A Mutant Poliovirus Containing a Novel Proteolytic Cleavage Site in VP3 Is Altered in Viral Maturation

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A six-amino-acid insertion containing a Q-G amino acid pair was introduced into the carboxy terminus of the capsid protein VP3 (between residues 236 and 237). Transfection of monkey cells with full-length poliovirus cDNA containing the insertion described above yields a mutant virus (Se1-1C-02) in which cleavage occurs almost entirely at the inserted Q-G amino acid pair instead of at the wild-type VP3-VP1 cleavage site. Mutant Se1-1C-02 is delayed in the kinetics of virus production at 39°C and exhibits a defect in VP0 cleavage into VP2 and VP4 at 39°C. Sucrose gradient analysis of HeLa cell extracts prepared from cells infected by Se1-1C-02 at 39°C shows an accumulation of fast-sedimenting replication-packaging complexes and a significant amount of uncleaved VP0 present in fractions containing mature virions. Our data provide in vivo evidence for the importance of determinants other than the conserved amino acid pair (Q-G) for recognition and cleavage of the P1 precursor by proteinase 3CD and show that an alteration in the carboxy terminus of VP3 or the amino terminus of VP1 affects the process of viral maturation.

Poliomyelitis, once a fearsome disease averaging 21,000 cases per year in the United States before the advent of a vaccine, is caused by poliovirus, an enterovirus within the family *Picornaviridae* (24). Poliovirus is a small, plus-stranded RNA virus containing a genomic RNA that is 7.5 kilobases in length. The virus replicates in the oropharynx and lower gastrointestinal tract, extending in 1 to 4% of the cases to the nervous system. While it is not understood what factors are responsible for such limited, tissue-specific replication, the unique features of the virus life cycle (e.g., shutoff of host cell protein synthesis, replication of a protein-linked genomic RNA, and site-specific protein processing) must contribute to the unique biology of this virus. Within the host cell, the viral RNA is translated in a cap-independent manner (23, 32) into a single polyprotein. The polyprotein is processed into functional structural and nonstructural proteins by the viral proteinases 2A and 3C (12, 31). Initially, 2A cleaves between the P1 and P2 regions, a unimolecular, cotranslational event (21, 25, 26, 31). Nearly all other polyprotein cleavages are completed by proteinase 3C or one of its precursors (3CD) (16, 33).

Cleavage specificity depends in part on primary sequence. Poliovirus 3C cleaves exclusively between glutamine-glycine (Q-G) amino acid pairs (6, 19, 28), and 2A cleaves between tyrosine-glycine (Y-G) amino acid pairs (31). Amino acid residues occupying positions proximal to 3C-mediated cleavage sites may also be important (21, 34). The fourth residue (P4) proximal to Q-G cleavage sites is occupied by an aliphatic amino acid, usually alanine. Substitution of the naturally occurring P4 residue with other amino acids appears to interfere with cleavage in vitro (22, 34). It is unclear whether the P4 residue is recognized by proteinase 3C or whether this position is structurally crucial for presentation of the Q-G site.

Several observations suggest that 3C proteinase specificity depends on more than primary sequence. First, only 9 of the 13 Q-G amino acid pairs present in the polyprotein are cleaved. Second, the P1 region of the polyprotein requires a

nearly complete carboxy terminus to assume a conformation recognizable by 3C (35). Third, cellular proteins contain Q-G amino acid pairs but apparently are not cleaved by viral 3C proteinase (17). Finally, other picornaviral 3C proteinases recognize more than one amino acid pair. For example, coxsackievirus 3C proteinase recognizes Q-G and probably Q-N (20). On the basis of these observations and previously published experimental evidence (34), it has been proposed that the secondary and tertiary structures of the polyprotein in addition to primary sequence are important for 3C recognition and cleavage.

Interaction of 3C with polypeptide domains that are the result of a specifically folded P1 precursor appears to be more specific than are corresponding interactions with the P2 or P3 precursors. Rhinovirus 14 and coxsackievirus B3 3C proteinases recognize and cleave poliovirus nonstructural (P2 and P3) protein precursors but not structural (P1) protein precursors (5). Additionally, chimeric coxsackievirus and poliovirus 3C proteinases recognize various substrates of the nonstructural but not structural poliovirus precursor polypeptides (M. A. Lawson and B. L. Semler, unpublished observations). Further support for differences in proteinase-substrate specificities comes from the recent finding that poliovirus requires 3D sequences, probably in the form of the precursor 3CD (16, 33), to carry out efficient P1 processing but not P2 or P3 processing.

Of particular interest is our recent observation suggesting that 3D sequences may be required to specifically recognize important structural domains in the P1 precursor. In a previous study aimed at assessing the critical site-recognition domains required for efficient processing of the P1 precursor by proteinase 3CD (34), a series of four- to six-amino-acid insertions was constructed in the P1 region. Amino acid insertions disrupting the β -barrel structures predicted by X-ray crystallography (14) interfered with processing in vitro, while insertions in terminal extensions or loops between β -barrels did not interfere with processing. One of the six-amino-acid insertions, in the carboxy terminus of VP3, displayed a novel processing phenotype. The insertion contained a Q-G amino acid pair and was posi-

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FIG. 1. Simplified map of poliovirus translation, illustrating the nature of the altered VP3-VP1 cleavage site in Se1-1C-02. The amino acid sequence corresponds to the carboxy terminus of VP3 and the amino terminus of VP1 for wild-type (WT) PV1 and Se1-1C-02. The six amino acids inserted in Se1-1C-02 are underlined. The Q-G amino acid pairs are in italics, and the P4 amino acid residues are designated with asterisks. Solid arrows denote Q-G pairs cleaved in vivo; the open arrow denotes a wild-type Q-G amino acid pair cleaved rarely in cells infected with Se1-1C-02.

tioned between the P2 and P3 residues of the VP3-VP1 cleavage site, moving the alanine from the P4 position of the wild-type VP3-VP1 cleavage site to the P4 position of the inserted Q-G (Fig. 1). It appeared from the altered electrophoretic mobilities of both VP3 and VP1 that cleavage occurred almost exclusively at the inserted Q-G site and not at the wild-type VP3-VP1 cleavage site.

In this study, we describe the isolation of a mutant poliovirus containing the above six-amino-acid insertion near the carboxy terminus of VP3. We have examined the biosynthetic properties of this mutant (Se1-1C-02) in infected HeLa cell cultures to study the effects of altered viral capsid proteins on proteolytic processing and morphogenesis. Protein sequencing data revealed that Se1-1C-02 is cleaved almost exclusively at the inserted Q-G site just upstream from the wild-type Q-G pair at the VP3-VP1 junction of P1. Our data show that the mutant is temperature sensitive for virus growth and VP0 cleavage at 39°C. Sucrose gradient analysis of virus-specific structures isolated from infected cells shows an increased amount of fast-sedimenting replication complexes for the mutant when compared with those isolated from wild-type infected cells at 39°C. The gradient analysis also revealed the presence of an increased level of uncleaved VP0 in fractions cosedimenting with infectious 150S particles, suggesting a defect in one of the steps of virion morphogenesis.

MATERIALS AND METHODS

Construction of mutant plasmid pEV:236QG. The plasmid pVP3:236QG, a derivative of pT7-P1 described in reference 34, contains a 14-base-pair (bp) linker insertion at the *NheI* site at nucleotide 2470 in the poliovirus sequence. The insertion of the 14-bp linker, coupled with the additional 4-bp insertion resulting from the fill-in of the *NheI* site at nucleotide 2470, resulted in a total of 18 bp inserted into the polypeptide coding region. Plasmid pVP3:236QG was digested with restriction endonucleases *BsmI* and *SpeI*, and the 1,128-bp fragment corresponding to poliovirus sequences nucleotide 1513 through nucleotide 2641 (including the 14-bp linker insertion at position 2470) was gel purified. The full-length infectious poliovirus cDNA pEV104 (29) was digested with *BsmI* and *SpeI*. Three fragments (7,882, 1,341,

and 1,077 bp) were gel purified and ligated to the 1,128-bp fragment containing the linker insertion from pVP3:236QG. The 14-bp linker insertion contained the recognition sequence for restriction endonuclease *StuI* to facilitate the identification of recombinant plasmids containing the inserted sequence.

Transfections. Subconfluent COS-1 cell monolayers on 60-mm-diameter plates were transfected with 1 µg of pEV:236QG plasmid DNA and 19 µg of salmon sperm DNA as carrier by the calcium phosphate technique (9). At 4 h after transfection, the monolayers were treated with a 3-min glycerol boost (8) and overlaid with either Dulbecco modified Eagle medium containing 10% fetal calf serum or a semisolid medium consisting of Dulbecco modified Eagle medium, 6% fetal calf serum, and 0.45% agarose. The transfected monolayers were incubated at 37°C until cytopathic effects or plaques were detected.

Virus stock preparation and plaque assays. Mutant virus stocks were prepared by picking well-isolated plaques from the transfected COS-1 monolayers 4 to 6 days after transfection. The plaques were clonally purified by infecting HeLa cell monolayers, overlaying them with semisolid medium, and picking well-isolated plaques. The plaque-purified stock was expanded by two serial passages through HeLa cell monolayers and one passage through a HeLa Spinner culture. The titers of the stocks were determined on 60-mm-diameter plates of HeLa cell monolayers under semisolid medium at 37°C.

Sequencing of mutant viral RNA. Viral RNA was prepared by infection of 10-cm-diameter dishes of HeLa cell monolayers with wild-type and mutant virus at a multiplicity of infection of 25. After a 30-min adsorption period, the monolayers were overlaid with liquid medium (Dulbecco modified Eagle medium and 10% fetal calf serum) and incubated for 6 to 7 h at 37°C. The medium was then removed, and the monolayers were rinsed with phosphate-buffered saline. The cells were harvested in 2.5 ml of phosphate-buffered saline, pelleted, and suspended in 1.5 ml of TSM buffer (0.15 M NaCl, 5.0 mM MgCl₂, 10 mM Tris [pH 7.6]). The cells were repelleted, suspended in 0.2 ml of TSM plus 0.2% Nonidet P-40, and lysed with vigorous pipetting. The nuclei were pelleted, and the supernatant containing the cytoplasmic

extract was saved. A 0.2-ml portion of RNA extraction buffer (4% sodium dodecyl sulfate [SDS], 40 mM EDTA, 0.6 M NaCl, 0.1 M Tris [pH 8.0], 8 M urea) was added to the supernatant (4). The RNA was extracted with phenol-chloroform (1:1) and then ethanol precipitated. The viral RNA was sequenced by extension of a 20-nucleotide synthetic primer corresponding to nucleotides 2620 through 2640 of poliovirus RNA, using [32 P]dATP, dideoxynucleotides, and reverse transcriptase (11, 27).

One-step growth curves. HeLa cell monolayers on 60-mm-diameter plates were infected with poliovirus type 1 (PV1) or mutant Se1-1C-02 at a multiplicity of infection of 25. After a 30-min adsorption period, the monolayers were rinsed three times with phosphate-buffered saline and overlaid with liquid medium. The infected monolayers were incubated at 37 and 39°C. After 2, 3, 4, 5, and 6 h at 39°C and 3, 4, 5, 6, and 7 h at 37°C, cells and supernatants were harvested together and subjected to five cycles of freezing and thawing to release virus. Plaque assays were used to determine the PFUs per cell present at each time point.

Labeling infected cells with [35 S]methionine. HeLa cell monolayers on 60-mm-diameter plates were infected with PV1 or Se1-1C-02 at a multiplicity of infection of 20 to 30. Virus was allowed to adsorb for 30 min at room temperature, and then the cells were overlaid with 3 ml of methionine-free minimal essential medium plus 10% fetal calf serum. After 5 h of incubation at 37°C, 60 μ Ci of [35 S]methionine was added to each plate of infected cells, and the cells were incubated for 1 h at 37°C. The monolayers were then rinsed with phosphate-buffered saline, harvested from the plates, pelleted, and suspended in Laemmli sample buffer (18). For pulse-chase labeling, 5 ml of HeLa Spinner cultures (at a cell density of 5×10^6 cells per ml) were infected at a multiplicity of infection of 25 in methionine-free medium. After a 30-min adsorption at room temperature with occasional agitation, the infected cells were pelleted and suspended in 5 ml of methionine-free minimal essential medium with 10% fetal calf serum, 1% glutamine, and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.25). The infected cell suspensions were incubated at 37 and 39°C with constant stirring for 4 h. At this time, each culture of cells was given a pulse of 60 μ Ci of [35 S]methionine for 10 min and then a chase with 100 mM L-methionine. Portions of infected cells (10^6 cells) were then harvested immediately after the chase and every 15 min for 75 min. The harvested cells were pelleted, washed twice with phosphate-buffered saline, and suspended in Laemmli sample buffer. Lysates were boiled and analyzed on 12.5% polyacrylamide gels containing SDS (18). The autoradiograms of the gels were then scanned with an LKB Ultrascan 2 laser densitometer. To maximize the separation of 3C' and VP0 and to obtain more accurate quantitations of VP0/VP2 ratios, samples were loaded on 5 to 18% gradient polyacrylamide gels, and the resulting autoradiograms were scanned with a Beckman LKB Ultrascan 2 laser densitometer.

Purification and sequencing of VP1. HeLa Spinner cultures (25 ml) at a cell density of 5×10^6 cells per ml were infected with PV1 or Se1-1C-02 as described above. After 4 h of incubation at 37°C with constant stirring, 1 mCi of [35 S]methionine was added, and the infected cultures were labeled for 2.5 h. At 6.5 h after infection, the infected cells were pelleted, suspended in 3 ml of reticulocyte standard buffer plus Mg (10 mM Tris [pH 7.5], 10 mM NaCl, 1.5 mM MgCl_2), and subjected to five cycles of freezing and thawing. The cell debris and nuclei were pelleted by low-speed centrifugation. EDTA and SDS were then added to the

supernatants to final concentrations of 2 mM and 1%, respectively, and viral particles were pelleted by centrifugation in a Beckman 50 Ti rotor at 30,000 rpm for 3 h. The pellet was suspended in 0.2 ml of 0.1 buffer (0.1 M NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 0.5% SDS). The suspended pellets were then sedimented on 15 to 30% sucrose gradients (wt/wt in 0.1 buffer). Centrifugation was performed in an SW41 rotor at 28,000 rpm for 2.5 h. The gradients were fractionated into 0.4-ml fractions, and a portion of each fraction was counted in a liquid scintillation counter. Fractions representing the viral peaks were pooled and concentrated by centrifugation. The pellets were suspended in 0.2 ml of Laemmli sample buffer. Each sample was applied to a preparative SDS-polyacrylamide gel. After electrophoresis, the gels were dried and exposed to Kodak XAR-5 film. The exposed film was then used to identify the position of VP1 on the polyacrylamide gel (30). The band corresponding to VP1 was excised from the dried gel, minced, and rehydrated in electroelution sample buffer (0.01 M NH_4HCO_3 , 0.01% SDS). The protein was electroeluted in an electrophoretic sample concentrator (3), lyophilized, and suspended in 50 μ l of water. Each sample was then subjected to pulse-liquid protein sequencing, using an Applied Biosystems model 475-A sequencer (13). Fractions corresponding to 20 residues from the amino terminus of VP1 were collected and counted in a liquid scintillation counter (1).

Sucrose gradient analysis of virus-specific structures. HeLa Spinner cultures (15 ml) were infected with PV1 and Se1-1C-02 as described above. The cultures were incubated at 37 and 39°C for 4 h and were then labeled with 250 μ Ci of [35 S]methionine per 15 ml of culture. After 6.5 h, cells were pelleted, suspended in 1 ml of reticulocyte standard buffer plus Mg, and subjected to five cycles of freezing and thawing. The nuclei and cell debris were removed by centrifugation, and Triton X-100 was then added to the supernatant at a final concentration of 0.01%. One-half of each of the detergent-treated supernatants was then sedimented on 10 to 30% (wt/wt) sucrose gradients prepared in NET buffer (0.1 M NaCl, 0.04 M Tris hydrochloride [pH 7.5], 0.002 M EDTA) and 0.01% Triton X-100. Centrifugation was for 2.5 h in an SW41 rotor at 28,000 rpm. The sucrose gradients were fractionated into 0.4-ml fractions, and the fractions were analyzed by electrophoresis on SDS-polyacrylamide gels. The proteins present in the 150S viral peaks were then scanned with an LKB Ultrascan 2 laser densitometer.

RESULTS

Generation of mutant virus Se1-1C-02. A restriction fragment containing the 18-bp insertion in the carboxy terminus of VP3 was removed from the subgenomic transcription vector pVP3:236QG (34) and cloned into the full-length poliovirus infectious cDNA (pEV104) as described in Materials and Methods. Plasmid DNA from the resulting construct (pEV:236QG) was transfected into COS-1 cells at 37°C. After 5 days, infectious virus (Se1-1C-02) with a small plaque phenotype was recovered. The viral RNA was sequenced to ensure that the six-amino-acid insertion, containing an internal Q-G amino acid pair, was maintained (Fig. 1).

[35 S]methionine pulse-labeling of HeLa cells infected with PV1 or Se1-1C-02. Pulse-labeling experiments were used to examine the production of virus-specific proteins in infected HeLa cell monolayers. The profile of viral polypeptides synthesized in cells infected with Se1-1C-02 was normal at 37°C compared with that of the wild-type virus (Fig. 2), with the exception of the altered electrophoretic mobilities of the

FIG. 2. Pulse-labeling of HeLa cell monolayers infected with VP1 or Se1-1C-02. HeLa cell monolayers were infected at a multiplicity of infection of 25 and labeled with 60 μ Ci of [35 S]methionine at 4 h postinfection, and cells were harvested at 5 h postinfection. The extracts from infected cells were then boiled in Laemmli sample buffer and analyzed, using SDS-polyacrylamide gel electrophoresis. The figure shows an autoradiogram of a 12.5% polyacrylamide gel. Viral proteins are indicated on the left. Lanes: PV-1, cells infected with wild-type, transfection-derived poliovirus; 1C-02, cells infected with the mutant Se1-1C-02. The altered electrophoretic mobilities of VP1 (arrow) and VP3 (asterisk) are indicated.

structural proteins VP3 and VP1. These altered electrophoretic mobilities have been previously observed after *in vitro* translation and processing of P1 precursor polypeptides containing the lesion in Se1-1C-02 (34). The presence of capsid proteins (VP3 and VP1) with the altered electrophoretic mobilities seen in Fig. 2 suggests that cleavage of the inserted Q-G pair occurred *in vivo* as well. Cleavage at the inserted site adds six residues to the amino terminus of VP1 (Fig. 1), resulting in the observed slower mobility of the altered VP1 (Fig. 2, arrow). In addition, the alanine present at two residues from the carboxy terminus of wild-type VP3 would be replaced by an aspartic acid residue if Se1-1C-02 utilized the inserted Q-G site (Fig. 1). The substitution of the charged aspartic acid residue for the uncharged alanine

FIG. 3. Amino-terminal sequence analysis of [35 S]methionine-labeled VP1 isolated from purified virions. As described in Materials and Methods, HeLa cells infected with PV1 or Se1-1C-02 were labeled with [35 S]methionine. VP1 was then purified from virion particles by using SDS-polyacrylamide gel electrophoresis and subjected to 20 cycles of pulsed liquid-phase protein sequence analysis. The radioactivity released in each cycle was determined, and the peaks of radioactivity represent methionine residues present in the amino acid sequence of VP1. VP1 purified from wild-type (A) and Se1-1C-02 mutant (B) virions is shown. The total radioactivity released in each cycle is plotted as a function of residue number, with residue 1 being the amino-terminal residue. The radioactivity present in the first residue results from flowthrough of unbound [35 S]methionine-labeled VP1 and does not represent a methionine in that position.

probably alters the interaction of VP3 with SDS, resulting in the observed mobility shift of VP3 on an SDS-polyacrylamide gel (Fig. 2, asterisk).

Pulsed liquid-phase protein sequencing of VP1 recovered from purified PV1 or Se1-1C-02. To conclusively determine whether processing had occurred at the inserted Q-G site instead of at the wild-type VP3-VP1 cleavage site, VP1 was purified from mutant and wild-type infected cells that had been labeled with [35 S]methionine. The isolated polypeptides were then subjected to radiochemical microsequencing. Sequence analysis was carried out by liquid scintillation counting of fractions collected from 20 cycles on a pulsed liquid-phase automated protein sequencer. The peaks of radioactivity correspond to the methionine residues present near the amino terminus of VP1 (Fig. 3). The first fraction shown in each panel contains the flowthrough of unbound, labeled VP1 and does not represent a methionine in that position. For VP1 derived from wild-type virus, methionine is predicted at five and nine residues from the amino terminus, coinciding with the peaks of radioactivity in Fig. 3A. Cleavage at the inserted Q-G site in Se1-1C-02 would add six residues to the amino terminus of VP1 (Fig. 1). The methi-

FIG. 4. Kinetics of virus growth at 37 and 39°C. HeLa cell monolayers were infected at a multiplicity of infection of 25, and cells and supernatant were harvested at the indicated times postinfection. Plaque assays were then used to determine the number of infectious particles per cell as described in Materials and Methods. The time of harvest was plotted as a function of the logarithm of PFU per cell. HeLa cell monolayers infected with PV1 or Se1-1C-02 at 37°C (A) and 39°C (B) are shown. ○, PV1; ●, Se1-1C-02.

online residues are shifted to positions 11 and 15, thus confirming cleavage at the inserted site (Fig. 3B).

Analysis of viral growth at 37 and 39°C. We characterized the properties of Se1-1C-02 by first examining viral growth at 37 and 39°C. HeLa cells were infected with PV1 and Se1-1C-02, and cells and supernatant were harvested at 2, 3, 4, 5, and 6 h postinfection at 39°C and 3, 4, 5, 6, and 7 h postinfection at 37°C. The results of these one-step growth experiments are shown in Fig. 4. The kinetics of virus production in cells infected with Se1-1C-02 was only slightly reduced from that of wild-type virus at 37°C (Fig. 4A). However, the mutant showed a delay in growth and an approximate 20-fold decrease in maximum viral yield at 39°C (Fig. 4B).

[³⁵S]methionine pulse-chase labeling of HeLa cells infected with PV1 or Se1-1C-02. The highly specific interaction between the P1 precursor and viral proteinase 3CD (34) suggested that the temperature-sensitive defect in the growth of the mutant Se1-1C-02 results from a delay in 3CD-mediated P1 processing. To investigate such a possibility, we carried out pulse-chase labeling experiments of virus-specific polypeptides. The experiments were carried out as described in Materials and Methods. Extracts from infected cells labeled with [³⁵S]methionine were analyzed by SDS-polyacrylamide gel electrophoresis. When HeLa cells infected by wild-type PV1 or Se1-1C-02 were incubated at 37°C, there were no differences in the processing kinetics of the VP0-VP3 and inserted VP3-VP1 (3CD-mediated) cleavage sites (Fig. 5A). However, there were slightly delayed kinetics of VP0 cleavage into VP2 and VP4. Laser densitometry (Fig. 5C) shows that VP0 cleavage is two to three times slower in HeLa cells infected with the mutant Se1-1C-02 than in HeLa cells infected with wild-type PV1 at 37°C. At 39°C, VP0 cleavage into VP2 and VP4 in HeLa cells infected with Se1-1C-02 is significantly delayed (Fig. 5B). Laser densitometry (Fig. 5D) shows that VP0 cleavage in HeLa cells infected with the mutant Se1-1C-02 at 39°C is three- to fivefold slower than in HeLa cells infected with PV1 at 39°C. To quantitate the delay in VP0 cleavage, Se1-1C-02 VP0/VP2 ratios were compared with that of the wild-type for several different pulse-chase experiments at 37 and 39°C (Fig. 5A and B). Thus, the kinetics of 3CD-mediated processing of the P1 region are not affected. Instead, it appears that the defect in growth of mutant Se1-1C-02 is due to reduced cleavage of VP0 to VP4 and VP2, possibly as a result of altered viral morphogenesis.

Sucrose gradient analysis of extracts from HeLa cells infected with PV1 or Se1-1C-02 at 37 and 39°C. To determine whether a defect in Se1-1C-02 viral maturation resulted in the accumulation of an intermediate in the virion assembly process, infected cell extracts were analyzed by sedimentation on sucrose gradients. HeLa Spinner cultures were infected with PV1 and Se1-1C-02 at 37 and 39°C, labeled at 4 h postinfection with [³⁵S]methionine, and harvested as described in Materials and Methods. Each fraction from the gradients was analyzed by electrophoresis on SDS-polyacrylamide gels. Extracts prepared from cells infected at 37°C (Fig. 6A and B) had a sedimentation profile that contained, from the bottom to the top of the gradient, a viral peak (150S) (lanes 3 through 6), a procapsid peak (74S) (lanes 14 through 17), and a peak corresponding to small complexes of viral proteins or free viral proteins (lanes 22 through 24). No significant differences in the profiles obtained from mutant and wild-type infected cells were seen. When extracts prepared from cells infected at 39°C were analyzed, a fast-sedimenting peak containing capsid and other viral proteins (lanes 1 and 2), possibly representing membrane-bound replication-packaging complexes (see below), was observed in addition to the three peaks mentioned above for both PV1 and Se1-1C-02 (Fig. 6C and D). However, the fast-sedimenting complexes were present in larger quantities in cells infected with Se1-1C-02 than in wild-type infected cells. The viral peak obtained from extracts of cells infected with Se1-1C-02 contained a significant fraction of uncleaved VP0 and other viral proteins such as 3CD. Laser densitometer scanning of the viral peaks from extracts infected with PV1 and Se1-1C-02 showed that the ratio of uncleaved VP0 to VP2 is three to four times greater for the mutant at 39°C than for the wild type at 39°C (Fig. 6G and H). These viral peaks from the wild-type and mutant Se1-1C-02 samples both contained highly infectious particles, unlike the fractions at the top of the gradient (data not shown).

The altered electrophoretic mobility of VP1 from the mutant Se1-1C-02 can be clearly seen in the viral peak (150S) in Fig. 6B and D. Also present in the Se1-1C-02 viral peak is a protein migrating slightly faster than the altered VP1, comigrating with wild-type VP1 in the marker lane (Fig. 6B and D). The viral protein migrating at the same position as wild-type VP1 in the Se1-1C-02 viral peak is present at approximately 1/30 the level of the altered VP1, as shown by laser densitometry (Fig. 6F and H). This protein may represent the product of a small fraction of cleavage at the

FIG. 5. Pulse-chase analysis of protein processing by PV1 and Se1-1C-O2 at 37 and 39°C. HeLa cell suspension cultures were infected at a multiplicity of infection of 25 with PV1 or Se1-1C-O2, pulsed for 10 min at 4 h after infection with 60 μ Ci of [35 S]methionine, and chased with L-methionine. Numbers above the lanes in panels A and B represent minutes after the chase that cells were harvested. The extracts from harvested cells were boiled in Laemmli sample buffer, and viral proteins were analyzed on SDS-polyacrylamide gels. Polypeptide profiles from HeLa suspension cultures infected at 37°C (A) and 39°C (B) are shown. M, Marker lane of [35 S]methionine-labeled poliovirus proteins from HeLa cell monolayers infected with wild-type poliovirus. Shown below each pulse-chase analysis is the corresponding densitometry scan. Scans of lanes from cells harvested 75 min after the L-methionine chase for PV1 and Se1-1C-O2 at 37°C (C) and at 39°C (D) are shown. WT, Wild type.

FIG. 6. Sucrose gradient analysis of virus-specific structures. HeLa suspension cultures were infected at a multiplicity of infection of 25 with PV1 or Se1-1C-02 at 37 or 39°C and labeled with 250 μ Ci of [35 S]methionine at 4 h after infection, and cells were harvested at 6.5 h after infection. Infected cells were suspended in NET buffer (0.1 M NaCl, 0.04 M Tris hydrochloride [pH 7.5], 0.002 M EDTA) and 0.01% Triton X-100 and prepared for sucrose gradient analysis as described in Materials and Methods. HeLa cells infected with (A) PV1 at 37°C, (B) Se1-1C-02 at 37°C, (C) PV1 at 39°C, and (D) Se1-1C-02 at 39°C are shown. M, Marker lane of [35 S]methionine-labeled poliovirus proteins from HeLa cell monolayers infected with transfection-derived PV1. Gradient fraction numbers are indicated at the tops of panels A through D. The figure shows autoradiograms of 12.5% polyacrylamide gels. Viral proteins are identified on the left. The fourth gradient fraction for PV1 and Se1-1C-02 at 37 and 39°C was scanned with the LKB Ultrascan 2 laser densitometer. On the facing page are shown the densitometry tracings for (E) PV1 at 37°C, (F) Se1-1C-02 at 37°C, (G) PV1 at 39°C, and (H) Se1-1C-02 at 39°C. WT, Wild type.

wild-type VP3-VP1 cleavage site in Se1-1C-02. It should be noted that the above observation is consistent with the protein sequencing data. The 35 S-radioactivity present in residues 5 and 9, which correspond to methionine residues in

wild-type VP1, is slightly higher than the immediately surrounding background radioactivity (Fig. 3B). The background radioactivity just before the first peak of radioactivity (residue 11) is 2 to 4% of the first peak, whereas the

FIG. 6—Continued.

radioactive label present in residues 5 and 9 is 5 to 7% of the first peak. The increase in radioactivity present in residues 5 and 9 may result from a small percentage (3%) of wild-type VP1 present in the sequencing reaction.

DISCUSSION

Poliovirus proteinases 3C and 3CD cleave specifically at Q-G amino acid pairs, presumably recognizing primary sequence as well as secondary and tertiary structures. There are no highly conserved residues surrounding the poliovirus Q-G cleavage sites, except at the P4 position, which is occupied by a small subset of amino acids (alanine, valine, proline, and threonine). Evidence from *in vitro* experiments suggests that the P4 residue is important for recognition and cleavage (22, 34). The results from the present study provide *in vivo* evidence to support the *in vitro* studies. Mutant Se1-1C-02 contains a six-amino-acid insertion (including a Q-G amino acid pair) between the P2 and P3 residues of the VP3-VP1 cleavage site. The insertion replaces the alanine in the P4 position of the wild-type VP3-VP1 cleavage site with a glycine while maintaining an alanine in the P4 position of the inserted Q-G. Our amino acid sequencing data demonstrated that the P1 precursor encoded by Se1-1C-02 is cleaved almost entirely at the inserted Q-G pair. On the basis of the proximity of the inserted Q-G to the wild-type VP3-VP1 cleavage site and the predicted tertiary structure of the region, the three-dimensional location of the inserted and wild-type Q-G amino acid pairs should not be significantly different (14, 34). Therefore, the preference for cleavage of the inserted Q-G must depend to a large extent on the presence of the alanine in its P4 position.

Although the importance of the P4 residue has been demonstrated, its role is not understood. It is possible that

the P4 position is structurally crucial for presentation (or orientation) of the Q-G in the active site of 3CD. The amino acids present in the P4 position (alanine, valine, proline, and threonine) bear no significant homology aside from the aliphatic nature of three of the four residues (alanine, valine, and proline). The variability of the residues present in the P2 and P3 positions suggests that the P4 residue does not serve an entirely structural function. If the P4 residue provides an important structural component of the cleavage site, then the P2 and P3 residues should also be conserved for structural reasons, and they are not. In addition, substitution of the threonine present in the P4 position of the 3C-3D cleavage site with an alanine increases the rate of cleavage *in vitro* of a synthetic peptide bearing that cleavage site and surrounding sequences (22). This evidence supports specific recognition of the P4 residue by 3C, perhaps relying on electrostatic interactions of the enzyme with the aliphatic side chains surrounding the cleavage site of the substrate.

Wild-type processing kinetics of the Q-G sites present in the polyprotein encoded by Se1-1C-02 in infected cells provides additional evidence that the integrity of the β -barrel structures in the P1 region is important for recognition and cleavage by 3CD. *In vitro* experiments have previously shown that interruption of the β -barrel structures in the P1 region with linker insertions interfered with cleavage of that region, while insertions in loops or terminal extensions did not (34). The *in vitro* study analyzed processing in a qualitative manner but did not examine processing kinetics of altered P1 precursors. *In vivo* processing kinetics, which are useful for detecting subtle changes in enzyme activity, have been examined in the context of the viral replication cycle. In cells infected with Se1-1C-02, wild-type processing kinetics for the VP0-VP1 and VP3-VP1 (3CD-mediated) cleavage

sites were observed. This study provides *in vivo* confirmation that maintaining the integrity of secondary structure motifs in the P1 region will allow faithful processing of the capsid precursor.

Although processing of the 3CD-mediated cleavage sites was unaffected in cells infected with mutant virus Se1-1C-02, cleavage of VP0 into VP4 and VP2 was significantly delayed at 39°C. VP0 cleavage occurs after or concurrently with viral RNA packaging, possibly by an RNA catalysis mechanism (2), and is the final step in the formation of the mature virion. Therefore, the observed decrease in Se1-1C-02 growth at 39°C probably results from a defect in some step of viral maturation rather than processing. The alterations in VP3 and VP1 may cause slight changes in the conformation of the protomer and ultimately the 14S pentamers, interfering with either RNA packaging or VP0 cleavage. Assuming that the viral RNA is somehow involved in VP0 cleavage, it is likely that correct orientation of the capsid proteins in the protomer is crucial for VP0 cleavage. In support of this suggestion, we have recently isolated another poliovirus mutant that contains a six-amino-acid insertion near the amino terminus of VP1. This virus is also impaired for VP0 cleavage at 39°C (W. S. Blair and B. L. Semler, unpublished observations).

To investigate the specific defect in Se1-1C-02 maturation, extracts from infected cells were analyzed by sedimentation on sucrose gradients. The sedimentation profile for extracts prepared at 37°C showed a viral peak, a procapsid peak, and smaller viral protein complexes at the top of the gradient. Se1-1C-02 exhibited the same profile as did the wild type at 37°C. An additional peak near the bottom of the gradient (Fig. 6C and D, lanes 1 and 2) was observed in the sedimentation profile of infected extracts prepared at 39°C. The peak consisted of viral capsid proteins, including a large amount of uncleaved VP0, and other viral proteins such as 3CD. Although present in both Se1-1C-02 and PV1, the peak is much more prominent in Se1-1C-02. It is possible that the peak represents a membrane-bound RNA replication complex or a membrane-bound viral packaging intermediate. In support of this possibility, slot blot analysis showed that poliovirus-specific RNA was present in sucrose gradient fractions containing the fast-sedimenting replication-packaging complexes (W. S. Blair and B. L. Semler, unpublished observations). The two processes (RNA replication and packaging) appear to be coupled (15) and probably occur in the same cellular location. A delay in Se1-1C-02 packaging may result in an accumulation of such an intermediate.

The sedimentation profile of extracts from cells infected with Se1-1C-02 at 39°C also showed a larger amount of uncleaved VP0 in the 150S peak corresponding to mature virions. It is possible that infectious Se1-1C-02 viral particles may have a reduced amount of VP0 cleavage after RNA packaging, resulting in the accumulation of a provirionlike structure (7, 10). Other observations consistent with previously described characteristics of the provirion (SDS and 3 M CsCl lability) (7, 10, 25) suggest that the origin of uncleaved VP0 present in the Se1-1C-02 viral peak is a result of cosedimentation of provirions with mature virus particles. When extracts from HeLa cells infected with Se1-1C-02 or wild-type virus at 39°C were sedimented on sucrose gradients containing 0.5% SDS, the fast-sedimenting replication complexes were not present, and only a small amount of VP0 was present in the 150S viral peak. Additionally, disruption of the fast-sedimenting replication complexes coupled with the disappearance of uncleaved VP0 in the viral peak also occurred when Se1-1C-02 or wild-type viral extracts pre-

pared at 39°C were centrifuged on CsCl gradients in the absence of SDS. The 74S procapsids were present in both cases mentioned above (W. S. Blair and B. L. Semler, unpublished observations).

Alternatively, the increased amount of VP0 present in the Se1-1C-02 infectious particle peak may result from the presence of membrane-bound replication-packaging complexes containing immature viral particles that cosediment with the viral (150S) peak. The replication-packaging complexes, stalled at different stages of replication or packaging, may have a wide range of sedimentation coefficients, depending on the number and particular nature of association of the poliovirus proteins in the complex. Consistent with this explanation is the presence of nonstructural poliovirus proteins, such as 3CD, in the 150S viral peak. In addition, although VP0 appears to be specifically associated with procapsid structures (Fig. 6D, lanes 12 through 15), the rather random distribution of this protein (as well as other nonstructural proteins) throughout the remaining fractions of the sucrose gradient suggests that there is no specific association with infectious particles sedimenting at 150S (Fig. 6D, lanes 4 through 6).

It is unlikely that the mutant phenotypes observed for Se1-1C-02 (mentioned above) are the result of second site mutations introduced during the manipulation of the viral cDNA or preparation of viral stocks for the following reasons. (i) The 3CD-mediated processing phenotype of Se1-1C-02 *in vivo* is identical to the *in vitro* expression from its subgenomic predecessor pVP3:236QG (34). (ii) The mutant virus was entirely reconstructed in a different plasmid background, and the identical mutant phenotypes were observed. (iii) The mutant phenotypes were also observed in two independently derived plaque isolates of the originally constructed Se1-1C-02.

The altered properties of Se1-1C-02 have been useful for examining selected aspects of the poliovirus replication cycle. Our data show that a six-amino-acid insertion can be tolerated in the capsid protein region and that cleavage of an inserted Q-G amino acid pair can occur. The selective cleavage of the inserted Q-G supports recent models regarding the determinants necessary for the processing of the polyprotein by proteinase 3C (and 3CD). The temperature-sensitive defect in virion maturation that results in reduced cleavage of VP0 at 39°C may be a consequence of improper folding or assembly of morphogenetic precursors to mature virions. Such a temperature-sensitive defect appears to cause the accumulation of fast-sedimenting replication complexes, the further study of which could provide an important link in the regulatory coupling of RNA replication and virion assembly.

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