

Cleavage of Human Cytomegalovirus Protease pUL80a at Internal and Cryptic Sites Is Not Essential but Enhances Infectivity

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The cytomegalovirus (CMV) maturational protease, assemblin, contains an “internal” (I) cleavage site absent from its homologs in other herpesviruses. Blocking this site for cleavage did not prevent replication of the resulting I⁻ mutant virus. However, cells infected with the I⁻ virus showed increased amounts of a fragment produced by cleavage at the nearby “cryptic” (C) site, suggesting that its replication may bypass the I-site block by using the C site as an alternate cleavage pathway. To test this and further examine the biological importance of these cleavages, we constructed two additional virus mutants—one blocked for C-site cleavage and another blocked for both I- and C-site cleavage. Infectivity comparisons with the parental wild-type virus showed that the I⁻ mutant was the least affected for virus production, whereas infectivity of the C⁻ mutant was reduced by ≈40% and when both sites were blocked virus infectivity was reduced by nearly 90%, providing the first evidence that these cleavages have biological significance. We also present and discuss evidence suggesting that I-site cleavage destabilizes assemblin and its fragments, whereas C-site cleavage does not.

Herpesviruses encode a maturational protease that is essential for the production of infectious progeny. It functions during capsid maturation, and its activity is required to eliminate internal scaffolding proteins from the procapsid in preparation for incorporating the viral DNA. The enzyme is synthesized as an active precursor that cleaves itself at least twice (22, 32, 49). The first cleavage is near its carboxyl end, at a sequence called the maturational (M) site, and serves to break interactions of the scaffolding proteins with the inner surface of the capsid shell (3, 14, 25, 30, 43, 49, 50). The second cleavage is toward the amino end of the precursor, at a sequence called the release (R) site, and separates the 28-kDa proteolytic portion of the precursor from its carboxyl two-thirds (32, 49). Both M- and R-site cleavage are required to produce infectious virus (15, 34; also see reviews in references 6, 17, 19, 39, and 42 and references therein).

In cytomegalovirus (CMV) the proteolytic portion of the precursor is called assemblin (49). Structural and enzymatic characterizations have established that it and its homologs in other herpesviruses constitute a new and unusual type of serine protease, having a Ser-His-His catalytic triad instead of the typical Ser-His-Asp/Glu (7, 12, 26, 36–38, 40, 45); requiring dimerization to become active (13, 33), with two separate catalytic sites; and showing a low substrate turnover rate relative to “classical” serine proteases (8, 44).

Human CMV (HCMV) assemblin contains two additional cleavage sites (Fig. 1) that are absent from most of its homologs and whose functions are unknown. One is called the internal (I) site and is indicated by X-ray crystallography to be

in an unstructured loop near the active site. Its cleavage breaks the molecule into fragments A_n (15.5 kDa) and A_c (12.5 kDa), which remain associated as an active enzyme (1, 8, 24). The second, called the cryptic (C) site, is also in an unstructured loop but situated closer to the major α-helix of the enzyme dimer interface. Its cleavage produces fragment C_n (22.7 kDa) in both plasmid-transfected and virus-infected cells (10, 29). Although normally present in low abundance, C_n accumulates in both transfected (29) and mutant virus-infected (10) cells when I-site cleavage is blocked.

In an effort to determine the biological importance and function of the I- and C-site cleavages, we began by making a mutant virus blocked for I-site cleavage (I⁻; site changed from VEA ↓ AT to VEVAT). Although the I⁻ virus replicated well, it produced an increased amount of fragment C_n, raising the possibility that blocked cleavage at the I site might be circumvented by using C-site cleavage as an alternate processing pathway (10, 29). To test this and further investigate the role of these sites, we have made a second mutant virus blocked for C-site cleavage and a third blocked for both I- and C-site cleavage.

All three mutants have been compared with the parental wild-type (Wt) virus for growth in cell culture, production of extracellular virus particles, assemblin cleavage at the I and C sites, and infectivity. Our data show that, although neither cleavage is absolutely essential for virus replication, blocking either one reduces virus titer and blocking both has an approximately additive effect, reducing virus titer by ≈90%. Our findings indicate that the I and C sites are important for HCMV replication and that they are more likely to be functionally different than redundant.

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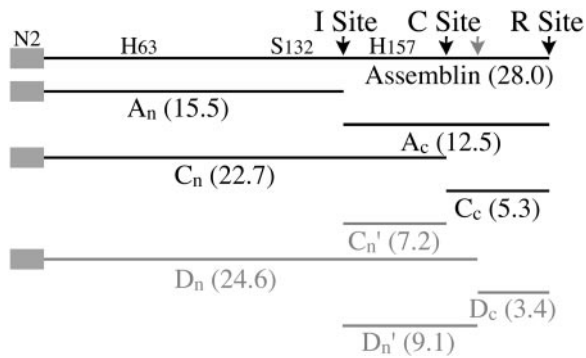


FIG. 1. Schematic of assemblin cleavage. The topmost line represents assemblin; residues comprising the catalytic triad (H63, S132, and H157) are shown above it, together with arrows indicating the internal (I), cryptic (C), and release (R) cleavage sites. Fragments resulting from I-site cleavage (A_n and A_c) and from C-site cleavage (C_n and C_c) are shown below assemblin (sizes are in kilodaltons, in parentheses). The putative dimer (D) cleavage site is indicated by a gray arrow above the top line. Lines and lettering in light gray indicate unidentified fragment C_n' and other fragments that could result from cleavage at the putative D site. N2 is the 15-amino-acid sequence used to produce antipeptide antiserum anti-N2 (23).

MATERIALS AND METHODS

Cells, virus, and microscopy. Human foreskin fibroblast (HFF) cells (18) and human retinal pigment endothelial (HRPE) cells transformed with the reverse transcriptase subunit of human telomerase (hTERT-RPE; catalog no. C4000-1; BD Biosciences Clontech, Palo Alto, CA) were grown in Dulbecco's high-glucose modified Eagle's medium containing penicillin (100 U/ml), streptomycin sulfate (10 μ g/ml), and fetal calf serum (10%) (culture medium).

The parental virus was reconstituted from an enhanced green fluorescent protein (EGFP)-tagged HCMV (strain AD169)-bacmid (AD169 Δ US2-11-EGFP-loxP) (16) obtained from G. Hahn, M. Messerle, and U. Koszinowski.

Fluorescence microscopy was done with a Nikon Eclipse TE200 inverted microscope equipped with a Sony DKC5000 imaging system. Contrast of the photomicrographs was adjusted in Adobe Photoshop.

Construction of mutant HCMV-bacmids (BACs). The general procedures used have been described before (4, 10). Mutations of the C site (A209V) and of both the C and I sites (A143V/A209V) were first made and tested by expression in bacteria and mammalian cells to verify that they block cleavage (confirmatory data not shown from transfection-Western immunoassay experiments).

The C-site mutation (A209V) was introduced into a DNA fragment by PCR amplification from plasmid MH22 using primers eb4a (GCGTCTAGAATT CATATGACGATGGACGAGCAGC) and sm4 (CTGTTGCCCAACAGGC CGTAGCTGTCTGAGCGAAAGGGATCGCCCGAC \underline{C} CGTTCGACAGCA GTGC), and the resulting mutant PCR product was used to replace the wild-type sequence in MH22, via *Apa*I and *Bgl*II sites, yielding SM5 for expression in *Escherichia coli* (*Bgl*II site underlined and point mutation underlined and italicized in sm4). The A209V mutation was then subcloned in a *Pml*I/*Spe*I fragment into the UL80a gene in LM13R (48), and then in a *Pml*I/*Bsu*36I fragment into the UL80a-containing, genomic *Not*I fragment in the BAC transfer vector pSTKS11. The I-site mutation was combined with the C-site mutation in the resulting plasmid using mutagenic primers as in the work of Chan et al. (10). The nucleotide sequence of the resulting PCR-derived *Not*I fragment was confirmed.

The mutant UL80a genes were electroporated into CBTS bacteria (10) for recombination with the resident HCMV-bacmid (AD169 Δ US2-11-EGFP-loxP) (16). Recombinant bacmids were identified, and the mutations were verified by DNA sequence analysis. The I-, C-, and double I/C-site mutant HCMV-bacmids were designated A143V (I^-) (10), A209V (C^-), and A143V/A209V (I^-/C^-), respectively. These DNAs were transfected into HRPE cells, which were maintained for approximately 10 days and then cocultivated with HFF cells for another 2 or 3 weeks, at which time the viral cytopathic effects of wild-type HCMV-BAC-derived virus are generally extensive (10). Cells and medium were collected together from cultures showing strong cytopathic effects and stored at -80°C as primary virus stocks.

Infectivity assays. Endpoint dilution assays were done essentially as described by Chan et al. (10), by adding HFF cells (suspended to give $\approx 25\%$ confluence) to quadruplet, 10-fold, serial dilutions of clarified infected-cell culture medium in 96-well plates (100 μ l virus plus 100 μ l cell suspension). Cells were monitored by microscopy for viral cytopathic effect. The culture medium was aspirated and replaced every 2 weeks.

Plaque assays were done by adding 10-fold serial dilutions of clarified (14,000 \times g, 2 min, room temperature) infected-cell culture medium to 6-cm petri dish cultures of subconfluent HFF cells (100 μ l inoculum/5 ml culture volume). The next day the medium was aspirated and the cell layers were overlaid with 5 ml of plaque assay medium, prepared by combining equal volumes of agarose (1%, melted in water and cooled to 45°C) and $2\times$ culture medium at 45°C . The overlay, $\approx 40^\circ\text{C}$ when added to the cultures, solidified at room temperature in 30 to 60 min, and the dishes were returned to the 37°C incubator and observed periodically for up to 6 weeks. A second overlay (5 ml) of the same composition was added to the cultures 3 weeks after infection to nourish the cell layer. Virus titers were calculated as the average number of GFP-expressing cell clusters ("plaques") per dish at the two highest dilutions, corrected for the dilution factor and original volume.

Recovery of extracellular virus particles. Extracellular particles were recovered from the culture medium of infected cells by rate-velocity sedimentation in 15 to 50% gradients of sucrose prepared in 40 mM phosphate buffer containing 150 mM NaCl, pH 7.4, as described before (10, 28). Particles were concentrated by diluting the gradient sample 1:1 with the same buffer and pelleting at 35,000 rpm, 4°C , for 2 h in an SW55Ti rotor (Beckman, Palo Alto, CA). After the liquid was removed from each tube, the pelleted particles were collected into protein sample buffer (3 parts NuPAGE buffer [NP0007; Invitrogen, Carlsbad, CA] and 2 parts 1 M dithiothreitol) and frozen at -80°C until analyzed.

Protein analysis by PAGE and Western immunoassay. Proteins were separated electrophoretically in 4 to 12% polyacrylamide gels (NP0323; Invitrogen), using 2-(*N*-morpholino)ethanesulfonic acid (MES) electrode buffer (NP0002; Invitrogen) containing sodium dodecyl sulfate (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]). Proteins were stained with SYPRO Ruby protein gel stain (SYPRO-R, S12000; Molecular Probes, Eugene, OR) and imaged with a Kodak Gel Logic 200 detection system, using 1D Image Analysis, version 3.6, quantification software.

Western immunoassays were done essentially as described by Towbin et al. (46) but using an electrotransfer unit (EI9051 XCellIII Blot Module) and transfer buffer (NP0006-1) from Invitrogen. After electrotransfer, the resulting membrane (polyvinylidene difluoride, LC2002; Invitrogen) was blocked (5% bovine serum albumin, 0.01 M Tris, 0.9% NaCl, pH 7.4) and probed with rabbit anti-peptide antisera, followed by ^{125}I -protein A (NEX-46L; Perkin-Elmer, Boston, MA) as the secondary reagent (9). Images of the probed membrane were acquired by using a Fuji BAS1000 phosphorimager or by fluorography using Biomax MS film and an intensifying screen (Kodak, Rochester, NY). Quantification of Western immunoassays was done by phosphorimaging and with the use of Fuji ImageGauge version 3.45 software. Antipeptide antisera, anti-N2 and anti-mCP, were prepared by immunizing rabbits with synthetic peptides representing the amino-terminal 15 residues of assemblin and the carboxy-terminal 15 residues of the minor capsid protein (mCP, pUL85) as described before (20, 23).

PCR amplification of virion DNA. The UL80a sequence was PCR amplified by standard methods, using 1 μ l of sucrose gradient-purified virions (see above) per reaction and primers eb11 (forward; AGTTCGCGGTACAGATGAGG) and eb12 (reverse; TTGCGAGCCTTGACGACTTG).

RESULTS

All three mutant viruses replicate in HFF cells. Mutations blocking cleavage of assemblin at the I site (I^-), the C site (C^-), and at both sites (I^-/C^-) were introduced into HCMV-bacmids as described in Materials and Methods. The mutant bacmids and that of the wild-type parent were separately transfected into HRPE cells, which were then cocultured with HFF cells. Extensive cytopathic effects consistent with HCMV infection (e.g., slow progression, gradual cell rounding, and detachment) were evident within 3 to 4 weeks. Virus stocks prepared from each culture were used to infect subconfluent HFF cells in 10-cm petri dishes. The spread and pattern of GFP fluorescence (marker encoded by parental bacmid) and the progression of cytopathic effects during the first 6 days were

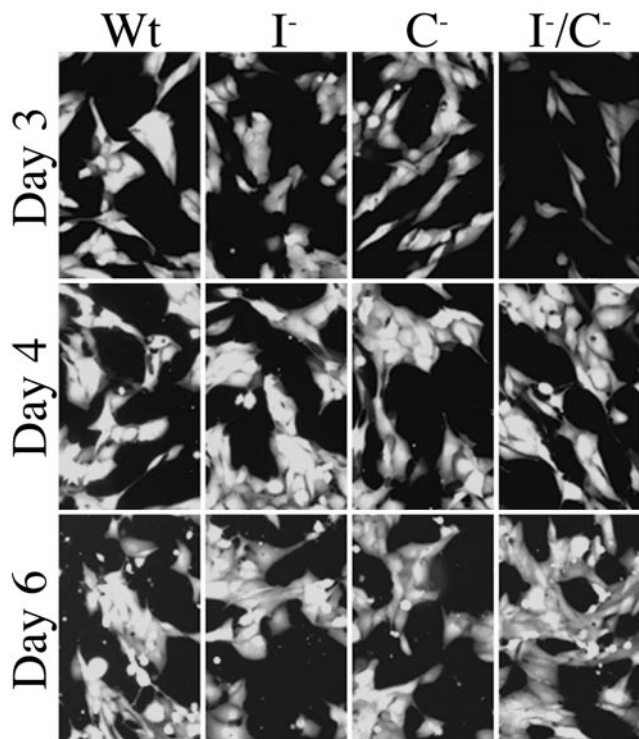


FIG. 2. Spread and cytopathic effects of I- and C-site mutants similar to those of wild-type HCMV. HFF cells were infected with GFP-tagged Wt virus or mutants blocked for cleavage at the I (I⁻) or C (C⁻) site or both (I⁻/C⁻). At 3, 4, and 6 days after infection the cells were examined by fluorescence microscopy. Shown here is a collage of images recorded at ×100 magnification.

comparable in all four cultures (Fig. 2). However, extensive cell rounding, detachment, and rupture took longer for the I⁻/C⁻ mutant (data not shown).

Mutants produce extracellular particles in ratios and with protein compositions similar to those of wild-type virus. We next compared the production of extracellular virus particles by the mutants with that by wild-type virus. Approximately 2 weeks after infection, virus particles were recovered from the culture medium of each dish. The abundance and relative amounts of the three extracellular particle types—noninfectious enveloped particles (NIEPs), virions, and dense bodies (see reference 27)—appeared similar for the four viruses by visual inspection of the light-scattering bands in the gradients. Virions and NIEPs were collected for each virus. A 30-μl portion of each virion band was frozen at -80°C for DNA analyses; the rest of the particle preparations were separately concentrated by ultracentrifugation, solubilized in protein sample buffer, and stored at -80°C until analyzed.

When the protein compositions of virions and NIEPs produced by cells infected with wild-type or the mutant viruses were compared following SDS-PAGE and staining with SYPRO-R, no gross differences were evident (Fig. 3). The amount of major capsid protein (MCP, pUL86; directly proportional to number of particles) varied less than 15% between the eight preparations, indicating that each had similar numbers of particles.

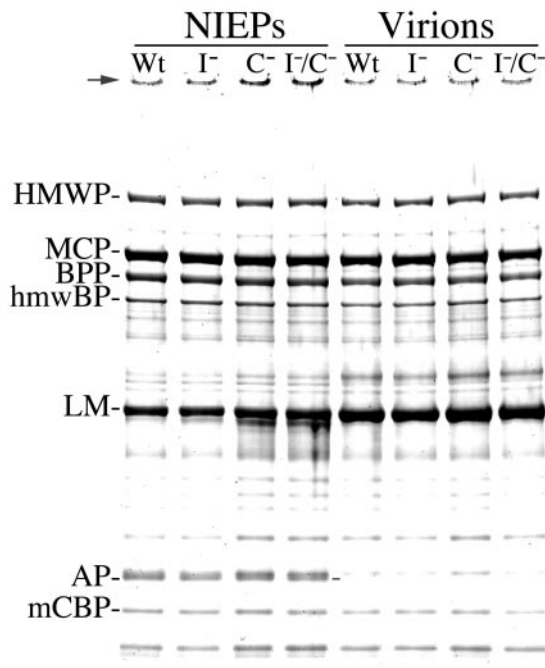


FIG. 3. Protein compositions of NIEPs and virions from I⁻, C⁻, and I⁻/C⁻ mutants are similar to those of wild-type virus. NIEPs and virions were recovered and analyzed by SDS-PAGE as described in Materials and Methods and Results. Shown here is an image of the resulting gel after staining with SYPRO-R. Abbreviations at top for wild-type and mutant viruses are as in Fig. 2; protein abbreviations to left are for high-molecular-weight protein (HMWP, pUL48), major capsid protein (MCP, pUL86), basic phosphoprotein (BPP, pp150, pUL32), HMWP-binding protein (hmwBP, pUL47), lower matrix protein (LM, pp65, pUL83), assembly protein (AP, pUL80.5), and minor capsid protein binding protein (mCBP, pUL46). AP migrates just faster than a protein present in both NIEPs and virions. Arrow at top left indicates bottom of gel sample well.

UL80a sequence verified in DNA from wild-type and mutant virions. The presence of the intended mutations was confirmed by using DNA in the virion preparations obtained above to PCR amplify a 2,831-bp sequence encoding the I and C sites. The resulting PCR product from each virus was gel purified and cleaved with MluI (Fig. 4, lanes 2 to 5) or with NruI (Fig. 4, lanes 6 to 9). The point mutation used to change the I site created a new NruI site that is absent in the wild-type sequence. Similarly, the C-site mutation destroyed an MluI site present in the wild-type sequence. The PCR product for wild-type virion DNA was cleaved by MluI but not NruI (Fig. 4, lanes 2 and 6), DNA from the I-site mutant was cleaved by both enzymes (Fig. 4, lanes 3 and 7), DNA from the C-site mutant was cleaved by neither (Fig. 4, lanes 4 and 8), and DNA from the double I-/C-site mutant was cleaved by NruI but not MluI (Fig. 4, lanes 5 and 9). These patterns verify the intended site-specific changes in each of the mutants.

Sequence analysis of the entire UL80a open reading frame, PCR amplified from each of the four virion DNAs, showed the wild-type sequence to be as expected (10, 11) and the three mutants to contain no changes other than the intended point mutations (data not shown from automated sequencing).

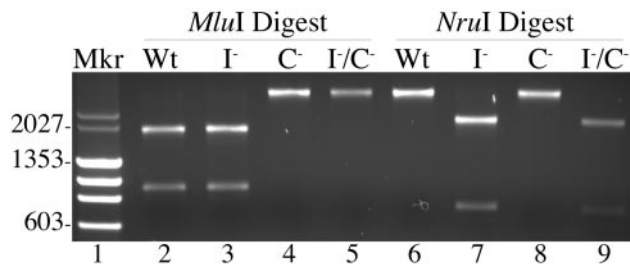


FIG. 4. Intended mutations demonstrated in DNA of extracellular virions. A sequence encoding the assemblin I and C sites was PCR amplified from wild-type and mutant virions and cleaved with either *Mlu*I or *Nru*I, and the resulting digests were subjected to agarose gel electrophoresis. Shown here is an image of the resolved fragments stained with ethidium bromide. Virus abbreviations at the top are as in Fig. 2, and the sizes (base pairs) of marker fragments (Mkr) in lane 1 are indicated on the left.

Proteins detected in mutant virus-infected cells and in extracellular particles are consistent with intended blocked cleavage sites. Assemblin is cleaved at its I and C sites in HFF cells infected with wild-type HCMV-BAC-derived virus (10). Of the five fragments that could be formed by combinations of these two cleavages (Fig. 1), only A_n , A_c , and C_n have been reported (1, 8, 10, 23, 29, 47). To verify that the new C^- and I^-/C^- mutants are blocked for the intended cleavages, nuclear and cytoplasmic fractions were prepared from wild-type- and mutant virus-infected cells by treatment with NP-40, as described before (18). The resulting fractions were subjected to SDS-PAGE followed by Western immunoassay with anti-N2 (N2, Fig. 1), to detect assemblin and fragments containing its amino end (e.g., C_n and A_n). Results of the assay show that (i) assemblin was cleaved at both the I and C sites in cells infected with wild-type virus, giving rise to fragments A_n and C_n , respectively (Fig. 5A, lane 1) (10, 29); (ii) I-site cleavage was blocked in the I^- mutant, as indicated by the absence of fragment A_n (Fig. 5A, lane 2); (iii) C-site cleavage was blocked in the C^- mutant, as indicated by the absence of fragment C_n (Fig. 5A, lane 3); and (iv) the double I^-/C^- mutant showed neither A_n nor C_n , indicating that both I- and C-site cleavages were effectively blocked (Fig. 5A, lane 4). Thus, all three mutations blocked cleavage of the intended sites.

Also notable in these data are that (i) assemblin and its cleavage products were more abundant in the nuclear fraction than in the cytoplasmic fraction (Fig. 5A); (ii) when I-site cleavage was possible (i.e., wild type and C^- mutant), comparatively little assemblin, C_n , and A_n were detected (Fig. 5A, lanes 1 and 3); however, when I-site cleavage was blocked (i.e., I^- and I^-/C^- mutants), the mutant forms of C_n and assemblin accumulated (Fig. 5A, lanes 2 and 4); and (iii) when C-site cleavage was blocked (e.g., C^- and I^-/C^- mutants), a small amount of an additional band (≈ 25 kDa) was detected just ahead of assemblin that was not seen in wild-type or I^- virus (Fig. 5A, lanes 3 and 4; see asterisk). The size of this protein and its reactivity with anti-N2 are compatible with it being the 24.6-kDa amino fragment D_n that would result from cleavage at the sequence VDA↓L, 19 amino acids after the C site (VDA↓S) (Fig. 1) (also see reference 29). This putative cleavage site is within the major helix of the assemblin dimer interface (45), suggesting that it may be the counterpart of the

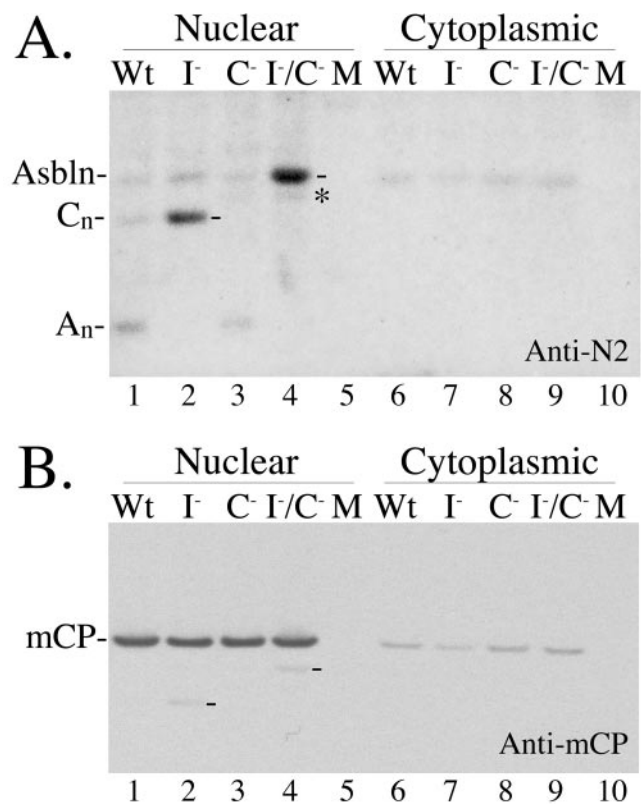


FIG. 5. Expected protease cleavages blocked in C^- and I^-/C^- mutants. HFF cells infected with Wt or one of the three mutant viruses (I^- , C^- , and I^-/C^-) were separated into nuclear and cytoplasmic fractions and subjected to SDS-PAGE and Western immunoassay, as described in Materials and Methods and Results. The membrane was probed with anti-N2 (A) (phosphorimage) and subsequently with anti-mCP (B) (autoradiogram). Abbreviations to left of images are for assemblin (Asbln), the C- and I-site cleavage products C_n and A_n (Fig. 1), and the minor capsid protein (mCP). Dashes to right of C_n and assemblin in panel A, lanes 2 and 4, are shown in panel B for reference in aligning the images. The asterisk denotes a putative D-site fragment, D_n (Fig. 1).

Kaposi's sarcoma-associated herpesvirus dimer (D) site, whose cleavage inactivates the Kaposi's sarcoma-associated herpesvirus assemblin homolog (35).

The relative steady-state amounts of the fragments detected in Fig. 5A were determined by measuring the intensities of the relevant bands and normalizing those values to that of mCP—a fixed-copy-number capsid shell protein (2). mCP amounts were obtained by probing over the same membrane with anti-mCP and quantifying the bands shown in Fig. 5B by phosphorimaging. From these measurements we calculated that (i) about three times the amount of C_n was present in cells infected with the I^- mutant as in cells infected with wild-type virus, (ii) about five times the amount of assemblin was present in cells infected with the I^-/C^- mutant, and (iii) the total amount of assemblin and related products was about the same for the I^- and I^-/C^- mutants but was 26% lower for wild type and $\approx 62\%$ lower for the C^- mutant (Table 1). Thus, more assemblin and cleavage products accumulated when the I site was blocked (I^- and I^-/C^- mutants) than when it could be cleaved (i.e., wild type and C^- mutant).

TABLE 1. Relative amounts of assemblin and cleavage products in NP-40 nuclear fraction of cells infected with wild-type and I⁻, C⁻, and I⁻/C⁻ mutant viruses

Protein ^a	Amt in nuclear fraction ^b			
	Wt ^c	I ⁻	C ⁻	I ⁻ /C ⁻
Assemblin	1.0	1.3	1.0	4.8
C _n	1.1	3.6	—	—
A _n	1.5	—	0.8	—

^a Proteins detected by Western immunoassay shown in Fig. 5A, all as described in Results and Materials and Methods.

^b Relative amount of protein calculated as units of photostimulated luminescence (psl) for the indicated protein band in Fig. 5A, divided by psl for the mCP band in the corresponding lane of Fig. 5B. The resulting values were then normalized to that of Wt assemblin. Dashes indicate absence of detected band in that preparation.

^c Viruses abbreviated as in Fig. 2.

Assemblin and its cleavage products are also present in NIEPs but not in virions or dense bodies (10). To determine whether this distribution is altered in the C⁻ and I⁻/C⁻ mutants, extracellular particles were prepared from cultures infected with wild-type virus or with one of the three mutants. NIEPs, virions, and dense bodies from all four viruses were then subjected to Western immunoassay, using anti-N2 to detect assemblin-related products. Although the intensities of anti-N2-reactive proteins were too weak to quantify with confidence, it can be seen that NIEPs from the I⁻ mutant contain assemblin (Fig. 6A, lanes 2 and 4). As in the nuclear fractions (Fig. 5A, lanes 1 to 5), there was more assemblin-related material in NIEPs from the I⁻ and I⁻/C⁻ mutants than in those from wild-type virus and the C⁻ mutant (Fig. 6A, lanes 1 and 3). Neither assemblin nor antigenically related fragments were detected in the virion and dense body preparations (Fig. 6A, lanes 5 to 12), consistent with previous findings reported for wild-type and the I⁻ mutant viruses (10). The membrane shown in Fig. 6A was probed again with anti-mCP, as done above, to determine the relative amount of NIEPs and virions in each preparation (dense bodies contain neither mCP nor the other capsid proteins; see reference 27) and as a means of normalizing the amount of assemblin-related proteins to the number of capsids (i.e., constant ratio of mCP/capsid). Calculations made from the membrane shown in Fig. 6B indicate that there was less than 10% variation in the amount of particles between the eight preparations (Fig. 6B). Thus, the lower amount of assemblin and related proteins in wild-type and C⁻ mutant NIEPs and the failure to detect assemblin-related proteins in virions were not due to there being less of these particles.

Mutants blocked for I- or C-site cleavage or both yield lower titers of infectious virus. A time course assay of virus production was done to compare the infectivities of wild-type and the three mutant viruses. In order to infect the cells with comparable multiplicities, stocks of all four viruses were tested by an endpoint titration in 96-well plates. Based on the relative titers obtained in this first assay (1.0 [Wt]:0.6 [I⁻]:0.3 [C⁻]:0.05 [I⁻/C⁻]), 10-cm-petri dish cultures of HFF cells were infected with one of the four viruses, at approximately equal multiplicities. One day after infection the inoculum was aspirated from each dish and replaced with 11 ml of fresh medium. A 1-ml sample

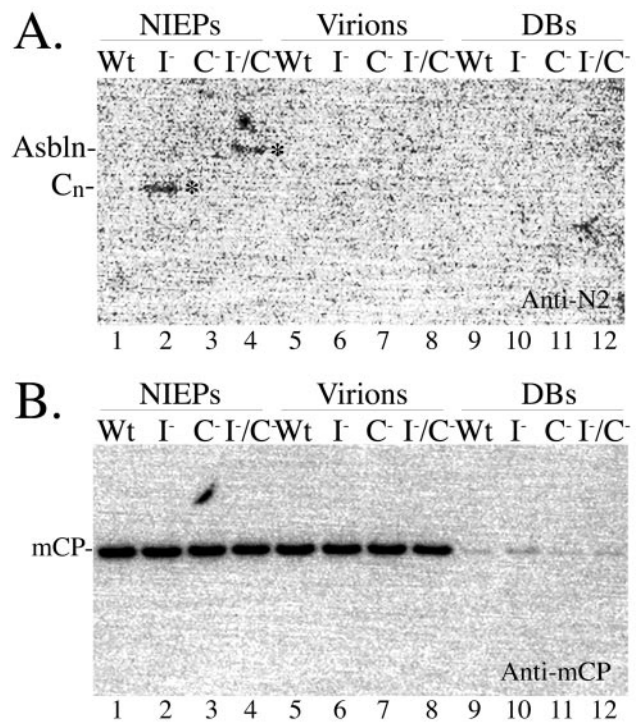


FIG. 6. NIEPs but not virions contain assemblin-related products. Extracellular virus particles were recovered from cells infected with Wt or one of the three mutant viruses (I⁻, C⁻, and I⁻/C⁻) and analyzed by Western immunoassay following SDS-PAGE. The membrane was probed with anti-N2 (A) and subsequently with anti-mCP (B). The small amount of mCP present in the dense body preparations (DBs) is due to minor contamination with NIEPs and virions resulting from the single banding in sucrose used here, in contrast to the more complete separation achieved by multiple bandings in glycerol-tartrate gradients (27; unpublished observations). Shown here are phosphorimages of the membrane after the two sequential probes. Abbreviations and labeling are as in Fig. 5.

was immediately taken from each culture, clarified (14,000 × g for 2 min at room temperature), serially diluted (10-fold to 10⁻⁸), and frozen at -80°C until assayed. Similarly prepared samples were taken on the following 8 days, each time with replacement of the 1-ml sample volume with fresh medium.

To gauge the range of titers in the resulting samples for each virus on each day, a second endpoint dilution assay was done using 100-fold serial dilutions. The final titer of the I⁻/C⁻ mutant ($\approx 5 \times 10^4$), even in this survey assay, was notably lower than those of the other three viruses ($\approx 10^5$ for the Wt, I⁻, and C⁻ viruses). To obtain more accurate measurements, appropriate 10-fold dilutions of the samples were tested by plaque assay, as described in Materials and Methods. Results of the assay showed that the final titers of the I⁻ (1.6×10^6), C⁻ (1.0×10^6), and I⁻/C⁻ (0.3×10^6) mutants were 20%, 50%, and 85% lower, respectively, than that of the parental wild-type virus (2.0×10^6) (Fig. 7). Although there was some variability in the absolute values calculated and the standard deviations of specific samples (e.g., Fig. 7 legend), the titers of the mutant viruses were consistently lower than those of wild-type virus and consistently in the rank order of I⁻ > C⁻ > I⁻/C⁻, as can be seen on day 8 of the time course experiment where

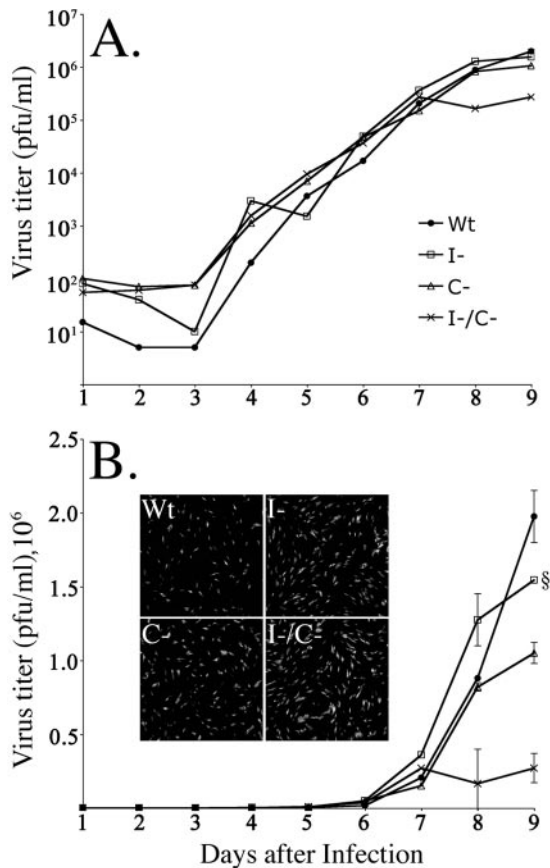


FIG. 7. Mutations blocking cleavage of the I or C site, or both, reduce virus titer. Plaque assays were done for samples taken over a 9-day period following infection with the Wt or mutant viruses (I^- , C^- , and I^-/C^-), as described in Results and Materials and Methods. Shown here are logarithmic (A) and arithmetic (B) plots of the data. Panel B inset shows fluorescence (i.e., GFP reporter expressed from viral genome) images of representative fields from the infected cultures 3 days after addition of virus and reveals comparatively fewer GFP-expressing cells (i.e., infected) in the culture infected with wild-type virus. §, standard deviations (indicated by vertical bars at each data point) for the titers of day 9 I^- virus [$\pm(0.8 \times 10^6)$] and day 8 Wt virus [$\pm(0.4 \times 10^6)$] were higher than those for all other determinations and are not shown because they obscure other data.

there is no overlap of titers (\pm standard deviation) for the mutants (Fig. 7B; Table 2).

These data also indicate that the rate of infectivity increase differed for the four viruses between days 3 and 9 (Fig. 7). Wild-type virus increased continuously, after a lag resulting from its slightly lower relative multiplicity of infection (e.g., Fig. 7A, day 1 titer, and 7B, inset). The I^- and C^- mutants increased at about the same rate as wild type from days 3 to 7 but then slowed on days 8 and 9 (Fig. 7B). The I^-/C^- mutant increased at a rate similar to that of the others through day 6 and then slowed somewhat by day 7 and was unchanged on days 8 and 9 (Fig. 7B). We interpret the plateauing of infectivity for the I^-/C^- mutant as being more consistent with a reduced efficiency of virus production or a reduced stability of infectious virus than with a delay in reaching maximal titer.

TABLE 2. Summary comparison of wild-type and mutant virus titers

Expt	Virus titer (% of wild type)			
	Wild type	I^-	C^-	I^-/C^-
Titer stock (end point) ^a	100	60	30	5
Survey time course (end point) ^b	100	100	100	50
Time course plaque assay ^c				
Data from day 8	44 ^d	65	41	8
Data from day 9	100	78 ^e	53	14

^a 10-fold dilutions of stock tested.

^b 100-fold dilutions of each time point tested.

^c 10-fold dilutions tested; range based on results of "survey" titration above; data from Fig. 7.

^d Values calculated as percentage of wild-type virus titer on day 9, due to lag resulting from relatively lower multiplicity of infection of wild-type virus inoculum (Fig. 7 and legend).

^e Standard deviation was high for this data point (legend to Fig. 7).

DISCUSSION

Restriction of I-site cleavage to the cytomegalovirus subgroup of herpesvirus assemblin homologs and the even more stringent restriction of C-site cleavage to HCMV assemblin suggest that one or both cleavages may have a species-specific function. As a means of identifying that function, we have used mutant viruses to uncover leads from their phenotype. In our initial study we showed that a mutant virus having a noncleavable I-site (I^- mutant) replicated relatively well. But our conclusion that this cleavage is not essential for virus replication was qualified by the finding that the I^- mutant showed increased C-site cleavage, as was also found when the protease was expressed in transient-transfection assays (29), possibly reflecting use of that site as an alternate or escape processing pathway (10, 29). We now show, however, that blocking either site alone adversely affects virus replication and that, when both are blocked, output of infectious virus is diminished by nearly 90%. Thus, although these cleavages are not absolutely essential for HCMV replication, they substantially increase its efficiency in producing infectious virus.

We did observe some variability in the magnitude of titer differences between the four viruses from experiment to experiment, but relative titers were reproducibly wild-type $> I^- > C^- > I^-/C^-$, highest to lowest, and the reduced titer of the I^- mutant versus wild-type virus determined here by plaque assay is consistent with the difference estimated before by endpoint dilution (10). The additive impact of the two mutations and the finding that neither site fully compensates for mutation of the other favor the interpretation that the I and C sites have different functions.

We have speculated that I-site cleavage may be involved with eliminating assemblin from the virion capsid (10). This followed from the observation that HCMV virions contain neither assemblin nor detectable fragments of it, which is in contrast to the presence of the assemblin homolog VP24 in virions of herpes simplex virus (21, 39, 41). A reason for this difference was suggested to be a requirement within the HCMV capsid for additional room to accommodate its 51% longer DNA. Our finding that the I^- , C^- , and most notably the I^-/C^- mutants all produced lower yields of infectious virus is compatible with the prediction that DNA packaging would be less efficient if elimination of assemblin from the capsid were

impeded. The reduced titers of these mutants could also be accounted for by a destabilizing effect of these mutations on infectious virus particles. Although this interpretation could reconcile the lower titer of the I⁻/C⁻ mutant (Fig. 7) with its more normal production of extracellular particles (Fig. 3 and 6), its appeal is weakened by the comparatively stable titer of this mutant over the last 3 days of the time course assay (Fig. 7).

A related explanation for the I- and C-site cleavages in HCMV is that they may be needed to prevent assemblin from acting outside of the capsid. If released from the capsid as an active enzyme, assemblin could potentially attack capsid assembly intermediates (e.g., pAP-MCP complexes; see references 19 and 50) prematurely and interfere with capsid formation. Thus, although assemblin cleavage may not be required to enable DNA packaging per se (i.e., a separate mechanism may allow assemblin to leave capsid), it may be needed as a consequence of it (e.g., to inactivate free protease). This would be unnecessary in HSV, since the protease remains sequestered inside the DNA-containing capsid. This explanation suggests that the reduced production of infectious virus observed with the I⁻/C⁻ mutant (Fig. 7) could be due to the presence of active noncleavable assemblin outside the capsid, where it interferes with procapsid formation. Parenthetically, assemblin blocked for I- and C-site cleavage, as in the I⁻/C⁻ mutant, remains an active enzyme (references 5, 13, and 31 and unpublished findings).

We also noted that the relative stability of assemblin and its cleavage products in virus-infected cells is differentially influenced by these mutations. Whereas I-site cleavage destabilized assemblin and resulting fragments, C-site cleavage did not (Fig. 5). Similar effects were observed with protease expressed alone in transfections (29). Thus, even though I-site cleavage does not inactivate the protease (24), it may mark the enzyme for accelerated degradation, minimizing the possibility that it could attack nascent procapsid subunits. The effect of C-site cleavage on assemblin has not been determined; however, it does not appear to share the putative destabilizing effect of I-site cleavage since its product, C_n, is relatively stable (Fig. 5, lane 2). If C-site cleavage is inactivating, like that of the dimer (D) site in the Kaposi's sarcoma-associated herpesvirus assemblin homolog (35), then it could provide an alternate or additional means of ensuring the functional silence of assemblin released from maturing HCMV capsids.

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REFERENCES

- Baum, E. Z., G. A. Beberitz, J. D. Hulmes, V. P. Muzithras, T. R. Jones, and Y. Gluzman. 1993. Expression and analysis of the human cytomegalovirus UL80-encoded protease: identification of autoproteolytic sites. *J. Virol.* **67**: 497-506.
- Baxter, M. K., and W. Gibson. 1997. Presented at the 22nd International Herpesvirus Workshop, La Jolla, Calif.
- Beudet-Miller, M., R. Zhang, J. Durkin, W. Gibson, A. D. Kwong, and Z. Hong. 1996. Viral specific interaction between the human cytomegalovirus major capsid protein and the C terminus of the precursor assembly protein. *J. Virol.* **70**:8081-8088.
- Borst, E. M., G. Hahn, U. H. Koszinowski, and M. Messerle. 1999. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in *Escherichia coli*: a new approach for construction of HCMV mutants. *J. Virol.* **73**:8320-8329.
- Brignole, E. J., and W. Gibson. 2004. Presented at the Herpesvirus Symposium, Philadelphia, Pa.
- Brown, J. C., M. A. McVoy, and F. L. Homa. 2002. Packaging DNA into herpesvirus capsids, p. 111-153. *In* A. Holzenberg and E. Bogner (ed.), Structure-function relationships of human pathogenic viruses. Kluwer Academic/Plenum Publishers, New York, N.Y.
- Buisson, M., J. F. Hernandez, D. Lascoux, G. Schoehn, E. Forest, G. Arlaud, J. M. Seigneurin, R. W. Ruigrok, and W. P. Burmeister. 2002. The crystal structure of the Epstein-Barr virus protease shows rearrangement of the processed C terminus. *J. Mol. Biol.* **324**:89-103.
- Burck, P. J., D. H. Berg, T. P. Luk, L. M. Sassmannshausen, M. Wakulchik, D. P. Smith, H. M. Hsiung, G. W. Becker, W. Gibson, and E. C. Villarreal. 1994. Human cytomegalovirus maturational proteinase: expression in *Escherichia coli*, purification, and enzymatic characterization by using peptide substrate mimics of natural cleavage sites. *J. Virol.* **68**:2937-2946.
- Burnette, N. 1981. "Western blotting": electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**: 195-203.
- Chan, C. K., E. J. Brignole, and W. Gibson. 2002. Cytomegalovirus assemblin (pUL80a): cleavage at internal site not essential for virus growth; protease absent from virions. *J. Virol.* **76**:8667-8674.
- Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell. 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154**:125-169.
- Chen, P., H. Tsuge, R. J. Almassy, C. L. Gribskov, S. Katoh, D. L. Vanderpool, S. A. Margosiak, C. Pinko, D. A. Matthews, and C.-C. Kan. 1996. Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad. *Cell* **86**:835-843.
- Darke, P. L., J. L. Cole, L. Waxman, D. L. Hall, M. K. Sardana, and L. C. Kuo. 1996. Active human cytomegalovirus protease is a dimer. *J. Biol. Chem.* **271**:7445-7449.
- Desai, P., and S. Person. 1996. Molecular interactions between the HSV-1 capsid proteins as measured by the yeast two-hybrid system. *Virology* **220**: 516-521.
- Desai, P., and S. Person. 1999. Second site mutations in the N-terminus of the major capsid protein (VP5) overcome a block at the maturation cleavage site of the capsid scaffold proteins of herpes simplex virus type 1. *Virology* **261**:357-366.
- Falk, C. S., M. Mach, D. J. Schendel, E. H. Weiss, I. Hilgert, and G. Hahn. 2002. NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. *J. Immunol.* **169**:3257-3266.
- Gibson, W. 2005. Assembly and maturation of the capsid. *In* M. J. Reddehase (ed.), Cytomegalovirus: pathogenesis, molecular biology and infection control. Horizon Scientific Press, Norwich, United Kingdom.
- Gibson, W. 1981. Structural and nonstructural proteins of strain Colburn cytomegalovirus. *Virology* **111**:516-537.
- Gibson, W. 1996. Structure and assembly of the virion. *Intervirology* **39**:389-400.
- Gibson, W., M. K. Baxter, and K. S. Clopper. 1996. Cytomegalovirus "missing" capsid protein identified as heat-aggregable product of human cytomegalovirus UL46. *J. Virol.* **70**:7454-7461.
- Gibson, W., and B. Roizman. 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. *J. Virol.* **10**:1044-1052.
- Gibson, W., A. R. Welch, and M. R. T. Hall. 1995. Assemblin, a herpes virus serine maturational proteinase and new molecular target for antivirals. *Perspect. Drug Discov. Des.* **2**:413-426.
- Hall, M. R. T., and W. Gibson. 1996. Cytomegalovirus assemblin: the amino and carboxyl domains of the proteinase form active enzyme when separately cloned and coexpressed in eukaryotic cells. *J. Virol.* **70**:5395-5404.
- Holwerda, B. C., A. J. Wittwer, K. L. Duffin, C. Smith, M. V. Toth, L. S. Carr, R. C. Wiegand, and M. L. Bryant. 1994. Activity of two-chain recombinant human cytomegalovirus protease. *J. Biol. Chem.* **269**:25911-25915.
- Hong, Z., M. Beudet-Miller, J. Burkin, R. Zhang, and A. D. Kwong. 1996. Identification of a minimal hydrophobic domain in the herpes simplex virus type 1 scaffolding protein which is required for interaction with the major capsid protein. *J. Virol.* **70**:533-540.
- Hoog, S. S., W. W. Smith, X. Qiu, C. A. Janson, B. Hellmig, M. S. McQueney, K. O'Donnell, D. O'Shannessy, A. G. DiLella, C. Debouck, and S. S. Abdel-Meguid. 1997. Active site cavity of herpesvirus proteases revealed by the

- crystal structure of herpes simplex virus protease/inhibitor complex. *Biochemistry* **36**:14023–14029.
27. **Irmiere, A., and W. Gibson.** 1983. Isolation and characterization of a non-infectious virion-like particle released from cells infected with human strains of cytomegalovirus. *Virology* **130**:118–133.
 28. **Irmiere, A., and W. Gibson.** 1985. Isolation of human cytomegalovirus intranuclear capsids, characterization of their protein constituents, and demonstration that the B-capsid assembly protein is also abundant in noninfectious enveloped particles. *J. Virol.* **56**:277–283.
 29. **Jones, T. R., L. Sun, G. A. Beberitz, V. P. Muzithras, H.-J. Kim, S. H. Johnston, and E. Z. Baum.** 1994. Proteolytic activity of human cytomegalovirus UL80 proteinase cleavage site mutants. *J. Virol.* **68**:3742–3752.
 30. **Kennard, J., F. J. Rixon, I. S. McDougall, J. D. Tatman, and V. G. Preston.** 1995. The 25 amino acid residues at the carboxy terminus of the herpes simplex virus type 1 UL26.5 protein are required for the formation of the capsid shell around the scaffold. *J. Gen. Virol.* **76**:1611–1621.
 31. **LaFemina, R. L., K. Bakshi, W. J. Long, B. Pramanik, C. A. Veloski, B. S. Wolanski, A. I. Marcy, and D. J. Hazuda.** 1996. Characterization of a soluble stable human cytomegalovirus protease and inhibition by M-site peptide mimics. *J. Virol.* **70**:4819–4824.
 32. **Liu, F., and B. Roizman.** 1992. Differentiation of multiple domains in the herpes simplex virus 1 protease encoded by the UL26 gene. *Proc. Natl. Acad. Sci. USA* **89**:2076–2080.
 33. **Margosiak, S. A., D. L. Vanderpool, W. Sisson, C. Pinko, and C. C. Kan.** 1996. Dimerization of the human cytomegalovirus protease—kinetic and biochemical characterization of the catalytic homodimer. *Biochemistry* **35**:5300–5307.
 34. **Matusick-Kumar, L., P. J. McCann III, B. J. Robertson, W. W. Newcomb, J. C. Brown, and M. Gao.** 1995. Release of the catalytic domain N₀ from the herpes simplex virus type 1 protease is required for viral growth. *J. Virol.* **69**:7113–7121.
 35. **Pray, T. R., A. M. Nomura, M. W. Pennington, and C. S. Craik.** 1999. Auto-inactivation by cleavage within the dimer interface of Kaposi's sarcoma-associated herpesvirus protease. *J. Mol. Biol.* **289**:197–203.
 36. **Qiu, X., J. S. Culp, A. G. DiLella, B. Hellmig, S. S. Hoog, D. A. Janson, W. W. Smith, and S. S. Abdel-Meguid.** 1996. Unique fold and active site in cytomegalovirus protease. *Nature* **383**:275–279.
 37. **Qiu, X., C. A. Janson, J. S. Culp, S. B. Richardson, C. Debouck, W. W. Smith, and S. S. Abdel-Meguid.** 1997. Crystal structure of varicella-zoster virus protease. *Proc. Natl. Acad. Sci. USA* **94**:2874–2879.
 38. **Reiling, K. K., T. R. Pray, C. S. Craik, and R. M. Stroud.** 2000. Functional consequences of the Kaposi's sarcoma-associated herpesvirus protease structure: regulation of activity and dimerization by conserved structural elements. *Biochemistry* **39**:12796–12803.
 39. **Rixon, F. J.** 1993. Structure and assembly of herpesviruses. *Semin. Virol.* **4**:135–144.
 40. **Shieh, H.-S., R. G. Kurumbail, A. M. Stevens, R. A. Stegeman, E. J. Sturman, J. Y. Pak, A. J. Wittwer, M. O. Palmier, R. C. Wiegand, B. C. Holwerda, and W. C. Stallings.** 1996. Three-dimensional structure of human cytomegalovirus protease. *Nature* **383**:279–282.
 41. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. *J. Virol.* **9**:143–159.
 42. **Steven, A. C., and P. G. Spear.** 1997. Herpesvirus capsid assembly and envelopment, p. 312–351. *In* W. Chiu, R. M. Burnett, and R. L. Garcea (ed.), *Structural biology of viruses*. Oxford University Press, New York, N.Y.
 43. **Thomsen, D. R., W. W. Newcomb, J. C. Brown, and F. L. Homa.** 1995. Assembly of the herpes simplex virus capsid: requirement for the carboxyl-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes. *J. Virol.* **69**:3690–3703.
 44. **Tong, L.** 2002. Viral proteases. *Chem. Rev.* **102**:4609–4626.
 45. **Tong, L., C. Qian, M. J. Massariol, P. R. Bonneau, M. G. Cordingly, and L. Lagacé.** 1996. A new serine-protease fold revealed by the crystal structure of human cytomegalovirus protease. *Nature* **383**:272–275.
 46. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
 47. **Welch, A. R., L. M. McNally, M. R. Hall, and W. Gibson.** 1993. Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine. *J. Virol.* **67**:7360–7372.
 48. **Welch, A. R., E. C. Villarreal, and W. Gibson.** 1995. Cytomegalovirus protein substrates are not cleaved by the herpes simplex type 1 proteinase. *J. Virol.* **69**:341–347.
 49. **Welch, A. R., A. S. Woods, L. M. McNally, R. J. Cotter, and W. Gibson.** 1991. A herpesvirus maturational protease, assemblin: identification of its gene, putative active site domain, and cleavage site. *Proc. Natl. Acad. Sci. USA* **88**:10792–10796.
 50. **Wood, L. J., M. K. Baxter, S. M. Plafker, and W. Gibson.** 1997. Human cytomegalovirus capsid assembly protein precursor (pUL80.5) interacts with itself and with the major capsid protein (pUL86) through two different domains. *J. Virol.* **71**:179–190.