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Na, an Autoproteolytic Product of the Herpes Simplex Virus Type 1 Protease, Can Functionally Substitute for the Assembly Protein ICP35

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The herpes simplex virus type 1 (HSV-1) protease and its substrate, the assembly protein ICP35, are involved in virion maturation. Both proteins are encoded by a single open reading frame but are translated independently from 3′-coterminal mRNAs of different sizes and are in frame. The herpesvirus shell assembles around an internal scaffold which is subsequently lost during packaging of the viral genome. The scaffold is composed of ICP35, which is the major component, and autoproteolytically processed forms of the viral protease containing sequences common to ICP35 (Nb). In the baculovirus system, HSV-1 intact capsids can be formed in the presence of the protease or ICP35, indicating that the protease may substitute for ICP35 (Thomsen et al., J. Virol. 68:2442–2457, 1994). This is further supported by the fact that ICP35, in contrast to the protease, is not absolutely essential for viral growth. The processed intermediate of the protease analogous to ICP35 is the 388-amino-acid (aa) protein, Na, which is an N-terminal 59-aa extension of the 329-aa ICP35. To directly examine whether Na can functionally substitute for ICP35 during viral replication, we first constructed a mutant virus, *NaΔ35*, in which 35 aa from the N terminus of Na were deleted. Phenotypic analysis of the mutant showed that this deletion had no effect on protease function. The function of Na was further examined by construction of a plasmid expressing Na alone and testing its ability to complement the growth of the mutant *Prb* virus in the absence of ICP35. Our results demonstrate that Na can functionally substitute for ICP35 during viral replication.

The herpes simplex virus type 1 (HSV-1) protease and ICP35, encoded by the UL26 and UL26.5 genes, respectively (15, 22), are involved in capsid assembly and virion maturation (2, 10–12, 26, 28). Both genes have their own promoters and are translated independently from 3′-coterminal mRNAs of different sizes (15). The UL26 gene encodes 635 amino acids (aa), and UL26.5 encodes 329 aa, initiating at Met-307 of the UL26 amino acid sequence. Genetic and functional analyses demonstrated that UL26 encodes a protease, designated Pra, and UL26.5 encodes ICP35, which shares amino acid sequence identity with the C-terminal 329 aa of Pra (14, 15). Pra undergoes auto-processing at two sites, the R and M sites, generating the catalytic domain N_o, Nb, and a C-terminal 25-aa peptide (Fig. 1) (8, 15–17, 25, 33). Auto-processing of Pra at only the R site generates Na, an N-terminal 59-aa extension of ICP35. The HSV-1 protease is also responsible for the processing of ICP35 c,d to ICP35 e,f (Fig. 1).

The HSV-1 protease is a serine protease, and its active site has been mapped to residue Ser-129 (8, 16). Its requirement for proper capsid assembly confirmed that the protease is essential for viral replication (10). Although the full-length protease Pra, the catalytic domain N_o, and the R and M cleavage site mutant proteases are proteolytically active in the absence of capsid structures (1, 5, 8, 15, 17, 21, 27, 30, 31, 34, 35), our recent data suggested that formation of functional capsids capable of packaging viral DNA requires proteolytic cleavage events which occur during or after capsid assembly (29).

It has been postulated that ICP35 provides a function analogous to that of the scaffold protein of double-stranded DNA bacteriophage T4 because of its transient association with capsids: the processed forms of ICP35 (ICP35 e,f) and the protease (N_o and Nb) are present in B capsids but absent in the DNA-containing mature virions (3, 11, 12). However, the function of Na in capsid assembly is unclear. Based on the fact that capsid structures were observed when either the protease or ICP35 was omitted but not when both were omitted (6, 7, 10, 18, 30, 31), we and others have postulated that Na and ICP35 can functionally substitute for each other under certain conditions (18, 31). This hypothesis was further supported by our recent finding that in the absence of the ICP35 but in the presence of full-length protease (Pra), the mutant virus (Δ*ICP35*) survives (18).

In this report, we directly address the question of whether or not Na can substitute for ICP35. We first constructed a mutant virus, *NaΔ35*, in which 35 aa of the N terminus was deleted from Na, and also directly examined whether Na, expressed from transfected cells, was able to complement the growth of the mutant *Prb* virus in the absence of ICP35. Our results demonstrate that Na can functionally substitute for ICP35 during viral replication.

Phenotype of the mutant *NaΔ35* virus. (i) Construction of the mutant *NaΔ35* virus. To determine whether the N-terminal 59 aa of Na contribute to any functions of the HSV-1 protease, we constructed a mutant *NaΔ35* virus (Fig. 1). Two considerations were taken into account during construction of this mutant virus. First, the minimal length of a peptide substrate for the enzyme is 13 aa (P5/P8′) (4, 9), indicating that an

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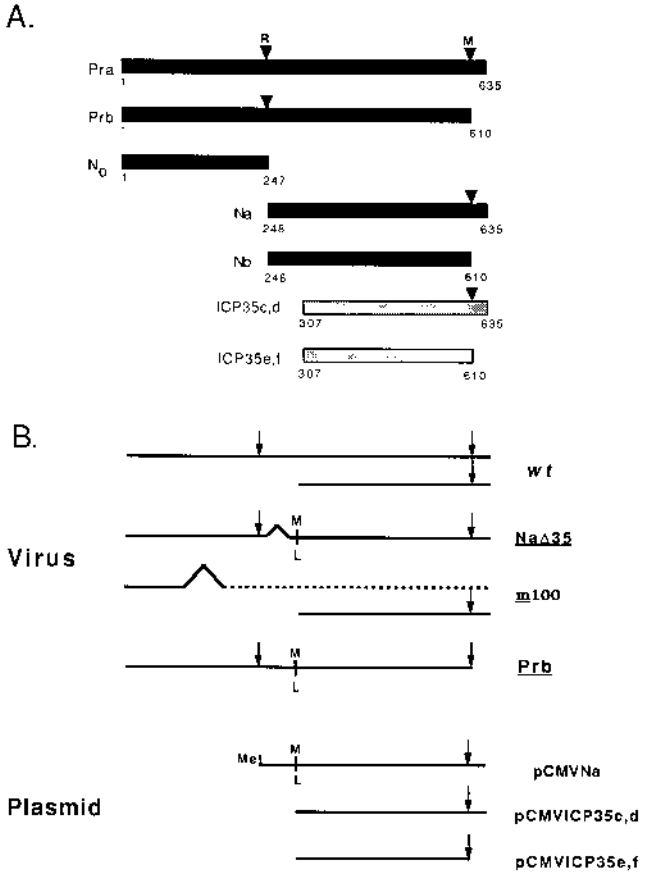

FIG. 1. (A) Polypeptide products of UL26 and UL26.5 ORFs. The HSV-1 protease (Pra), substrate (ICP35 c,d), and cleavage products Prb, N_o (VP24), Na, Nb (VP21), and ICP35 e,f (VP22a) are described in the text. The cleavage sites (R and M sites) of Pra, Prb, Na, and ICP35 c,d are indicated by arrows. The UL26 amino acid numbers of the N and C termini of each protein are indicated. (B) The open reading frames of HSV-1 wt and mutant viruses as well as plasmids used for this study. Arrows indicate cleavage sites. The dashed line indicates a frameshift in the ORF of the catalytic domain of the protease rendering it inactive while leaving the ICP35 ORF unaffected. M over L indicates that the methionine at 307 (start codon for ICP35) is changed to leucine. The ATG start codon and M307L mutation were engineered in the coding sequence of Na in the plasmid pIM19 (13). Plasmids pCMVICP35 c,d, and pCMVICP35 e,f, were constructed by replacing the *Xba*I/*Kpn*I sites of pcDNA3.1- (Invitrogen Inc. San Diego, Calif.) with the 1,050-bp *Xba*I/*Kpn*I fragments of pSVICP35 c,d and pSVICP35 e,f (24), respectively. pCMVNa was constructed by replacing the *Bam*HI/*Hin*dIII sites of pcDNA3.1- with the 1,195-bp *Bgl*II/*Hin*dIII fragment of pSVNa. pSVNa was constructed by ligating the *Bgl*II fragment encoding Na (pre-VP21) (13) into the *Bam*HI/*Bgl*II sites of pJ3Ω. The mutation corresponding to the Met-307-to-Leu change in pCMVNa was confirmed by DNA sequencing.

extended recognition sequence may be required for efficient release of N_o. Since release of N_o is essential for viral replication (13), the entire 59 aa from the N terminus of Na could not be deleted. We, therefore, decided to make our Na deletion as close to the initiation codon of ICP35 as possible. Secondly, our previous results demonstrated that the Met-to-Leu change at residue 307 of the protease open reading frame (ORF) eliminated the synthesis of ICP35 but did not affect any protease functions (10). This mutation is necessary for the *NaΔ35* virus, so that the function of truncated Na expressed from the mutant virus can be examined in the absence of ICP35. A mutation bearing a deletion from aa 269 to 303 as well as a Met-to-Leu change at the residue 307 of the protease was introduced into the HSV-1 genome by marker transfer (Fig. 1).

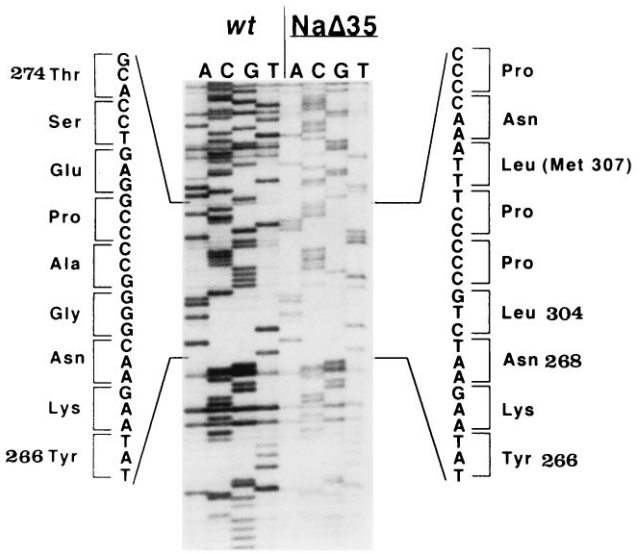

FIG. 2. DNA sequencing of the NaΔ35 mutation. The *Sph*I-*Pst*I fragment representing HSV-1 base pairs from 50042 to 52274 of the mutant *NaΔ35* viral DNA was cloned and the expected mutation region was sequenced. The plasmid BR4057 (20) encoding wt protease and ICP35 was used for comparison. The analysis revealed that DNA sequences corresponding to amino acid residues 269 to 303 of the protease were deleted from the mutant viral DNA and that Met-307 had been changed to Leu.

The mutant was plaque purified and propagated in ICP35-expressing 35J cells (18).

To verify the genetic structure of the recombinant virus, the *Sph*I-*Pst*I fragment representing HSV-1 from bp 50042 to 52274 of the mutant *NaΔ35* viral DNA was cloned and the expected mutation region was sequenced. Sequence analysis demonstrated that DNA sequences corresponding to amino acid residues 269 to 303 of the protease were deleted from the mutant viral DNA and that Met-307 had been changed to Leu (Fig. 2).

(ii) Na expressed from *NaΔ35*. To confirm that the mutant *NaΔ35* virus expressed the predicated mutant Na polypeptide, extracts of mutant- and wild-type (wt)-infected cells were examined by Western blots (Fig. 3). Since the amino acid sequence of ICP35 is identical to the C-terminal region of the protease, the ICP35-specific monoclonal antibody MCA406 also reacted with several autoprocessed protease products. In wt-infected cells, ICP35 c,d was *trans*-cleaved into ICP35 e,f by the protease (Fig. 3, lanes 1 and 2). In this particular experiment, except for full-length protease Pra, other autoproteolytic products were not apparent because the amount of the pro-

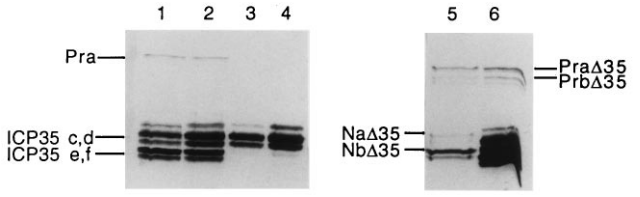

FIG. 3. Western blot analysis of HSV-1 protease-related polypeptides in wt (lanes 1 and 2), *m*100 (lanes 3 and 4), or *NaΔ35*-infected cells (lanes 5 and 6). Vero cells (lanes 1, 3, and 5) or 35J cells (lanes 2, 4, and 6) were infected at a multiplicity of infection of 5 PFU/cell and harvested at 16 h postinfection. Polypeptides from infected cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with the monoclonal antibody MCA406, specific for ICP35.

TABLE 1. Growth of wt and mutant viruses on Vero and 35J cells[a]

Virus and cell types	Yield (PFU/cell) from titration in:	
	35J	Vero[b]
wt (KOS1.1)		
Vero	85	180
35J	150	95
Prb		
Vero	0.0005	<0.0005
35J	160	<0.005
Δ*ICP35*		
Vero	0.65	<0.0005
35J	105	0.005
*Na*Δ*35*		
Vero	0.95	<0.0005
35J	135	<0.005

[a] Vero and 35J cells were infected with viruses at a multiplicity of infection of 2, incubated at 37°C for 18 h, and harvested. Titers of progeny viruses were determined in Vero and 35J cells.

[b] Mutant viruses Δ*ICP35* and *Na*Δ*35* formed very small plaques on Vero cells. To determine the level of reversion, only large plaques were counted.

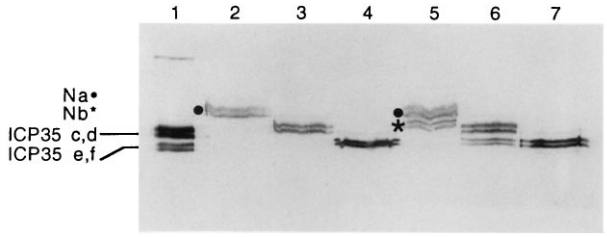

FIG. 4. Expression and processing of Na in transfected cells. Vero cells were transfected with plasmids encoding Na (lane 2), ICP35 c,d (lane 3), ICP35 e,f (lane 4), Na plus N_o (lane 5), ICP35 c,d plus N_o (lane 6), and ICP35 e,f plus N_o (lane 7). Polypeptides were separated by a polyacrylamide gel from cells at 24 h posttransfection, transferred to a nitrocellulose membrane, and reacted with monoclonal antibody MCA406 specific for ICP35. wt virus-infected Vero cell lysate was used as a control (lane 1).

tease produced in HSV-1-infected cells was much less than that of ICP35 (10, 23, 27). The protease deletion mutant *m*100 was used as a negative control. In *m*100-infected Vero and ICP35-expressing 35J cells, ICP35 c,d expressed from the mutant virus or the cell line was not *trans*-cleaved into ICP35 e,f (Fig. 3, lanes 3 and 4). To more clearly visualize protein products in *Na*Δ*35*-infected cells, lanes 5 and 6 (Fig. 3) were separated from the rest of the Western blot membrane and allowed to develop for an extended period. In *Na*Δ*35*-infected Vero cells, as expected, ICP35 was not produced because of the Met-to-Leu change at the initiation codon (Fig. 3, lane 5). However, truncated forms of mutant Pra, Prb, Na, and Nb were detected. In *Na*Δ*35*-infected 35J cells, ICP35 c,d expressed from the cell line was *trans*-cleaved into ICP35 e,f (Fig. 3, lane 6). We, therefore, conclude that the mutant protease bearing a deletion from aa 269 to 303 and a Met-to-Leu change at aa 307 retains both the auto-proteolytic and *trans*-proteolytic activities.

Growth properties of *Na*Δ*35*. We previously reported that the growth of the ICP35 deletion mutant Δ*ICP35* in Vero cells was severely restricted (18). Similarly, when the growth characteristics of the mutant *Na*Δ*35* were examined in Vero cells, it was found that the mutant virus was able to form plaques, but they were much smaller than those of wt virus, indicative of reduced viral yield (results not shown). To quantify the growth deficiency of *Na*Δ*35*, both Vero and 35J cells were infected with wt or *Na*Δ*35* at a multiplicity of infection of 2 PFU per cell, and viral yields were determined by plaque assay on 35J cells. The growth of the mutant virus Δ*ICP35* was directly compared with that of *Na*Δ*35*. The *Prb* mutant virus (20) was used as a negative control for viral replication in Vero cells. As shown in Table 1, all viruses grew well in 35J cells, but the *Prb* virus did not replicate in Vero cells at the lowest dilution tested. Similar to Δ*ICP35*, the yield of *Na*Δ*35*-infected Vero cells was almost 100-fold lower than that of wt virus and this growth defect was fully compensated for when ICP35 was supplied in *trans* (20) (Table 1, in *Na*Δ*35*- or Δ*ICP35*-infected 35J cells). Since the yield of *Na*Δ*35* virus in 35J cells was comparable to those of wt, Prb, or Δ*ICP35* virus, it suggests that deletion of 35 aa from the full length protease does not exhibit a *trans*-dominant phenotype. Our results demonstrate that the truncated form of Na expressed from *Na*Δ*35* virus retains all essential functions of wt Na.

Na can functionally substitute for ICP35. The growth properties of *Na*Δ*35* strongly suggest that Na may functionally substitute for ICP35 during viral replication. To more directly address this question, we constructed a plasmid expressing Na alone under the control of the human cytomegalovirus immediate-early promoter, and we examined whether Na expressed in transfected Vero cells could support the growth of the *Prb* virus (Fig. 1B). For the same reason we constructed a Met-307-to-Leu change in *Na*Δ*35*, the initiation codon Met-307 of ICP35 was changed to Leu in the Na construct (pCMVNa, Fig. 1B). In the *Prb* virus, in addition to the Met-307-to-Leu change, a stop codon was engineered at position 611, eliminating the synthesis of the C-terminal 25 aa of the protease (Fig. 1B) (20). This mutant does not grow in Vero cells and requires an ICP35-expressing cell line 35J for its propagation.

We first examined the expression of Na from transfected cells. The plasmid encoding Na (pCMVNa) was transfected into Vero cells, and the lysate was prepared two days after transfection and then analyzed by Western blots (Fig. 4, lane 2). Plasmids encoding ICP35 c,d and ICP35 e,f were used as controls (Fig. 4, lanes 3 and 4). All three polypeptides were expressed at the expected size when compared to those from the extract of wt virus-infected cells (Fig. 4, lane 1). To determine whether Na could be processed to Nb, the plasmid pCMVNa was cotransfected with the catalytic domain N_o (pSVN$_o$) (10). As expected, Na was *trans*-cleaved into Nb and as a control ICP35 c,d was *trans*-cleaved into ICP35 e,f (Fig. 4, lanes 5 and 6).

Prior to *trans*-complementation of *Prb* virus by Na, we first examined the nuclear localization of Na by indirect immunofluorescence. Na, like ICP35 (10, 24), exclusively localized to the nucleus when expressed in transfected cells (results not shown). *trans*-Complementation experiments were then performed to determine whether Na can complement the defect of the *Prb* virus. Plasmids encoding ICP35 c,d and ICP35 e,f were used as positive and negative controls, respectively. As shown in Table 2, Na, like ICP35 c,d, could sufficiently complement the growth of the mutant virus *Prb*. Similar results were also obtained when Na, ICP35 c,d, and ICP35 e,f were expressed from the simian virus 40 early promoter (data not shown). The high background of viral yield in pUC18 control transfection in experiment 2 of Table 2 was apparently due to virions that were not removed by low pH treatment after a 1-h absorption. Infected cell lysates from the same complementation experiments were also examined for wt recombinants by looking for the ability of the mutant virus to form plaques in

TABLE 2. Ability of Na plasmids to complement *Prb* virus

Plasmid transfected[a] and expt no.	Virus yield (PFU/ml)[b] from titration in		Complementation index[c]
	35J	Vero	
pUC18			
1	2.0×10^1	<20	1
2	1.1×10^4	<20	1
pCMVICP35 c,d			
1	8.2×10^5	<20	41,000
2	1.4×10^6	<20	127
pCMVICP35 e,f			
1	3.0×10^1	<20	1.5
2	1.2×10^4	<20	1.1
pCMVNa			
1	3.2×10^5	<20	16,000
2	4.6×10^5	<20	42

[a] Vero cells were transfected with the plasmids indicated. At 20 h posttransfection the 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.

[c] Expressed as virus yield in 35J cells relative to transfection with pUC18 DNA.

Vero cells. The titers of viruses in Vero cells from these experiments were not detectable at the lowest dilution tested (Table 2). We, therefore, conclude that the enhancement of growth of the mutant virus *Prb* by Na and ICP35 c,d was not due to recombination and that Na can functionally substitute for ICP35 during viral replication.

Since ICP35 shares amino acid sequence identity with the C-terminal 329 aa of Pra, it is not surprising that Na can functionally substitute for ICP35 during capsid assembly (18, 31) and viral growth. However, if the protease (Pra) possesses all the necessary functions of the assembly protein ICP35, why does HSV-1 produce ICP35 and why is virus growth severely restricted when ICP35 is deleted (18)? There are several key points we would like to address in order to answer these questions. First, the amount of the protease produced during HSV-1 infection is much less than that of ICP35. Although we do not know whether the auto-processing of the protease occurs in *cis* or in *trans*, our recent results clearly demonstrated that *trans*-cleavage of the protease could lead to the assembly of functional capsids and the production of infectious virus (29). It is conceivable that if large amounts of the protease were produced, it might dramatically increase the *trans*- and auto-processing before the enzymes reach the capsids. This "premature cleavage" could abort the production of infectious virus. Secondly, the fact that Na substitutes for ICP35 does not necessarily mean that the full-length protease can substitute for ICP35. In fact, the R site mutant, *A247S*, which expresses only full-length protease, does not support viral replication, suggesting that Na only substitutes for ICP35 after its release from the full-length protease (20). Therefore, we believe that one function of Na, as a part of the full-length protease, is to ensure that the catalytic domain of the protease and the substrate ICP35 localize to the specific site of capsid assembly. Several lines of evidence support this hypothesis. One is that the full-length protease, like ICP35, is predominantly localized in the nucleus, while the catalytic domain N_o shows equal intensity of cytoplasmic and nuclear distributions, suggesting that the function of Na, at least in part, is to direct N_o to the nucleus (10). Another is that the C-terminal 25 aa of ICP35 interact with VP5 and are involved in the formation of sealed capsids (13, 20, 32). It is quite possible that the identical 25 aa of the protease has the same function and facilitates localization of these capsid proteins to the site where assembly occurs. Thirdly, the N-terminal 59 aa of Na, which is absent in ICP35, most likely functions as a tether between the nuclear localization signal of ICP35 and the catalytic domain of the protease, N_o. It may also serve as a spacer providing access to the R site for proteolysis, since our previous results demonstrated that release of N_o is required for proper capsid assembly (19). Additionally, the DNA sequence encoding these 59 aa also serves as the promoter for expression of ICP35 (14).

We have demonstrated that Na can functionally replace ICP35 during viral growth. During wt HSV-1 infection the protease and ICP35 are regulated and translated separately. This may be necessary since only uncleaved ICP35 is capable of participating in capsid assembly (13, 32). Due to its close proximity to the catalytic domain of the protease, Na might be prematurely processed and therefore unable to participate in functional capsid assembly. Consequently, although they may be able to serve a similar function, efficient viral replication requires regulated expression of ICP35 and Na. It may also be possible that the amount of Na produced during infection is the determinant of its ability to substitute for ICP35.

A question that remains to be answered in this study is whether the size and symmetry of B capsids is altered when ICP35 is replaced with Na. The experiments performed above do not properly address this question because the amount of Na expressed from transfected cells is insufficient for capsid analysis. Isolation of a cell line expressing Na should provide an answer to this question.

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