recombinant vaccinia virus vectors and were analyzed for ability to *trans* complement a capsid gene-deficient poliovirus RNA genome delivered to the recombinant vaccinia virus-infected cells by coinfection with a preparation of poliovirus particles containing only the defective genomes (PV-defSM). In the *trans* complementation assay (Fig. 3), the VP3-G001V mutant precursor failed to complement the defective genome, whereas the VP1- Δ 1-4 mutant complemented at levels similar to those of the wild-type P1 precursor. The VP1-G001V precursor complemented the defective genome at levels markedly below those of either wild-type P1 or the VP1- Δ 1-4 precursor. The levels of complementation observed

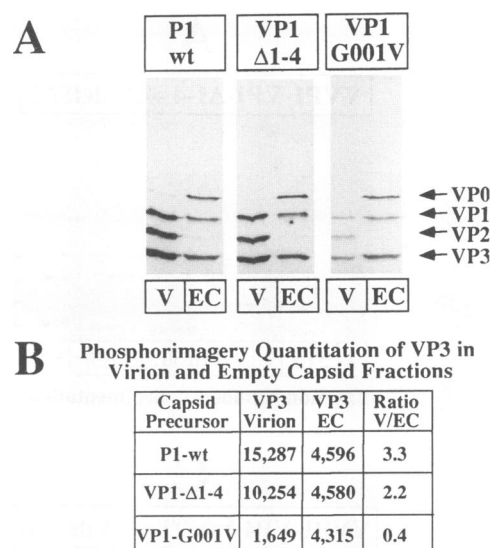


FIG. 7. Quantitation of poliovirus capsid-specific proteins distributed in virion and empty capsid fractions. To quantitatively compare the relative distribution of poliovirus capsid specific proteins derived from different P1 precursors into empty capsid-corresponding and virion-corresponding fractions, sucrose density gradient fractionations were conducted with lysates from cells coinfecting with VVP1 and PVdefSM, VVP1-VP1- Δ 1-4 and PVdefSM, and VVP1-VP1-G001V and PVdefSM. A sucrose density gradient fractionation of a lysate of poliovirus-infected cells was performed to allow identification of virion and empty capsid fractions. Capsid-specific proteins were immunoprecipitated from fractions corresponding to the virion and empty capsid peaks from the poliovirus gradient. The virion and empty capsid samples for each gradient were pooled and analyzed by SDS-PAGE. (A) Autoradiogram of the SDS-PAGE analysis of the pooled immunoprecipitated proteins from empty capsid (EC) and virion (V) fractions derived from each precursor. The migrations of capsid proteins VP0, VP1, VP2, and VP3 are indicated. (B) The VP3 proteins in each lane of the gels shown in panel A were quantitated by phosphorimagery. The values resulting from this analysis are presented along with the ratio of VP3 derived from each precursor present in virion fractions to that present in empty capsid fractions (V/EC).

in Fig. 3 were reflected in the differences in proteolytic processing, assembly, and encapsidation observed upon analysis of the products of cleavage of the mutant precursors. Mutant P1 polyproteins containing QV cleavage sites in place of QG sites were proteolytically processed at the altered sites by the viral protease 3CD, although processing at the altered sites was hindered. The VP0, VP3, and VP1 capsid proteins resulting from complete cleavage of P1 precursors with the VP3-G001V substitution did not assemble stable virions or subviral intermediates. The VP0, VP3, and VP1 proteins generated upon complete cleavage of P1 precursors with the VP1-G001V mutation were found to assemble empty capsids, but formation of virions was inhibited, and empty capsids derived from this mutant accumulated in abundance over virions in contrast to particles derived from wild-type P1. Interestingly, the defect in virion formation observed for the VP1-G001V mutant was less evident when the first four amino acids of VP1 were deleted (VP1- Δ 1-4). These results demonstrate that the glycine-to-valine substitution at the amino terminus of VP1 rendered capsid protomers defective for RNA encapsidation and that this effect was partially overcome by deletion of the first four amino-terminal amino acids of VP1.

We were initially surprised to find that the P1 capsid precursors with QV cleavage sites were proteolytically processed at the altered sites, even with slower kinetics. The strict conservation of the QG cleavage sites in the poliovirus polyprotein (12, 25) suggested a limited flexibility for site recognition by poliovirus 3C^{pro} enzyme or proteolytically active polyproteins that contain 3C^{pro}, such as 3CD. A previous study of proteolytic processing of EMCV capsid precursor by using a cell-free *in vitro* system found that a QV-substituted site at the VP3-VP1 junction (QG is wild type) was not cleaved by 3C^{pro}, nor were other mutant sites containing larger hydrophobic side chains at the P1' position (34). The current study shows that the poliovirus 3CD protease can accommodate a valine side chain at the P1' position of the scissile bond, although processing at the QV sites occurs more slowly. This flexibility in the poliovirus P1 cleavage sites supports a previous study which suggested that a QM dipeptide created by deletion of the first four amino acids of VP1 could serve as the cleavage site between VP3 and VP1 (24). It is possible that the QG site mutations constructed for this study diverted proteolytic cleavage to an alternative, more favorable site in nearby sequences. Amino acid pairs that would constitute alternative cleavage sites, however, are not readily apparent in the surrounding sequences. Obvious mobility shifts for processed proteins derived from the VP3-G001V and VP1-G001V precursors, which might have indicated aberrant processing, were not observed. As previously reported by Kirkegaard and Nelsen, the VP1 protein derived from cleavage of the VP1-Δ1-4 precursor did have an altered mobility; however, an altered mobility for the VP3 protein derived from this mutant was not observed (24). If the slower migration of VP1 derived from the VP1-Δ1-4 precursor was the result of additional sequences present because processing had occurred at an alternative site in the carboxy-terminal portion of VP3, a similar shift in migration might have been expected for VP1 derived from the VP1G001V mutant if mutations at the QG cleavage site diverted proteolytic cleavage to an alternative bond. These studies suggest that a greater flexibility exists for substitutions of the P1' position of the QG cleavage sites in the poliovirus P1 precursor than for those of the EMCV P1 precursor. Alternatively, cleavage of the poliovirus P1 precursor *in vivo* may be more efficient and more tolerant of cleavage site substitutions.

A previous report suggested that processing of the poliovirus P1 precursor protein is ordered, with processing occurring first at the VP3-VP1 bond, releasing VP1, followed by cleavage of the VP0-VP3 (1ABC) intermediate (37). Evidence for this cleavage order was based on the observance of a VP0-VP3 (1ABC) intermediate but not a VP3-VP1 (1CD) intermediate from poliovirus-infected cells. Previous studies of picornaviral capsid precursor processing *in vitro* indicated that an obligatory order of cleavage does not exist for EMCV capsid precursor (34). The results of the present study demonstrate that cleavage at the QG site between VP3 and VP1 is not obligatory for proteolytic processing to take place at the QG site between VP0 and VP3 in the poliovirus P1 precursor, because a VP3-VP1 intermediate was readily detected as a product of cleavage of the VP1-G001V mutant precursor, demonstrating release of VP0 without prior cleavage between VP3 and VP1. In addition, the overall rate of cleavage of P1 precursors to either partially (VP0-VP3 and VP1 or VP0 and VP3-VP1) or completely (VP0, VP3, and VP1) cleaved products was similar for all of the mutant precursors and wild-type P1, providing further evidence that a less favorable cleavage site at one of the two locations did not significantly delay or preclude cleavage at the other site (data not shown). Whether the cleavage intermediates VP0-VP3 (1ABC) and VP3-VP1

(1CD) are substrates for 3CD protease or simply end products is not clear. The cleavage intermediates might be products of an abortive processing event which leaves cleavage intermediates that cannot serve as substrates for the enzyme. *In vitro*, the 3CD polyprotein has been identified as the protease responsible for efficient complete proteolytic cleavage of P1 to VP0, VP3, and VP1 (20, 42), whereas 3C^{pro} cleaves the P1 precursor only at the cleavage site between VP3 and VP1 (30). The *in vitro* cleavage of P1 to VP0-VP3 (1ABC) and VP1 by 3C^{pro} occurs inefficiently and only at high enzyme concentrations (30). Whether this cleavage by 3C^{pro} between VP3 and VP1 occurs *in vivo* is unclear. An interesting speculation might be that cleavage of populations of P1 by mature 3C^{pro} rather than 3CD in poliovirus-infected cells results in production of VP0-VP3 and VP1 and accounts for the observation of the VP0-VP3 intermediate from poliovirus-infected cells. In support of this idea, we did observe a VP0-VP3 cleavage intermediate from cells coinfecting with VVP1 and PVdefSM, in which both 3CD and 3C^{pro} should be present (Fig. 4B); however, in Fig. 2A, a VP0-VP3 intermediate from cells coinfecting with VVP1 and VVP3, which expresses 3CD without further cleavage to 3C^{pro} and 3D^{pol} (1a, 35), was not as evident. Cleavage of the P1 precursor at both QG sites might occur in a single complex interaction event with 3CD enzyme, with incomplete cleavage leading to formation of substrates not recognized by the protease. Overall yields of completely processed proteins (VP0, VP3, and VP1) derived from each of the mutant precursors were reduced in comparison with those derived from wild-type P1, suggesting that cleavage intermediates were unstable or were not further processed. Completely processed proteins derived from the mutant precursors might have arisen from situations in which complete cleavage of the precursors occurred in a single interaction event with enzyme.

Although the mutations introduced at the QG sites in the P1 capsid precursors hindered cleavage of the precursors, complete cleavage of a population of each of the mutant precursors was evident by formation of VP3, which can be released only upon cleavage at both of the sites acted on by 3CD in a common precursor protein. The VP0, VP3, and VP1 proteins generated upon complete cleavage of the VP3-G001V mutant P1 polyproteins did not assemble empty capsids or virions at detectable levels. The VP0, VP3, and VP1 products of complete cleavage of the VP3-G001V precursor were also unstable, and this instability is similar to that we have observed in previous studies of capsid proteins derived from a nonmyristylated poliovirus P1 precursor which are also defective in assembly (2, 3). Assembly of 14S pentamers, empty capsids, and virions is most likely preceded by association of single copies of VP0, VP3, and VP1 to form 5S protomer or monomer building blocks of the capsid (38). The 5S protomers may assemble from VP0, VP3, and VP1 derived from a common P1 precursor immediately after cleavage. In the case of the cleavage site mutants described in this report, we cannot distinguish whether VP0, VP3, and VP1 which do arise from complete cleavage of the mutant P1 precursors are derived from events in which cleavage occurred at both the unaltered and mutant sites in a single interaction event with 3CD or whether products of complete cleavage arise from processing of VP0-VP3 or VP3-VP1 intermediates in subsequent interactions with 3CD. If formation of 5S protomers is dependent upon association of VP0, VP3, and VP1 derived from a common precursor immediately after cleavage, then a delay in cleavage at one of the two sites may preclude formation of 5S protomers and subsequently other capsid particles and virions. Thus, we might have expected capsid proteins derived from both the VP3-G001V and VP1-G001V precursors to be defec-

tive in assembly of capsid particles. Instead, VP0, VP3, and VP1 derived from the VP3-G001V precursor failed to assemble stable capsid particles, whereas those derived from the VP1-G001V precursor did assemble empty capsids. This difference implies that the phenotype of the VP3-G001V mutant is the result of a true assembly defect rather than being a consequence of the proteolytic cleavage defect precluding formation of 5S protomer subunits. The assembly defect of the VP3-G001V mutant might also imply that cleavage of the VP0-VP3 site first is required to generate functional protomers with properly arranged capsid proteins suitable for assembly. Alternatively, the assembly incompetence of VP0-VP3-VP1 protomers derived from the VP3-G001V mutant might be the result of the valine substitution at the amino terminus of VP3. The adjacent VP3 N termini from the five protomers within a common pentameric subunit form a β -cylinder structure which is apparently critical for weaving protomers together to form a pentamer (16). The valine substitution at the amino terminus of VP3 might interfere with formation of the β -cylinder and render capsid protomers incapable of forming stable pentamer subunits, thereby preventing further assembly.

In contrast to capsid protomers generated from complete proteolytic processing of the VP3-G001V mutant, protomers derived from the VP1-G001V mutant did assemble capsid protein particles. However, the glycine-to-valine substitution at the amino terminus of VP1 apparently interfered with the RNA encapsidation process. A reduced level of VP2 derived from the VP1-G001V mutant was observed in comparison with that derived from the VP1- Δ 1-4 or wild-type precursors. The reduced level of VP2 derived from this mutant was not the result of inefficient maturation cleavage of VP0 derived from this mutant, since an increased presence of provirion particles derived from this mutant was not observed. The reduction in VP2 was thus an indicator of a defect in RNA encapsidation. Interestingly, deletion of the first four amino acids of the VP1 amino terminus restored the capability of mutant protomers to form mature virions at levels near those of wild-type protomers, although accumulation of these particles was delayed in comparison with that of wild-type particles. The VP1- Δ 1-4 mutant has previously been demonstrated to form mature virions with slower kinetics at a nonpermissive temperature of 39.5°C, whereas empty capsids formed normally (23). Importantly, in our studies, the amino-terminal deletion in VP1 delayed accumulation of completely processed proteins in comparison with wild-type precursor. The delay in virion formation observed with the VP1- Δ 1-4 precursor by using the *trans* complementation system might reflect additional time required to generate sufficient pools of protomers for virion formation. Although this argument can certainly be applied to the VP1-G001V mutant, the difference in completely processed protein levels between the VP1-G001V and VP1- Δ 1-4 mutants was much less pronounced than differences in virion formation, and completely processed proteins derived from the VP1-G001V mutant assembled comparable levels of empty capsids (Fig. 7). The results of this study, then, provide further evidence that the amino terminus of VP1 plays an important role in the RNA encapsidation process. The amino-terminal residues of VP1, which were disordered in the three-dimensional structure of poliovirus, have been modeled as an amphipathic helical structure (10). Previous reports have also implicated this region in the processes of RNA release (10, 23). Thus, the amino terminus of VP1 may play an active role in manipulating the poliovirus RNA genome during the processes of encapsidation and uncoating, and mutations in this structure may interfere with the mechanisms involved in these processes.

In summary, the results of this study demonstrate that

conservation of the glycine residues at the P1' positions of the 3CD cleavage sites in the poliovirus P1 precursor is required for assembly functions in addition to efficient proteolytic processing of the precursor. A valine substitution at the amino terminus of VP1 interfered with the formation of RNA-containing virions, whereas empty capsids accumulated at levels similar to those in cells coinfecting with VVP1 and PVdefSM. These results suggest that the amino terminus of VP1 directly interacts with the RNA genome or influences the structure of another determinant required for the encapsidation process. Clearly, determinants in addition to the amino-terminal residues of VP1 are important for RNA encapsidation. Further studies will be needed to define the role of the amino-terminal residues of VP1 and other poliovirus capsid determinants in the encapsidation process.

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