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The C-Terminal 25 Amino Acids of the Protease and Its Substrate ICP35 of Herpes Simplex Virus Type 1 Are Involved in the Formation of Sealed Capsids

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The herpes simplex virus type 1 protease and its substrate, ICP35, are involved in the assembly of viral capsids. Both proteins are encoded by a single open reading frame from overlapping mRNAs. The protease is autoproteolytically processed at two sites. The protease cleaves itself at the C-terminal site (maturation site) and also cleaves ICP35 at an identical site, releasing a 25-amino-acid (aa) peptide from each protein. To determine whether these 25 aa play a role in capsid assembly, we constructed a mutant virus expressing only Prb, the protease without the C-terminal 25 aa. Phenotypic analysis of the Prb virus in the presence and absence of ICP35 shows the following: (i) Prb retains the functional activity of the wild-type protease which supports virus growth in the presence of ICP35; (ii) in contrast to the ICP35 null mutant ΔICP35 virus, the Prb virus fails to grow in the absence of ICP35; and (iii) *trans*-complementation experiments indicated that full-length ICP35 (ICP35 c,d), but not the cleaved form (ICP35 e,f), complements the growth of the Prb virus. The most striking phenotype of the Prb virus is that only unsealed aberrant capsid structures are observed by electron microscopy in mutant-infected Vero cells. Our results demonstrate that the growth of herpes simplex virus type 1 requires the C-terminal 25 aa of either the protease or its substrate, ICP35, and that the C-terminal 25 aa are involved in the formation of sealed capsids.

The herpes simplex virus type 1 (HSV-1) capsid is an icosahedral protein shell that consists of 162 capsomers, 150 of which are hexons and 12 of which are pentons (1, 31, 39, 43, 47). Genetic and structural studies have demonstrated that assembly of the capsid is an essential step for virion maturation. Three major groups of HSV-1 capsids, designated A, B, and C, can be isolated by sucrose gradient sedimentation from infected cell extracts (12). C capsids contain the entire viral genome and are able to mature into infectious virus. Both A and B capsids lack DNA, but they can be distinguished from one another by the presence of large amounts of the virus assembly protein, ICP35 (VP22a), in B capsids (12, 27, 28, 33). Pulse-chase experiments with equine herpesvirus 1 showed that B capsids are able to package virus DNA to form C capsids, indicating that these B capsids may be intermediates in virus assembly (33).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel analyses have shown that B capsids contain at least seven proteins, VP5, VP19c, Nb (VP21), ICP35 (VP22a), VP23, N_0 (VP24), and VP26 (1, 13, 31, 43). The major capsid protein, VP5, accounts for about 70% of the capsid mass and forms the hexons and pentons. There are 960 copies of VP5 per capsid. It appears that VP5 may have different conformations in the capsomers, because a set of monoclonal antibodies (MAb) specific for VP5 can discriminate between hexons and pentons (9, 23, 42, 47).

Three capsid proteins, N_0, Nb, and ICP35, are encoded by the overlapping genes UL26 and UL26.5 (5, 15, 19, 25, 34, 44). The HSV-1 protease (Pra) encoded by the UL26 gene is 635 amino acids (aa) in length. The catalytic domain and cleavage sites of the cytomegalovirus virus protease were first identified by Welch et al., and they also noted that these cleavage sites may be conserved for all herpesviruses (46). Pra is autoprocessed between Ala and Ser residues at two sites. Processing at the release site between aa 247 and 248 generates N_0 and Na, while processing at the maturation site between aa 610 and 611 generates Prb and the C-terminal 25 aa (5, 8, 20–22, 24) (Fig. 1). Genetic and functional analyses have clearly demonstrated that N_0, the N-terminal 247 aa of Pra, contains the catalytic domain (5, 8, 20–22, 34, 44). Since residue 307 (Met) of Pra is also the start codon for ICP35, the entire ICP35 protein shares amino acid sequence identity with the C-terminal 329 aa of Pra. Consequently, ICP35 c,d can be *trans* cleaved by the protease at aa 304 and 305, 25 aa from its C terminus, producing ICP35 e,f (5, 8) (Fig. 1). There are approximately 1,000 copies of ICP35 per B capsid, while Nb and N_0 are less abundant, at approximately 100 copies per capsid (27, 28). ICP35 is also phosphorylated in infected cells and migrates as multiple forms during SDS-polyacrylamide gel electrophoresis (PAGE) before and after being processed (3). An additional internal autocleavage site within the catalytic domain of cytomegalovirus protease has also been identified (2, 45).

Since both Pra and ICP35 c,d are cleaved by the protease during capsid assembly and the processed forms (Nb and ICP35 e,f) are present in B capsids but absent in mature virions, it has been postulated that ICP35 is analogous to the scaffold protein of double-stranded DNA bacteriophage (4). However, the function of Na in capsid assembly is not clear. Our recent results demonstrated that the function of the Na domain, at least in part, is to direct the catalytic domain, N_0,

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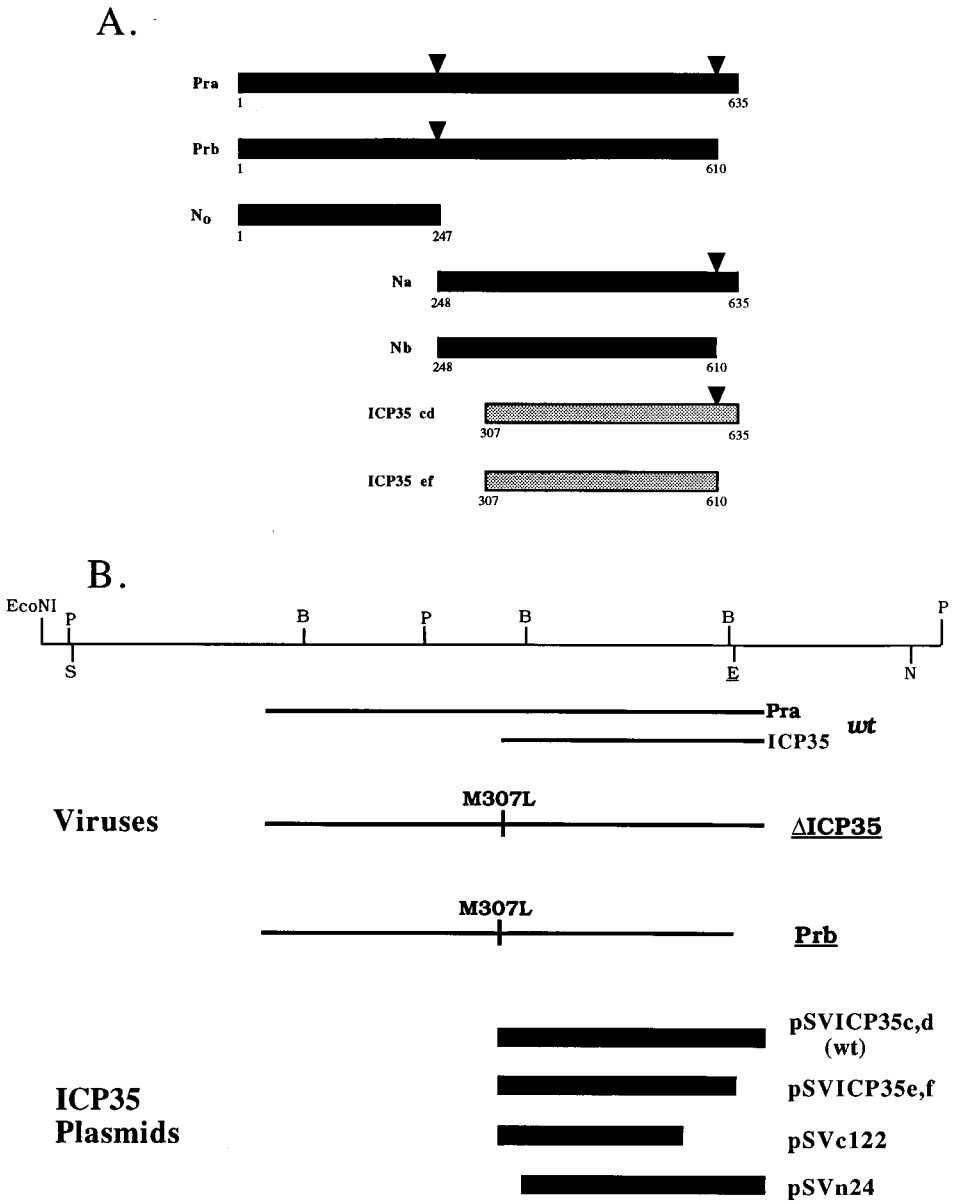

FIG. 1. (A) Polypeptide products of UL26 and UL26.5 open reading frames. The HSV-1 protease (Pra), substrate (ICP35 c,d), and cleavage products Prb, N_0 (VP24), Na, Nb (VP21), and ICP35 e,f (VP22a) are described in the text. The cleavage sites of Pra and ICP35 c,d are indicated by arrowheads. The UL26 amino acid numbers of N and C termini for each protein are indicated. (B) The UL26 and UL26.5 open reading frames of HSV-1 wt, ΔICP35, and Prb viral genomes, as well as locations of ICP35 gene mutations in this study. Plasmid designations are shown on the right. Restriction enzyme sites are shown on the top line. B, *Bam*HI; N, *Nhe*I; P, *Pst*I; S, *Sph*I; E, an *Eco*RI site generated only in the plasmid pSVICP35e,f and mutant Prb viral genome.

into the nucleus (9). In addition, we and others have shown that although the protease and ICP35 are essential for efficient virus growth, capsid structures were observed when either the protease or ICP35 was omitted, but not when both were omitted (7, 9, 23, 36–38, 40, 41). This led us to postulate that Na and ICP35 can functionally substitute for each other under certain conditions. This hypothesis is further supported by our recent data demonstrating that in the absence of ICP35, the mutant ΔICP35 virus, which expresses full-length Pra, can survive (23). However, these studies cannot rule out the possibility that there is another substrate for the virus protease or that this substrate, rather than ICP35, is essential for virus growth. If this is the case, one would not expect a mutation which inactivates any function of ICP35 to be lethal to the virus.

In this study, we report that we have developed a powerful genetic selection system for isolation of protease mutant Prb virus. Prb, a C-terminal 25-aa truncation of Pra, retains all the functions of the wild-type (wt) protease that supports virus growth. In contrast to the ICP35 null mutant ΔICP35 virus, the Prb virus can grow only in the presence of ICP35, indicating that Nb cannot functionally substitute for ICP35. The striking phenotype of the mutant Prb virus is that in the absence of the C-terminal 25 aa of the protease and ICP35, opened aberrant capsid structures are observed by electron microscopic analy-

sis. We conclude that the C-terminal 25-aa tail of either the protease or ICP35, but not both, is essential for the formation of sealed capsids.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained as described previously (16). The growth medium for the neomycin-resistant cell lines X4 and 35J (9, 23) included 250 μg of the antibiotic G418 per ml.

The HSV-1 wt strain KOS1.1 was propagated and assayed as described previously (16, 17). The mutant ΔICP35 and Prb viruses were propagated on ICP35-expressing 35J cells (23).

Plasmids. Plasmid pM307L encodes the HSV-1 protease with a Met-to-Leu change at residue 307, which corresponds to the initiation codon of ICP35 (9, 19, 20). Therefore, this mutation eliminates the synthesis of ICP35. Plasmid pRB4057 was a gift of F. Liu and B. Roizman (University of Chicago). The HSV-1 protease and ICP35 genes specified by pRB4057 carrying the wt *Eco*NI-*Kpn*I fragment are located between 0.325 and 0.55 map units (20). Plasmid pPrb/M307L was constructed as follows. A stop codon was introduced at position 611 of the UL26 open reading frame of pRB4260 (a gift of F. Liu and B. Roizman) by *dut/ung* mutagenesis (18). A mutagenic oligonucleotide of sequence CCTTGTCAACGCC**TAG**GAATTCGCACACGTGGAC was hybridized and extended to introduce a TAG stop codon (boldface) at residue 611 followed by an *Eco*RI restriction site (underlined). The introduction of this mutation was confirmed by restriction analysis, DNA sequencing, and Western immunoblot detection of the deletion product, Prb. The resulting *Xba*I-*Kpn*I fragment spanning the mutation was subcloned into pJ3Ω to generate pSVPrb (26). Plasmid pSVPrb/M307L was constructed by replacing the *Eco*47III-*Aat*II fragment of pSVPrb with that of pM307L. Finally, plasmid pPrb/M307L was constructed by replacing the *Eco*47III-*Asp*718 fragment of pRB4057 with that of pSVPrb/M307L. Thus, pSVPrb/M307L encodes Prb with the Met-to-Leu change at residue 307 (M307L). pUCICP35 was constructed by ligating the 2-kb *Hpa*I-*Hin*dIII fragment of pRB4057 into the *Sma*I-*Hin*dIII sites of pUC18. Thus, pUCICP35 encodes ICP35 expressed from its own promoter. pSVICP35 was constructed by ligating the *Xba*I-*Kpn*I fragment of pT7ICP35C (5) into the vector pJ3Ω. Thus, the expression of ICP35 from this construct is under the control of the simian virus 40 early promoter. pSVICP35e,f was constructed by replacing the 380-bp *Bsu*36I-*Asp*718 fragment of pSVICP35 with that of pSVPrb. pSVc122 was constructed by ligation of the *Xba*I-*Asp*718 fragment of vector pJ3Ω, the 386-bp *Xba*I-*Eag*I fragment of pSVICP35e,f, and the 683-bp *Eag*I-*Asp*718 of pT7513A (44). Thus, pSVc122 encodes a mutant ICP35 with a C-terminal 122-aa truncation. Plasmid pDel-ICP35 was constructed by standard PCR techniques. A mutagenic oligonucleotide of sequence AATTGG**CCATGG**CCTCCCATTACAAC CAGCTCG was hybridized and extended to introduce the ATG start codon (boldface) into an *Nco*I restriction site (underlined) at the position encoding residue 329 of the protease. The resulting *Nco*I-*Kpn*I fragment spanning the mutation was subcloned into pT7635K (44) to generate pDel-ICP35. Plasmid pSVn24 was constructed by ligating the 938-bp *Xba*I-*Asp*718 fragment of pDel-ICP35 into pJ3Ω. Thus, pSVn24 lacks codons for residues 2 to 24 of the ICP35 coding sequence.

Isolation of mutant Prb virus. The isolation of mutant Prb virus is illustrated in Fig. 2. Infectious *m*100 virus DNA and the linearized plasmid pPrb/M307L were cotransfected into X4 cells. Forty-eight hours after marker transfer, progeny viruses were harvested and used to inoculate ICP35-expressing 35J cells. Forty-eight plaques were picked and tested for the ability to grow on Vero and 35J cells. Seventeen failed to grow on Vero cells and required 35J cells for propagation. Two were plaque purified twice for initial characterization; one, designated Prb virus, was used for this study.

Analysis of virus DNA and proteins. Viral DNAs for Southern blotting were prepared as described previously (10) and probed with pRB4057 (20). For Western blot analysis of infected-cell lysates, cell monolayer cultures were infected with KOS1.1 or Prb virus at a multiplicity of infection (MOI) of 10 and harvested as indicated in the text. SDS-PAGE was performed as described previously (10, 44). Proteins were electrophoretically transferred to nitrocellulose filters. The detection of immune complexes on nitrocellulose filters by Western blot was conducted as specified by the manufacturer (Promega Biotec, Madison, Wis.). The anti-ICP35 MAb MCA406 (1:1,000 dilution) (Serotec) was used to detect ICP35 and protease-related products Pra, Prb, Na, and Nb.

Complementation of Prb virus in *trans*. The procedure for *trans* complementation has been described previously (9). Vero cells were transfected with 5 μg of wt or mutant ICP35 plasmid. At 20 h posttransfection, cells were infected with 3 PFU of Prb virus per cell and allowed to undergo a single cycle of infection. Virus yield on 35J cells was measured by plaque assay.

Electron microscopy. Infected cells to be examined by electron microscopy were centrifuged into a small (0.3-ml) pellet, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C, and postfixed with 2% OsO_4 for 1 h at 24°C. Then pellets were washed in water, dehydrated in graded concentrations of acetone, embedded in Epon 812, and cut into sections (~70-nm thick) with a Richert Ultracut E ultramicrotome. Sections were stained with a saturated solution of uranyl acetate in methanol (20 min at 60°C) and then with 0.25% lead citrate (2 min at 24°C) and examined with a JEOL 100CX transmission electron microscope operated at 80 keV.

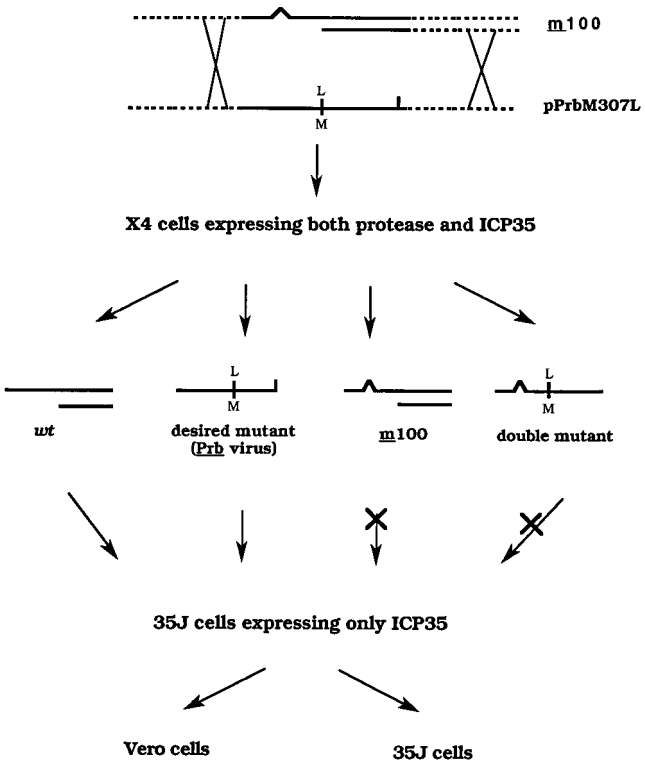

FIG. 2. Strategy used for isolation of mutant Prb virus. The Prb/M307L mutation was transferred to the viral genome accompanied by rescue of the *m*100 virus lesion. Plasmid pPrb/M307L was cotransfected with the infectious *m*100 viral genome into X4 cells. Viable progeny viruses, including parental *m*100 virus and recombinant virus due to homologous recombinations during cotransfection, were plated on 35J cells. Since 35J cells express ICP35 alone, only wt virus and the desired mutant Prb virus form plaques. Prb virus was further distinguished from wt virus by its failure to replicate efficiently on Vero cells.

Indirect immunofluorescence. Indirect immunofluorescence assays were performed as described previously (10). Primary antibodies, anti-ICP35 MAb MCA406 (1:50 dilution) and anti-VP5 MAb 8F5 (1:40 dilution) (42), were detected by using fluorescein-conjugated goat anti-mouse antibody (1:150 dilution).

RESULTS

The HSV-1 protease cleaves itself at the C-terminal site (maturation site) and also cleaves ICP35 at an identical site, releasing identical 25-aa peptides from both proteins. To determine whether these 25 aa play a role in capsid assembly, we constructed a mutant virus expressing only Prb, the protease without the C-terminal 25 aa (Fig. 1). Prior to construction of this mutant virus, we determined that Prb expressed from transfected cells retains all the functions of the protease by its ability to complement the growth of the protease deletion mutant *m*100 virus (11).

Strategy for isolation of mutant Prb virus. Since the protease and ICP35 are encoded by a single linear open reading frame, mutations in the ICP35 gene are normally present in the protease gene. We have developed a system to isolate and mutate each gene without altering the functions of the other. This ensured that the phenotype of the mutant reflects muta-

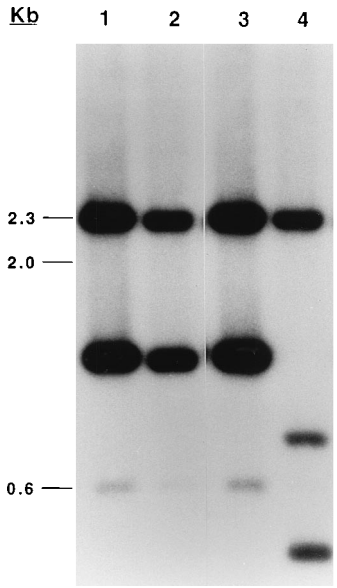

FIG. 3. Southern blot analysis of KOS1.1 and Prb viral genomes. Total DNAs from wt KOS1.1-infected cells (lanes 1 and 3) and Prb virus-infected cells (lanes 2 and 4) were digested with *Pst*I (lanes 1 and 2) and *Pst*I-*Eco*RI (lanes 3 and 4), respectively. Digested DNAs were separated on a 0.8% agarose gel, blotted onto a nylon membrane, and hybridized to ^{32}P-labeled plasmid pRB4057 (Fig. 1B). The locations of molecular size markers are shown on the left.

tions in the desired gene (9, 23). We recently reported the isolation of two cell lines, X4 and 35J, as well as a protease mutant virus, *m*100 (9, 23). X4 cells express both the HSV-1 protease and ICP35, while 35J cells express only ICP35 (9, 23). The protease deletion mutant *m*100 virus fails to produce the HSV-1 protease but does not alter expression of ICP35 (9). Along with this mutant virus, these cell lines provide a powerful genetic selection system for isolation of protease mutants. The procedure for isolation of mutant viruses involves transferring the protease mutation to the viral genome, accompanied by rescue of the *m*100 virus lesion. Figure 2 illustrates the strategy we used for isolation of Prb virus. Plasmid pPrb/M307L was cotransfected with the infectious mutant *m*100 viral genome into X4 cells, and progeny viruses were then plated on ICP35-expressing 35J cells. X4 cells support the growth of *m*100 virus and recombinants generated by homologous recombination from marker transfer. However, parental *m*100 virus, which represents the majority of progeny virus, should not grow on 35J cells. All surviving viruses should be recombinants, either Prb or wt virus. The Prb virus can be distinguished from wt virus by its ability to grow well on 35J cells, but not on Vero cells. Seventeen of 48 plaques picked from the marker transfer experiment displayed this mutant growth property. Two isolates were further plaque purified twice. Both mutant viruses were used for initial experiments and found to have the same phenotype. Further analyses were performed with one isolate, designated Prb virus.

Southern blotting analysis was performed to verify that the recombinant virus contained the appropriate mutation as a result of marker transfer from the pPrb/M307L plasmid (Fig. 3). Virus DNAs isolated from infected cells were digested with restriction enzymes and hybridized to ^{32}P-labeled plasmid pRB4057, which contains wt UL26 and UL26.5 genes. Both wt and Prb virus DNAs contained three identical *Pst*I fragments (2,242, 1,119, and 570 bp) (Fig. 3, lanes 1 and 2). A similar restriction enzyme digestion pattern was observed when wt virus DNA was digested with *Pst*I-*Eco*RI as there is no *Eco*RI site in this region (Fig. 3, lane 3). In contrast, the 1,119-bp fragment of Prb virus DNA was cleaved into 762- and 357-bp fragments by *Pst*I-*Eco*RI double digestion because of the extra *Eco*RI site that had been engineered after codon 610 (Fig. 3, lane 4). The 570-bp *Pst*I fragment was visible in all digests (Fig. 3, lanes 1 through 4) following longer exposure. We concluded, therefore, that the intended mutation had been introduced into the viral genome.

TABLE 1. Complementation of Prb virus by 35J cells

Virus	Titer (PFU/ml)[a]		Titer ratio[b]
	Vero	35J	
wt	9.8×10^8	6.2×10^8	1.6
Prb	$<2 \times 10^4$	1.2×10^9	$<1.7 \times 10^{-5}$

[a] Titers of virus stocks on the cell lines indicated were determined by plaque assay, and plaque numbers were counted 2 days p.i.
[b] Vero/35J.

Phenotypic characterization of mutant Prb virus. We reported previously that mutant ΔICP35 virus, expressing full-length Pra, formed extremely small plaques on nonpermissive Vero cells in the absence of ICP35 (23). To determine whether the C-terminal 25 aa of Pra in the absence of ICP35 are required for virus growth, the Prb virus was examined for its ability to grow on Vero cells and ICP35-expressing 35J cells. As shown in Table 1, Prb virus was unable to form plaques on Vero cells at the lowest dilution but grew efficiently on 35J cells. Therefore, deletion of the C-terminal 25 aa of Pra in the absence of ICP35 is lethal. To more quantitatively assess the growth defect of Prb virus, Vero and 35J cells were infected with wt or mutant virus at an MOI of 0.1 PFU per cell and harvested at various times up to 48 h postinfection (p.i.) (Fig. 4). Virus yield on 35J cells was determined by plaque assay. In this analysis, mutant ΔICP35 virus was used for direct comparison with Prb virus. As expected, both mutants yielded approximately wt levels of progeny virus when infections were carried out on 35J cells. In agreement with the results of our recent report, the growth of ΔICP35 virus on Vero cells was severely restricted, but a small amount of progeny virus was produced (23). In contrast, the amount of Prb virus produced in Vero cells was almost undetectable. These results demonstrate that the C-terminal 25 aa must be supplied from either Pra or ICP35 to support virus growth.

We next examined the expression and processing of protease-related polypeptides in Prb virus-infected cells. Vero and 35J cells were either mock infected or infected with virus, and cell extracts were prepared at 10 h p.i. Cell extracts were separated by SDS-PAGE and analyzed by Western blotting with a mouse MAb reactive with an epitope in ICP35 (5) (Fig. 5). Because the full-length protease, Pra, was produced in very small quantities and its autoproteolytic products are not always detected (7, 9, 23), Prb and Na were not apparent in wt-infected cells (Fig. 5, lanes 1 and 2). Prb was produced in Prb virus-infected Vero cells; it migrated slightly faster than Pra in wt-infected Vero cells (Fig. 5; compare lanes 2 and 4). Prb produced in mutant-infected Vero cells was processed at the N-terminal cleavage site, yielding Nb, but neither Na nor ICP35 was detected (Fig. 5; compare lanes 2 and 4). ICP35 c,d expressed in 35J cells was processed into ICP35 e,f following infection with mutant Prb virus (Fig. 5, lane 5). Since 35J cells expressed only ICP35, Pra and Na were not detected in Prb mutant virus-infected 35J cells (Fig. 5, lane 5). These results suggest that Prb retains all the activities of the protease.

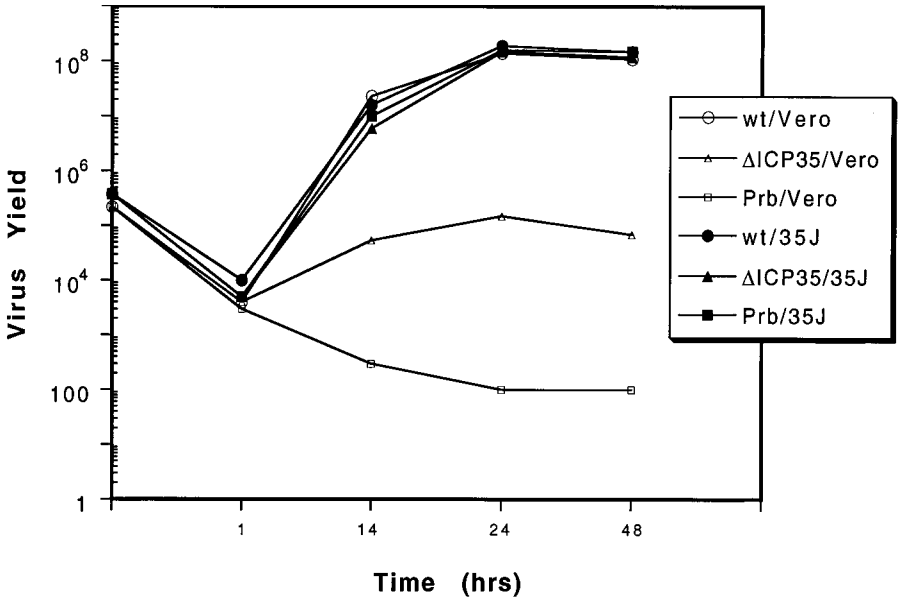

FIG. 4. Single-step growth analyses of wt, ΔICP35, and Prb viruses on Vero and 35J cells. Cells were infected at an MOI of 0.1 PFU per cell and harvested at various time points p.i. Progeny virus were titered on 35J cells. Cell numbers were determined by cell counts of a culture flask of each cell line prior to infection.

Growth of mutant Prb virus can be *trans* complemented by ICP35 c,d but not by ICP35 e,f. To identify functional regions of ICP35, we constructed several N- and C-terminal truncations in the ICP35 gene (Fig. 1). Previous attempts to examine the ability of truncated ICP35 molecules to complement the growth of ΔICP35 virus have not been conclusive because of the high background of ΔICP35 virus growth on Vero cells (11). The Prb virus allowed us to define the functional domain(s) of ICP35 since this mutant does not grow on Vero cells but retains all the protease functions necessary to support virus growth in the presence of ICP35. To determine whether any mutations in the ICP35 gene affect an essential function of ICP35, we examined (i) the expression and cellular distribution of mutant ICP35, (ii) the requirement of mutant ICP35 for proper conformation of capsid structures, and (iii) the ability of mutant ICP35 to complement the growth of Prb virus.

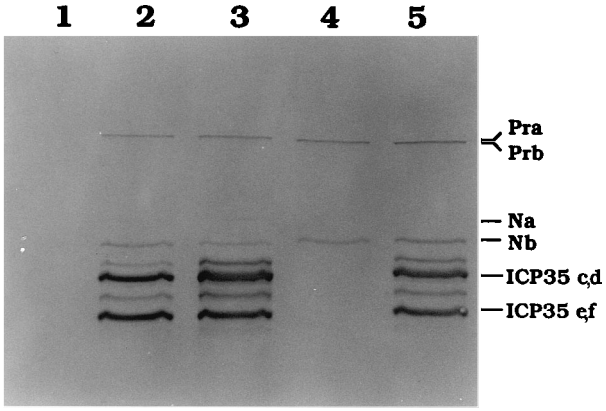

FIG. 5. Western blot analysis of HSV-1 protease-related polypeptides in wt- and Prb virus-infected cells. Vero (lanes 1, 2, and 4) and 35J cells (lanes 3 and 5) were mock infected (lane 1) or infected with wt (lanes 2 and 3) or Prb (lanes 4 and 5) virus. Total proteins were prepared at 10 h p.i., separated by SDS–12.5% PAGE, and transferred to a nitrocellulose filter. The filter was probed with a MAb, MCA406, specific for an epitope within ICP35. The positions of the indicated polypeptides are given on the right.

We first used immunofluorescence microscopy to examine the subcellular distribution of mutant ICP35 polypeptides expressed from plasmids in transfected Vero cells (Fig. 6) and to confirm that it was similar to that of wt ICP35. In agreement with the results of our previous report (9), wt ICP35 (ICP35 c,d) localized exclusively to the nucleus, not the nucleoli (Fig. 6A). Similarly, mutants ICP35 e,f (Fig. 6B) and *n*24 (which lacks the N-terminal 24-aa truncation [Fig. 1]) (results not shown) also localized to the cell nucleus. The numbers of immunofluorescence-positive cells expressing these plasmids in transfected cells were similar. Cells transfected with pSVc122 expressing ICP35 with a C-terminal 122-aa truncation were negative for antibody staining since the epitope recognized by MAb MCA406 is contained within the C-terminal 122 residues (11).

We previously observed the formation of capsid structures in both *m*100- and ΔICP35 virus-infected Vero cells. However, the assembly of hexons in these capsids was conformationally altered, as demonstrated by poor recognition by the VP5 hexon-specific MAb 8F5 (9, 23). Since Prb virus also lacked ICP35 expression, MAb 8F5 failed to recognize VP5 in Prb virus-infected Vero cells, as expected (results not shown). We also determined whether the detection of VP5 can be restored when wt and mutant ICP35 polypeptides are expressed from plasmids. Vero cells were transfected with ICP35 plasmids and then superinfected with Prb virus at 20 h posttransfection. An immunofluorescence assay was performed 6 h p.i. The reactivity of VP5 with MAb 8F5 was restored following expression of wt ICP35 c,d in Vero cells (Fig. 6C), suggesting that the assembly of VP5 into capsid structures was corrected when wt ICP35 c,d protein was provided in *trans*. However, no apparent positive immunofluorescence staining of capsids was observed when ICP35 e,f protein lacking the C-terminal 25 aa was expressed in Vero cells (Fig. 6D).

Because expression of a polypeptide from a transfected plasmid can also complement the growth of certain mutant viruses,

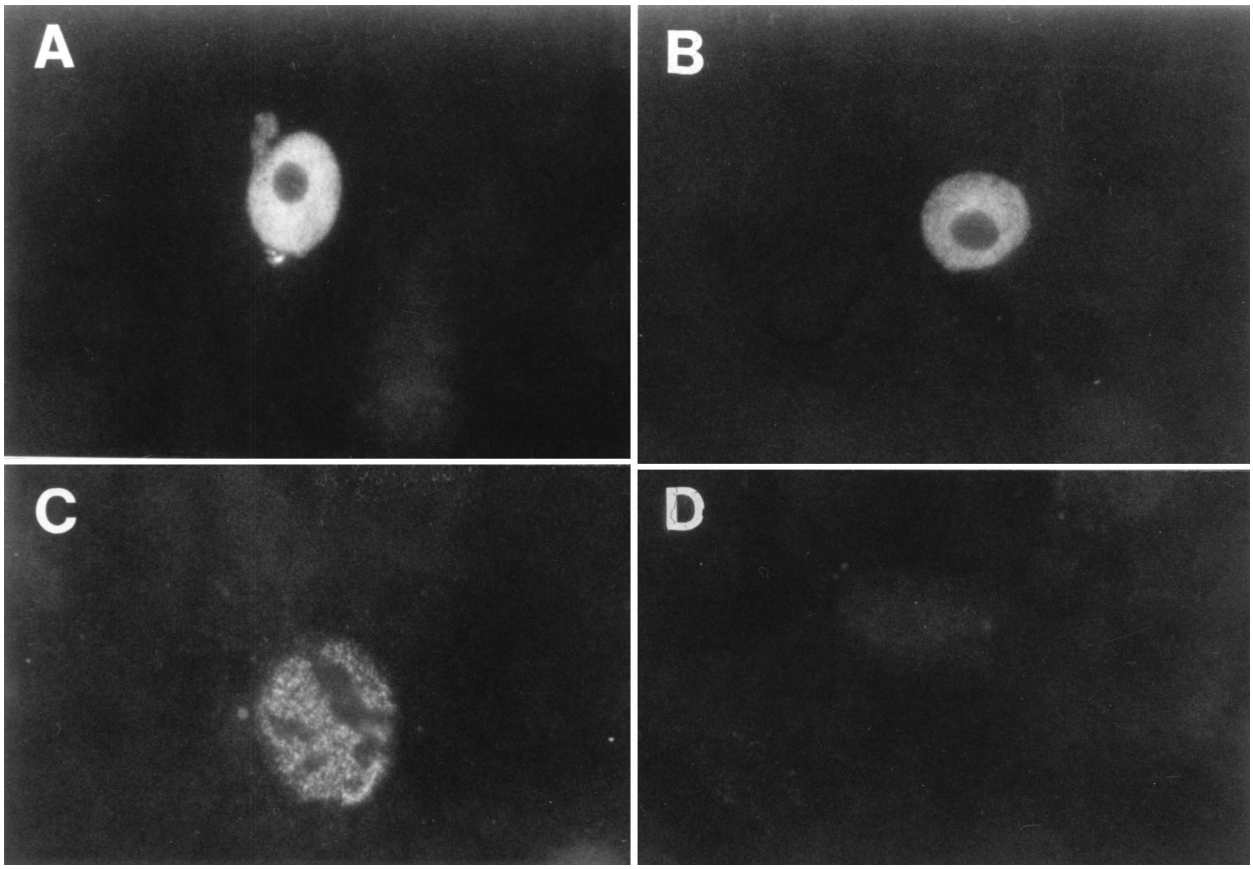

FIG. 6. Antibody recognition and subcellular distribution of ICP35 and VP5. Vero cells were transfected with pSVICP35 (wt) (A and C) or pSVICP35e,f (B and D). At 20 h posttransfection, cells were either processed for indirect immunofluorescence assay by using a MAb, MCA406, specific for ICP35 (A and B) or superinfected with Prb virus for 6 h and then processed for indirect immunofluorescence assay by using a MAb, 8F5, specific for VP5 (C and D).

including the HSV-1 protease mutant *ts*Prot.A (9), we determined whether any of our ICP35 mutants complemented the growth of Prb virus. Vero cells were transfected with vector DNA only or with wt or mutant ICP35 plasmids and then were superinfected with Prb virus 20 to 24 h after transfection. The yield of progeny virus on 35J cells was determined (Table 2).

TABLE 2. Abilities of mutant ICP35 plasmids to complement Prb virus

Plasmid transfected[a]	Expt no.	Virus yield (PFU/ml)[b]	Complementation index[c]
pUC18	1	1.6×10^3	1
	2	2.6×10^2	1
pSVICP35c,d	1	1.6×10^5	100
	2	2.0×10^4	77
pSVICP35e,f	1	3.0×10^3	1.9
	2	5.6×10^2	2.2
pSVn24	1	1.1×10^4	6.8
	2	1.4×10^3	5.4
pSVc122	1	1.0×10^3	0.6
	2	1.6×10^2	0.6

[a] Vero cells were transfected with the plasmid indicated. At 20 h posttransfection, cells were infected with 3 PFU of Prb virus per cell and incubated for a further 20 h before being harvested.
[b] Determined by plaque assay on 35J cells.
[c] Expressed as virus yield relative to transfection with pUC18 DNA.

As expected, transfection of the wt ICP35 c,d plasmid significantly increased the yield of mutant virus. The N-terminal truncation, ICP35n24, showed slight complementation of the growth of Prb virus (approximately 5 to 7% of the wt level), while two mutant ICP35s, ICP35 e,f and *c*122, inactivated an essential function(s) of ICP35. These results clearly demonstrated that lytic growth of HSV-1 requires the C-terminal 25 aa, either from the protease or from ICP35, but not necessarily from both.

Electron microscopic studies. To determine whether capsid structures were formed in Prb virus-infected Vero cells, thin sections of virus-infected cells were examined by electron microscopy. Vero and 35J cells were infected with wt or Prb virus, harvested, fixed with 2.5% glutaraldehyde at 16 h p.i., and prepared for electron microscopy as described in Materials and Methods. Cells infected with wt HSV-1 were found to contain the three expected capsid types, A, B, and C (Fig. 7A). No A-, B-, or C-like capsids were found in Prb virus-infected Vero cells; only aberrant capsids were observed. These structures were larger than wt B capsids (compare Fig. 7A and B) and very similar to those "sheet and spiral structures" of capsid shells observed in Vero cells infected with a mutant lacking both Pra and ICP35 (7). At a higher magnification, these aberrant capsids appeared to be unsealed (Fig. 7C, arrows). Therefore, we conclude that the C-terminal 25 aa of the protease and ICP35 are involved in the formation of sealed capsids.

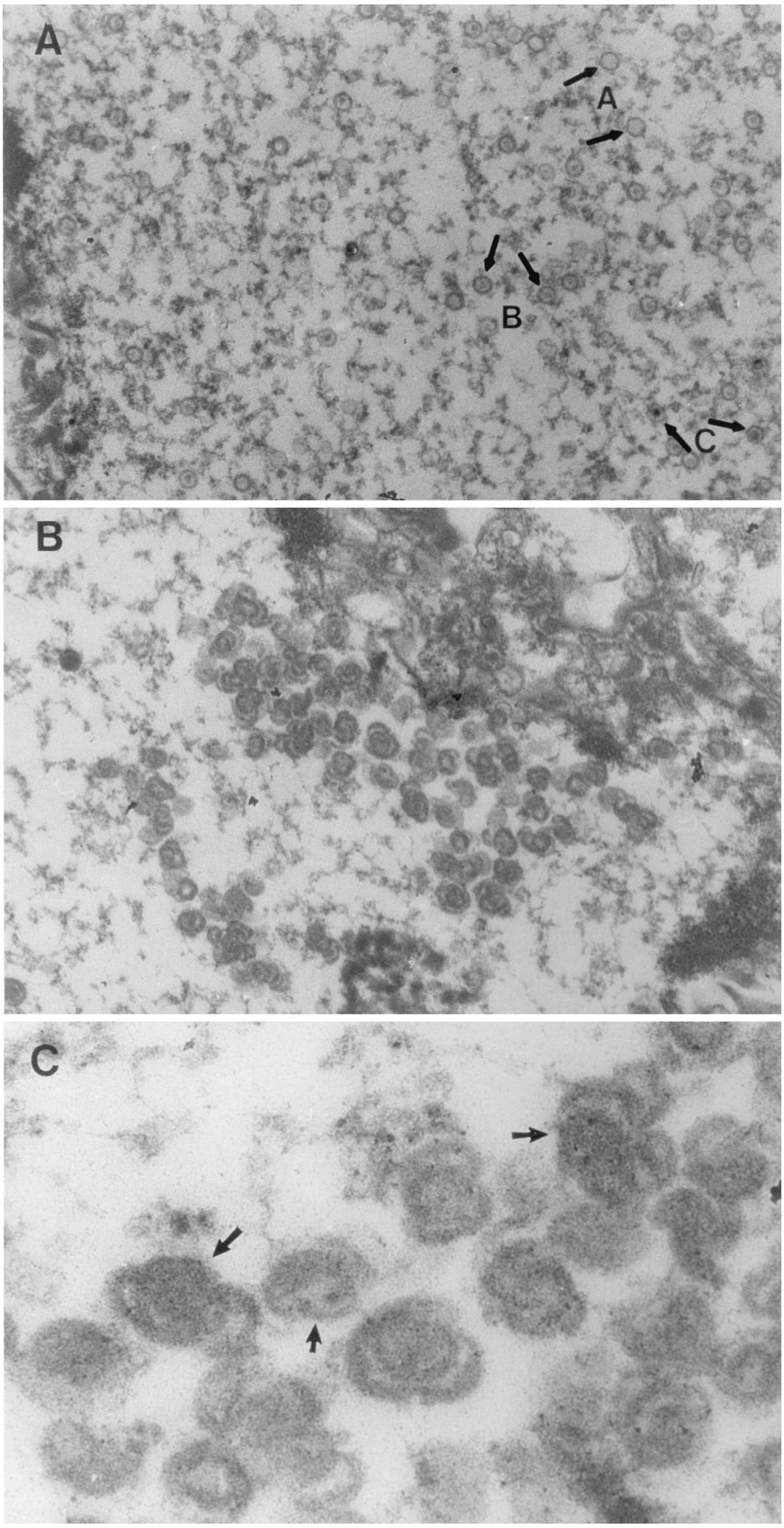

FIG. 7. Electron micrographs of thin sections of wt- and Prb virus-infected cells. Vero cells were infected with wt (A) or Prb virus (B and C) at an MOI of 10 PFU per cell. Cells were fixed and prepared at 16 h p.i., as described in Materials and Methods. Magnifications: ×32,500 (A and B) and ×107,250 (C).

DISCUSSION

Since both UL26 and UL26.5 gene products of HSV-1 are essential for efficient virus replication, it is necessary to isolate transformed cell lines which produce wt copies of UL26 and UL26.5 gene products for propagation of protease- and ICP35-deficient viruses (9, 23). However, the isolation of a mutant virus is still a time-consuming process because the recombinant virus usually represents less than 1% of total progeny after marker transfer. In-frame insertion of the *lacZ* gene into HSV-1 genes has proved to be a useful tool for the isolation of null mutants because recombinant viruses expressing β-galactosidase, the product of the *lacZ* gene, can be identified on the basis of their blue color in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (14). The ability to select for replication of only recombinant viruses, as described in this report, provides a powerful genetic system to rapidly discriminate between parental and mutant viruses. This was accomplished by rescuing the *m*100 virus lesion and simultaneously transferring the Prb mutation into the viral genome. In several similar experiments, we found that almost 100% of progeny viruses plated on 35J cells were recombinant, while the percentage of the desired mutant ranged from 5 to 30%. This genetic selection should be particularly useful for isolating *trans*-dominant mutants, such as cleavage site mutants, since the percentage of such mutants after marker transfer is usually extremely low (6). Since the open reading frames of Pra and ICP35 are colinear, one limitation for isolating ICP35 mutants in this particular procedure is that the mutation in ICP35 must not affect any function of the protease; otherwise, the mutant cannot grow on 35J cells. Desai et al. described a similar strategy for isolating gB mutant viruses (6).

Prb mutant virus was constructed with two goals in mind. The first was to gain insight into the role of the C-terminal 25 aa of Pra and ICP35. We have recently demonstrated that although ICP35 plays an important role during capsid assembly and virion maturation, it is not absolutely essential for virus replication (23). These findings raised an interesting question: why is the HSV-1 protease essential, while its substrate, ICP35, is not? There are two possible explanations for these results. One possibility is that another substrate for the HSV-1 protease (besides ICP35) is essential for virus growth. Another possibility is that Na, the C-terminal portion of the protease, functionally substitutes for ICP35. Several lines of evidence from our study and those of others strongly favor the latter hypothesis: (i) the C-terminal 25 aa of the protease are not required to support virus growth as long as ICP35 is provided, (ii) growth of ΔICP35 virus in protease-expressing MG22 cells is enhanced (data not shown), and (iii) the protease and ICP35 can substitute for each other in the formation of capsid structures in HSV-1 mutant-infected nonpermissive cells and in a recombinant baculovirus system (9, 23, 30, 40, 41). It should be pointed out that there is a fundamental difference between capsid assembly in HSV-1 mutant-infected cells and the recombinant baculovirus system. The amount of ICP35 produced in HSV-1-infected cells is approximately 10- to 15-fold greater than those of the protease and Na, while in a recombinant baculovirus system, similar quantities of ICP35, the protease, and Na are produced. Therefore, the quantity of protein may be a limiting factor for the compensatory function of Na for ICP35 in ΔICP35 virus-infected Vero cells.

The second goal in constructing mutant Prb virus was to create a virus that could be used to define the functional domains of ICP35. ΔICP35 mutant virus could not be used for this purpose because it formed small plaques on Vero cells; therefore, it was not possible to carry out *trans*-complementation experiments. Analysis of ICP35 function by *trans* complementation of Prb virus indicated that a small truncation from either the C or N terminus of ICP35 eliminated an essential function of this protein. This may not be surprising since the size and symmetry of B capsids are determined by ICP35 and Nb (7). In the case of lambda phage head assembly, the space occupied by scaffold protein determines the amount of virus DNA that can be packaged into capsids (4). Therefore, an alteration in the size of the scaffold may have drastic effects on virus replication. The coassembly of ICP35 molecules with other capsid proteins is the most likely means for ICP35 to give the correct size and shape to capsids. It has been reported that the protease in the presence of ICP35 stimulates the formation of scaffold-like structures (35). Chemical cross-linking of B capsids provided further evidence of physical interactions between ICP35 molecules and other capsid proteins (7). Both genetic and biochemical evidence suggests that ICP35 interacts with VP5 and VP19C (7, 23, 31). There appears to be a correlation between the conformational change of capsids and the function of ICP35. In all of the ICP35 mutants we examined, we found that if a mutant ICP35 can correct the capsid conformation in Prb virus-infected Vero cells, as determined by recognition with the VP5-specific MAb 8F5, it retains its ability to complement growth of the mutant virus. However, it is clear from our results that these essential functions of ICP35 can be genetically separated from the nuclear localization signal of ICP35, as both ICP35 e,f and *n*24, like wt ICP35 c,d, localized to the nucleus in transfected cells.

A striking characteristic of Prb virus is that following deletion of the C-terminal 25 aa of the protease in the absence of ICP35, only unsealed aberrant capsid structures are observed by electron microscopy. Mutation analyses of the protease and ICP35 have revealed three different phenotypes. (i) When both Pra and ICP35 have been deleted, only sheet and spiral structures, including VP5, VP19C, VP23, and VP26, or aberrant capsids are observed (7, 41). (ii) Results from our laboratory and others have demonstrated that sealed capsid structures are formed in the absence of Pra (N_0 and Na) or ICP35 (9, 23, 40, 41), suggesting that the integrity of capsid structures is maintained when either the protease or ICP35 has been omitted. (iii) Unsealed aberrant capsid structures are observed when the C-terminal 25 aa have been deleted from both proteins. Under an electron microscope, our unsealed capsids look similar to those sheet and spiral structures when both Pra and ICP35 have been omitted (7) and are larger than normal B capsids. The obvious explanation for these results is that the C-terminal 25 aa of Pra and ICP35 can functionally substitute for each other and are responsible for sealing capsids. It is important to point out that since ICP35 is not cleaved when Pra has been omitted, it is unlikely that processing of the 25 aa of ICP35 is required for these interactions; instead, the 25 aa themselves are critical for capsid closure (9).

It is particularly interesting to speculate about the potential functional roles of these 25 aa during the assembly of capsids. The C-terminal 25 aa are shared by both Pra and ICP35, but whether these proteins play the same role or different roles in capsid assembly is unknown. Although these residues apparently play an integral role in the assembly of capsids, our results did not provide any information on whether these 25 aa are required for the formation or maintenance of capsids. We and others have demonstrated that one function of ICP35 is to interact with the major capsid protein VP5 (23, 32). In ΔICP35 virus-infected Vero cells, VP5 lost its ability to localize into the nucleus and assemble into hexons (23). It is conceivable that the C-terminal 25 aa are responsible for these functions. In one scenario, ICP35 and VP5 interact in order to transport VP5 to

the nucleus. After nuclear transport, cleavage by the protease may cause a conformational change in ICP35, resulting in its release from VP5. This change may be required for the self-interaction of ICP35 e,f, which may also enable it to interact with a different domain of VP5 and/or other capsid proteins and serve as a scaffold. In this case, the C-terminal 25 aa are only required for the initiation of capsid formation.

Alternatively, the C-terminal 25 aa may be required for maintenance of capsids. The peptide may be retained not only in B capsids but also in mature virions after they have been cleaved from ICP35 c,d to serve as a "glue" which connects VP5 and other capsid proteins, such as VP19C, to the capsid shell. Direct examination for the presence of the C-terminal 25 aa in capsids may lend support to this hypothesis. However, preliminary attempts to demonstrate the presence of the 25-aa peptide in B capsids have been unsuccessful (29). It is important to point out that no matter how many proteins ICP35 e,f may interact with, these interactions are probably transient, since ICP35 e,f is not associated with capsids when mature virions are formed. On the other hand, interaction with the C-terminal 25 aa may be permanent, functioning as part of the capsid structure.

A required function for the formation of sealed capsids is retained within the C-terminal 25 aa of ICP35 and Pra. It is quite possible to manipulate these residues to define the precise role of each of these amino acids during capsid assembly. One genetic approach we are taking is to isolate substitution mutants within these C-terminal 25 aa and to subsequently select for second-site revertant mutants. Such revertants can be extremely helpful in defining intramolecular protein-protein interactions and elucidating the role of these C-terminal 25 aa in the process of virion maturation.

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