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Autoproteolysis of Herpes Simplex Virus Type 1 Protease Releases an Active Catalytic Domain Found in Intermediate Capsid Particles

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The UL26 gene of herpes simplex virus type 1 (HSV-1) encodes a 635-amino-acid protease that cleaves itself and the HSV-1 assembly protein ICP35cd (F. Liu and B. Roizman, J. Virol. 65:5149–5156, 1991). We previously examined the HSV protease by using an *Escherichia coli* expression system (I. C. Deckman, M. Hagen, and P. J. McCann III, J. Virol. 66:7362–7367, 1992) and identified two autoproteolytic cleavage sites between residues 247 and 248 and residues 610 and 611 of UL26 (C. L. DiIanni, D. A. Drier, I. C. Deckman, P. J. McCann III, F. Liu, B. Roizman, R. J. Colonno, and M. G. Cordingley, J. Biol. Chem. 268:2048–2051, 1993). In this study, a series of C-terminal truncations of the UL26 open reading frame was tested for cleavage activity in *E. coli*. Our results delimit the catalytic domain of the protease to the N-terminal 247 amino acids of UL26 corresponding to N_o, the amino-terminal product of protease autoprocessing. Autoprocessing of the full-length protease was found to be unnecessary for catalysis, since elimination of either or both cleavage sites by site-directed mutagenesis fails to prevent cleavage of ICP35cd or an unaltered protease autoprocessing site. Catalytic activity of the 247-amino-acid protease domain was confirmed in vitro by using a glutathione-*S*-transferase fusion protein. The fusion protease was induced to high levels of expression, affinity purified, and used to cleave purified ICP35cd in vitro, indicating that no other proteins are required. By using a set of domain-specific antisera, all of the HSV-1 protease cleavage products predicted from studies in *E. coli* were identified in HSV-1-infected cells. At least two protease autoprocessing products, in addition to fully processed ICP35cd (ICP35ef), were associated with intermediate B capsids in the nucleus of infected cells, suggesting a key role for proteolytic maturation of the protease and ICP35cd in HSV-1 capsid assembly.

Herpes simplex virus type 1 (HSV-1) is an enveloped double-stranded DNA virus with an icosahedral capsid that is assembled in the nucleus of infected cells. Herpesvirus capsid assembly occurs through the formation of capsid intermediates, B capsids, which accumulate in the nucleus prior to viral DNA encapsidation (10, 17). The interior of HSV-1 B capsids is occupied by a proteolytically processed form of ICP35 (infected cell protein 35) (15, 20). This protein, designated ICP35ef (3), is present in B capsids prior to encapsidation of the HSV-1 chromosome but is absent after DNA encapsidation is completed (8). This scenario is reminiscent of the involvement of bacteriophage scaffold proteins in phage prohead assembly and their removal upon DNA packaging and head maturation (4). HSV-1 also encodes the protease responsible for maturational cleavage of ICP35. In addition to cleavage of ICP35, the HSV protease carries out at least two autoproteolytic cleavages (6, 12). Although a functional protease gene product is required for HSV-1 replication (18), the role of ICP35 cleavage or autoproteolysis in HSV-1 capsid assembly has not been determined.

The protease and ICP35 are both encoded by the UL26 open reading frame but are translated independently from 3′-coterminal mRNAs of different sizes (11). HSV-1 protease is the product of complete translation of the 635-amino-acid open reading frame and is designated Pra (Fig. 1). Translation of ICP35 initiates at Met-307 of the predicted UL26 amino acid sequence (UL26.5) and thus shares sequence identity with the carboxyl-terminal 329 amino acids of Pra (11) (Fig. 1). Antibodies against ICP35 detect a family of proteins, designated ICP35a through ICP35f, in Western immunoblots (Western blots) of HSV-1-infected cell lysates (3). The 329-amino-acid full-length ICP35 protein corresponds to ICP35cd, a doublet of approximately 40 kDa in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). ICP35cd is cleaved by the HSV-1 protease at a site near its C terminus to yield a slightly smaller doublet, ICP35ef (12) (Fig. 1), which is the fully processed form of ICP35 found in B capsids (3, 8, 15, 20). The ICP35 proteins are modified by phosphorylation, and the formation of doublets (cd and ef) in SDS-PAGE might be due to differential posttranslational modifications (3).

In addition to processing ICP35cd, Pra cleaves itself autoproteolytically at two sites (6, 12). One site near its C terminus is identical to the C-terminal cleavage site of ICP35cd. Cleavage of Pra at this site gives rise to a slightly smaller protein designated Prb (12) (Fig. 1). Using an *Escherichia coli* expression system, Deckman and colleagues discovered a second autoproteolytic cleavage site within Pra that is N terminal to the initiation codon of ICP35cd (6) (Fig. 1). Direct amino acid sequencing of autoproteolytic cleavage products purified from *E. coli* revealed that the scissile bonds in Pra occurred between Ala-Ser dipeptide sequences located at positions Ala-247/Ser-248 and Ala-610/Ser-611 of UL26 (7). Cleavage at Ala-247/Ser-248 was evidenced by the accumulation of Na and its C-terminally cleaved counter-

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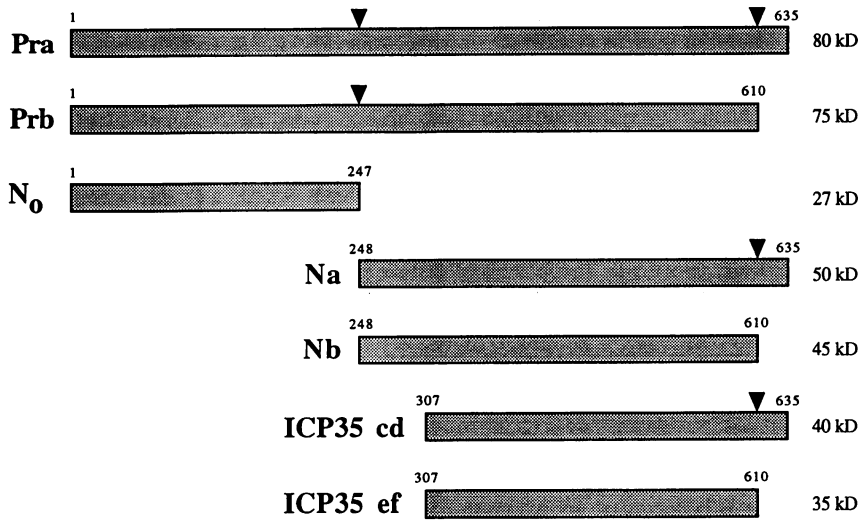

FIG. 1. HSV-1 protease processing in *E. coli*. The genesis of HSV-1 protease (Pra), substrate (ICP35cd), and cleavage products (Prb, N_o, Na, Nb, and ICP35ef) is described in the text. HSV-1 protease cleavage sites are indicated with arrowheads. The UL26 amino acid numbers of amino and carboxyl termini of each protein are indicated. Estimated molecular sizes are shown at the right.

part, Nb, in an HSV-1 protease-dependent manner (6) (Fig. 1). Na and Nb are both recognized by ICP35 antisera and are the C-terminal products of cleavage at amino acids 247 and 248 (Fig. 1). Na and Nb are similar in size and immunoreactivity to ICP35a and ICP35b, two members of the HSV-1 ICP35 family of proteins that migrate more slowly than full-length ICP35cd in SDS-PAGE (3).

Cleavage at Ala-247/Ser-248 of Pra is also predicted to yield a 247-amino-acid N-terminal cleavage product, referred to as N_o (Fig. 1), but the accumulation of N_o has not been demonstrated in *E. coli* or during HSV-1 infection. Nevertheless, the N-terminal 247 amino acids of UL26 were recently shown to direct proteolytic cleavage in transfected tissue culture cells (14). Simian cytomegalovirus (SCMV) encodes a protease that is homologous to Pra and carries out analogous N-terminal and C-terminal autoproteolytic cleavages. The SCMV N-terminal cleavage product analogous to N_o has been identified and was found to retain protease activity when expressed independently in the presence of substrate (23). Similar results have been demonstrated for human cytomegalovirus (HCMV) (1). It is likely, therefore, that N_o is an active form of the HSV-1 protease as well. A recent study has shown that VP24, a protein associated with B capsids, contains fragments with amino acid sequence identity to UL26 (upstream of Ala-247) and migrates in SDS-PAGE with the predicted size of N_o (5). The association of cleavage products of the protease (VP24) and ICP35cd (ICP35ef) with B capsids provides a link between HSV protease activity and capsid assembly. It has been suggested further that the B-capsid protein VP21 might be a slightly larger form of ICP35cd, perhaps Na or Nb (5), indicating that a third product of cleavage by the HSV-1 protease might also be involved in capsid assembly.

In this study, we mapped the catalytic domain of the HSV-1 protease in *E. coli* by using a set of C-terminal truncations of the protease gene. A specific antiserum was used to identify the catalytic domain of the protease, N_o, in *E. coli*, and site-directed mutagenesis of the cleavage sites demonstrated that autoprocessing of Pra is unnecessary for protease activity in *E. coli*. The 247-amino-acid catalytic domain was overexpressed as a fusion protein in *E. coli* and found to be active in vitro for the cleavage of ICP35cd. Finally, a panel of domain-specific antibodies against the HSV-1 protease and ICP35 was used to identify predicted cleavage products in HSV-1-infected cell extracts and in isolated B capsids. Our results show that in addition to ICP35ef, at least two autoprocessing products of the HSV-1 protease, including one corresponding to the catalytic domain, are associated with B capsids.

MATERIALS AND METHODS

Construction of plasmid mutants. Protease expression plasmids are designated by the convention pT7*nnn*Y, where *nnn* is the protein length in amino acids and Y indicates the antibiotic resistance. Point mutants are designated pT7X*nnn*Z, where X indicates the wild-type amino acid, *nnn* is the position of that amino acid, and Z is the mutant amino acid. pT7635K was constructed by ligating the 1,964-bp *Nco*I-to-*Eco*RI fragment of pT7635A (6) into the 584-bp *Nco*I-to-*Eco*RI deletion of pET9d (Novagen). pT7635C was constructed by ligating the 2,328-bp *Bgl*II-to-*Hin*dIII fragment of pT7635K into the 2,226-bp *Bcl*I-to-*Hin*dIII deletion of pLysS (21). Protease truncations were derived from pT7HSV (6). pT7193A was constructed by digestion of pT7HSV (pT7635A) with *Eco*RI and *Sac*I, treatment with mung bean nuclease, and self-ligation. pT7218C was constructed by digestion of pT7635C with *Hin*dIII, treatment with Klenow fragment, digestion with *Hpa*I, and self-ligation. pT7243C and pT7247C were constructed by polymerase chain reaction mutagenesis of pT7635C. pT7289A and pT7330A were constructed by linker insertion of two in-frame stop codons at the *Bst*EII and *Bam*HI sites, respectively, of pT7635A. pT7419K was constructed by digestion of pT7635K with *Drd*I, blunting, digestion with *Nco*I, and recloning of the resulting 1,252-bp fragment. pT7489K was constructed by linker insertion of two in-frame stop codons at the *Pst*I site of pT7635K. pT7513A was constructed by inserting the 1,986-bp *Xba*I-to-*Kpn*I fragment of pRB4217 (13) into *Xba*I-plus-*Kpn*I-treated pT7635A vector. pT7562A was constructed by digesting pT7635K with *Nco*I plus *Pvu*II and recloning the resulting 1,680-bp fragment into *Eco*RI

(blunt ended)-plus-*Nco*I-treated pT7635A. pT7ICP35A and pT7H148A were described previously (6). Point mutants pT7A247S and pT7A610S were made by polymerase chain reaction mutagenesis. Protease mutants were expressed alone and coexpressed with ICP35cd in *E. coli* BL21(DE3), using the system of Studier et al. (21), by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma).

Western immunoblot analysis. Antibodies for Western blots included a mouse monoclonal antibody (MAb) (MCA406; Bioproducts for Science-Serotec) reactive with sequences upstream of the C-terminal cleavage site of ICP35cd (ICP35 MAb) and a rabbit polyclonal antiserum raised against a synthetic peptide of the C-terminal 14 amino acids of UL26 (C-term pAb [6]). A polyclonal rabbit antiserum was raised against the glutathione-*S*-transferase (GST)-N_o fusion protein described in this report and cleared of GST-reactive antibodies by incubation with affinity-purified GST immobilized on nitrocellulose sheets (N-term rAb). Western blots were prepared by using standard procedures for SDS-PAGE and electrotransfer to nitrocellulose (9). The nitrocellulose filters were blocked with 5% nonfat milk (Carnation) in 25 mM Tris (pH 8.0)–50 mM NaCl–2.5 mM KCl prior to incubation with specific antisera. All Western blots were developed with alkaline phosphatase-conjugated swine anti-rabbit or rabbit anti-mouse secondary antibodies (DAKO Corp.).

Fusion protein expression and preparation. pGST247 was constructed by digestion of pT7247C with *Nco*I, treatment with Klenow fragment to fill in the overhang, and *Eco*RI digestion. The resulting insert was cloned into pGEX-2T (Pharmacia) treated with *Sma*I plus *Eco*RI. To confirm protease activity, pGST247 was coexpressed with pT7ICP35C in *E. coli* BL21(DE3) by induction with IPTG. pGST247 was overexpressed in *E. coli* BL21 for affinity purification.

Induced cells were resuspended in 50 mM Tris (pH 8.0)–2 mM dithiothreitol (DTT)–5 mM EDTA–25 mM NaCl–1 mg of lysozyme per ml–1% Triton X-100, incubated at room temperature for 5 min, and then placed on ice and lysed by sonication. The lysate was further treated in a Dounce homogenizer with 10 strokes of a B pestle and centrifuged at 23,000 × *g* for 30 min at 4°C in a Sorvall SS-34 rotor. This supernatant (SUP1) was used directly for affinity purification. The pellet (PEL1) was resuspended in 50 mM Tris (pH 8.0)–4 M urea–0.5 M NaCl–0.1 mM EDTA–1 mM DTT and treated again by Dounce homogenization and centrifugation. The supernatant (SUP2) was dialyzed against buffer A (50 mM Tris [pH 8.0], 0.1 mM EDTA, 1 mM DTT, 25 mM NaCl) and centrifuged at 18,500 × *g* for 5 min. This final supernatant (SUP3) was also used for affinity purification. All protein determinations were done by the method of Bradford (2) (Bio-Rad).

Affinity purification and thrombin cleavage. Glutathione-agarose beads (Sigma G4510) were equilibrated and washed in buffer A. Soluble protease fractions were bound to the beads by rocking for 15 min at 4°C. Bound fusion protease was separated by centrifugation at 400 × *g* for 5 min at 4°C and removal of the supernatant. Bound resin was washed four times with 5 to 10 bed volumes of buffer A. Fusion protease was eluted by resuspending the resin in 2 bed volumes of 50 mM Tris (pH 8.0)–5 mM reduced glutathione (Sigma G-4251). Eluted material was collected in the supernatant after centrifugation at 400 × *g* for 10 min. The elution step was repeated up to six times to ensure complete elution. Pooled eluates containing the fusion protease were dialyzed against 50 mM Tris (pH 8.0)–100 mM NaCl–0.1 mM EDTA–1 mM DTT–50% glycerol. Affinity-purified material was adjusted to 50 mM Tris (pH 8.6) and 12% glycerol by dialysis and incubated with human thrombin (10 U/mg of fusion protein; Sigma T-3010) for 1.5 h at 30°C to release N_o from GST.

ICP35cd purification and in vitro cleavage. Recombinant ICP35cd was purified from *E. coli* BL21(DE3) transformed with pT7ICP35cdC induced with 0.5 mM IPTG. The presence of ICP35cd was monitored during purification by Western blot analysis with the ICP35 MAb. Cells were resuspended in lysis buffer, incubated at room temperature with rocking for 5 min, and lysed by sonication on ice as described above. Debris was pelleted by centrifugation at 23,000 × *g* for 30 min at 4°C. The soluble supernatant fraction was adjusted to 25% ammonium sulfate, incubated on ice for 1 to 2 h, and centrifuged at 23,000 × *g* for 30 min at 4°C. The pellet was resuspended in one-half the lysis volume of a solution containing 20 mM Tris (pH 8.0), 0.1 mM EDTA, 1 mM DTT, and 25 mM NaCl and then adjusted to 1.7 M KCl. After being stirred for 1 to 2 h at 4°C, this material was centrifuged at 60,000 × *g* for 30 min, and the pellet was resuspended in 20 mM Tris (pH 8.0)–0.1 mM EDTA–1 mM DTT. This resuspended material was fractionated over DEAE-Sepharose, using a 0 to 1 M NaCl gradient elution. Fractions containing ICP35cd were pooled and dialyzed against 50 mM Tris (pH 8.0)–0.1 mM EDTA–1 mM DTT–10% glycerol and stored at −80°C. Cleavage of ICP35cd was carried out by incubation with GST or GST-N_o in 50 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.5)–1 mM DTT–0.1 mM EDTA–50 mM NaCl–100 μg of bovine serum albumin (BSA) per ml at 30°C.

Western blots and pulse-chase immunoprecipitation of HSV-1-infected cell proteins. BHK-21 cells were infected with 10 PFU of HSV-1(KOS) per cell. Cells were harvested at various times postinfection directly into SDS-PAGE sample buffer for analysis by Western blotting. For immunoprecipitation of radiolabeled proteins, BHK-21 cells grown in six-well clusters were infected with 10 PFU of HSV-1(Schooler) or HSV-2(Curtis) per cell or were mock infected. At 5 h postinfection, cells were incubated in methionine-free medium (Sigma M7270; supplemented with leucine and lysine) for 15 min prior to the addition of 75 μCi of [^{35}S]methionine (NEG-009T; DuPont/NEN) per ml. Cells were pulse-labeled for 15 min and then washed and chased in minimal essential medium (Earle's salts; GIBCO) plus 0.1 mM methionine (1,000×) for up to 4 h. Time point samples were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM $NaPO_4$ [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS). Lysates were clarified by centrifugation for 5 min at 15,000 × *g* at 4°C and then mixed 20:1 with MAb MCA 406 (anti-ICP35cd; Serotec) or a MAb raised against purified HSV N_o (6a). These mixtures were rocked at 4°C for 1 h, and then 20 μl of a 25% slurry of protein A–Sepharose CL-4B (Pharmacia) in RIPA buffer was added. These mixtures were rotated for 1 h at 4°C; then the Sepharose beads were pelleted by brief centrifugation and washed three times in RIPA buffer. After the final wash, the beads were resuspended in sample buffer and bound proteins were resolved by SDS-PAGE.

Sucrose density gradients. Vero cells were infected with 5 PFU of HSV-1(KOS) per cell for 18 h prior to the preparation of B capsids. Cells were resuspended in 10 mM Tris (pH 7.4)–0.1 mM EDTA–1 mM DTT, incubated on ice for 10 min, and disrupted with a Dounce homogenizer. Nuclei were pelleted by centrifugation at 1,000 × *g* for 10 min. The pellet

was resuspended in 10 mM Tris (pH 7.5)–1 mM EDTA–1 mM DTT and adjusted to 10% glycerol plus 1% Triton X-100. This suspension was frozen (dry ice) and thawed (37°C) three times with vigorous mixing. After centrifugation at 1,000 × *g*, the supernatant fraction was loaded on top of a 20 to 50% sucrose gradient developed in 10 mM Tris (pH 7.5)–100 mM NaCl–0.1 mM EDTA. Gradients were centrifuged for 1 h at 25,000 × *g* and fractionated with an Autodensiflow II (Buchler Instruments). B capsids were identified by the formation of a light-scattering band that corresponded to gradient fractions enriched for HSV-1 capsid proteins (data not shown). DNA was prepared for analysis by incubating aliquots of each fraction in 220 μl of 0.5% SDS–50 μg of proteinase K per ml for 1 h at 65°C. The DNA was denatured by addition of 5 μl of 10 M NaOH to each aliquot on ice. Samples were bound to GeneScreen (DuPont) by placing them in the wells of a Minifold II slot blot apparatus for 30 min prior to the application of vacuum to the apparatus. Slot blots were hybridized with a mixture of isolated HSV-1 *Eco*RI fragments (H, I, and J) radiolabeled by nick translation (19).

RESULTS

C-terminal truncation of the UL26 gene product. To define the smallest active domain of the HSV-1 protease, the 635-amino-acid UL26 gene product was truncated at its C terminus. The activities of several truncations were assessed by coexpression with ICP35cd substrate in an *E. coli* coexpression system (6). Substrate cleavage products were visualized by Western blot analysis of bacterial lysates with a mouse MAb immunoreactive with ICP35. These results showed that truncated proteins retaining the N-terminal 562, 513, 489, 419, and 247 amino acids of UL26 were capable of cleaving ICP35cd. Truncations retaining 330, 289, 243, 218, and 193 amino acids showed no proteolytic activity (Fig. 2A). The expression of each truncated protein was confirmed by replicate Western blots that were probed with a rabbit polyclonal antiserum raised against the N-terminal 247 amino acids of UL26 (N-term rAb; Materials and Methods). Proteins of the predicted molecular weight were detected in each case (Fig. 2B). Each of the active truncation mutants produced a second immunoreactive protein that comigrated with the protein expressed by a truncation at amino acid 247. This protein, designated N_o, corresponds to the predicted N-terminal product of autoprocessing at the Ala-247/Ser-248 cleavage site (Fig. 2B). This autoprocessing event releases an active catalytic domain, since the 247-amino-acid truncation was active for cleavage of ICP35cd. The 330- and 289-amino-acid truncations not only failed to cleave ICP35cd but also failed to autoprocess and release N_o (Fig. 2B). Since these truncations contained an intact catalytic domain, it is possible that they were misfolded during expression in *E. coli*. Alternatively, if cleavage of ICP35cd is carried out only by N_o, then autoprocessing would be required prior to cleavage of ICP35cd. Selective interference with this autoproteolytic cleavage site in the 330- and 289-amino-acid polypeptides might, therefore, prevent cleavage of ICP35cd.

Elimination of HSV-1 protease autoprocessing sites by site-directed mutagenesis. To determine whether autoprocessing of Pra plays any role in regulating the activity of the HSV-1 protease, specific amino acid substitutions were made at the P-1 position of both cleavage sites by site-directed mutagenesis. Cleavage activities of the resulting mutants were examined by expression in *E. coli*. Substitution of Ser for Ala-247 drastically reduced autoprocessing at the N-terminal cleavage site, as indicated by the absence of Na and Nb after several hours of induction (Fig. 3A and B). The Ser-247 mutant cleaved its unaltered C-terminal cleavage site, however, as shown by the efficient conversion of Pra to Prb, and it cleaved ICP35cd in the coexpression assay (Fig. 3B). Normal cleavage of these alternative sites indicated that the Ser-247 mutation did not have a deleterious effect on catalytic activity of the protease. A similar mutagenesis strategy was used to substitute Ser for Ala-610. This resulted in the formation of a nonfunctional C-terminal cleavage site, since Prb and Nb both failed to accumulate after several hours of induction (Fig. 3A and B). The Ser-610 mutant cleaved its unaltered N-terminal cleavage site efficiently, however, as shown by the accumulation of Na, and it cleaved ICP35cd efficiently in the coexpression assay (Fig. 3B). Thus, protease activity was not affected by elimination of the C-terminal cleavage site. A double mutant in which cleavage at both autoprocessing sites was eliminated (Ser-247 plus Ser-610) was also active for cleavage of ICP35cd (Fig. 3C). These results indicated that Pra is an active form of the protease

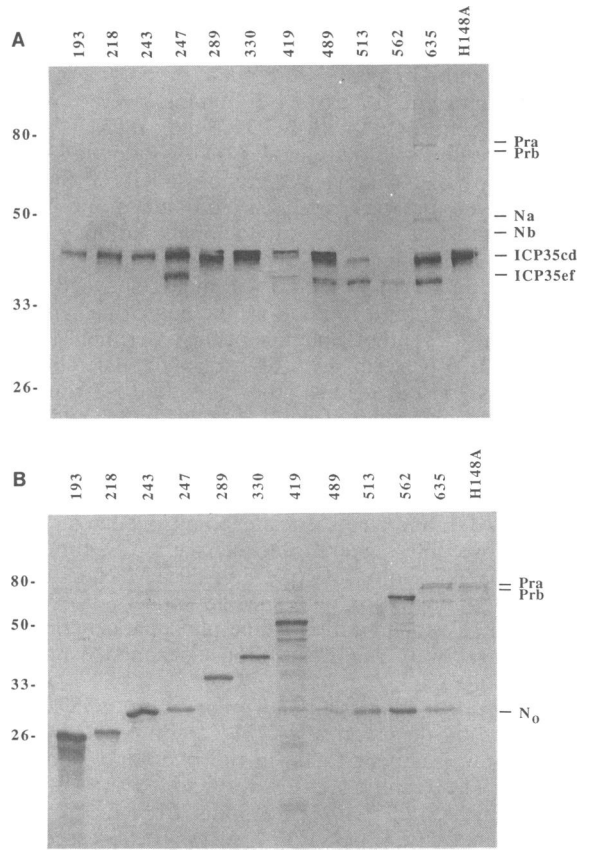

FIG. 2. (A) Cleavage by C-terminal truncations coexpressed with ICP35cd. Shown are Western blots of induced *E. coli* probed with ICP35 MAb. Protease truncations were coexpressed with ICP35cd for 5 h. The size of each mutant protease in numbers of amino acids is indicated above each lane. Positions of migration of ICP35cd substrate and cleaved ICP35ef are indicated at the right. Control lanes include wild-type, full-length protease (635) and an inactive point mutant, pT7H148A (H148A [6, 13]). Positions of molecular weight markers are in kilodaltons at the left. (B) Coexpressed C-terminal truncations probed with antiserum reactive with N_o. Shown are replicate Western blots (as described above) probed with UL26 N-term rAb.

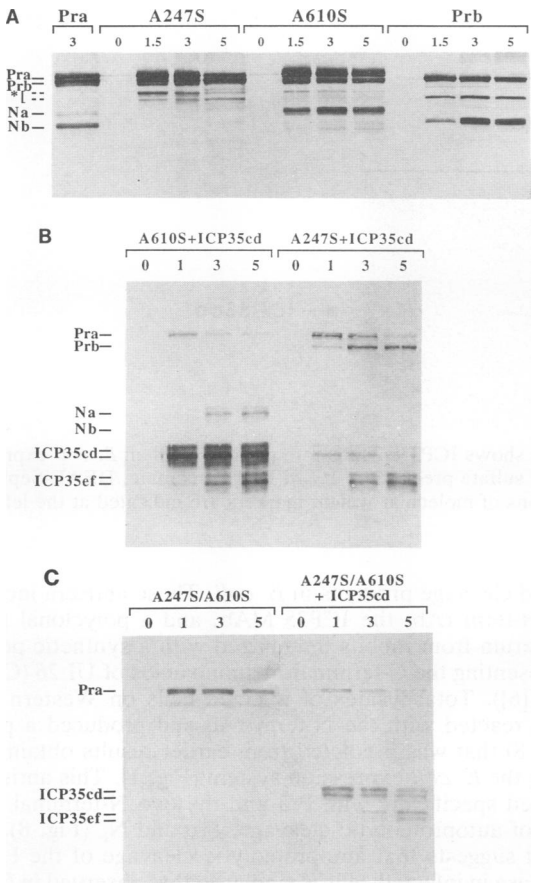

FIG. 3. (A) Autoprocessing by P-1 mutants. Shown are Western blots of induced cultures with single amino acid substitutions introduced at the P-1 position of the N-terminal (A247S) and C-terminal (A610S) autoprocessing sites or a stop codon after A610 (Prb). Cultures were induced for 0, 1.5, 3, and 5 h, and blots were probed with ICP35 MAb. A control sample is full-length, wild-type protease (Pra; 3-h time point). Positions of migration of Pra, Prb, Na, and Nb are shown at the left. Products of cryptic cleavage are indicated with an asterisk. (B) P-1 mutants coexpressed with ICP35cd. Shown are Western blots of P-1 mutants coexpressed with ICP35cd for 0, 1, 3, and 5 h. Blots were probed with ICP35 MAb. Cleavage of ICP35cd is indicated by the accumulation of ICP35ef (location shown at the left). (C) Double-cleavage-site mutant. Shown are Western blots of the double-cleavage-site mutant, A247S/A610S, expressed alone (left) or coexpressed with ICP35cd (right). Cleavage of ICP35cd is indicated by the accumulation of ICP35ef (location shown at the left).

and that there is no requirement in *E. coli* for cleavage of the full-length HSV-1 protease to activate the enzyme.

In addition to the efficient cleavage of their normal substrates, the two point mutants, Ser-247 and Ser-610, also displayed a notable alteration in their autoprocessing patterns. This was the accumulation of two novel immunoreactive species migrating between the positions of Prb and Na in SDS-PAGE (Ser-247; Fig. 3A). The size of these proteins suggests a cleavage within the central 50 amino acids of the protease catalytic domain. In the case of Ser-610, only the larger of these species accumulated significantly, suggesting that the smaller species results from proteolysis at the C-terminal cleavage site (Fig. 3A). Consistent with this conclusion, a single species produced by a 610-amino-acid

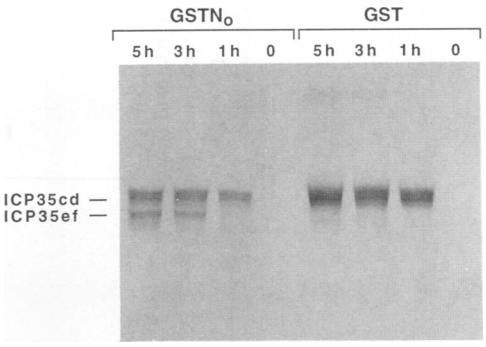

FIG. 4. Cleavage by GST-N_o fusion protease. Shown are Western blots of *E. coli* induced to coexpress GST-N_o fusion protein or GST with ICP35cd substrate. Cultures were induced for 0, 1, 3, and 5 h; blots were probed with ICP35 MAb. Specific cleavage of ICP35cd is indicated by the accumulation of ICP35ef.

truncation mutant (Prb) corresponds to the lower of the two novel species (Fig. 3A). Very low levels of accumulation of these species are occasionally detected upon expression of the wild-type protease alone, and coexpression of the mutants with ICP35cd caused a reduction of cleavage activity at the novel cleavage site (Fig. 3B). These findings suggest that the elimination of a preferred cleavage site in the P-1 mutants unmasked a cryptic cleavage site within the wild-type protease.

Overexpression, affinity purification, and in vitro activity of a protease fusion protein. Expression of truncated protease molecules proved that N_o, the N-terminal 247 amino acids of Pra, is required for HSV-1 protease activity in *E. coli*. To determine whether N_o alone is sufficient for catalytic activity in vitro, we employed a strategy for the overexpression and affinity purification of N_o with use of a GST fusion protein. The 247-amino-acid catalytic domain was fused at its N terminus to GST (GST-N_o), and the catalytic activity of GST-N_o was initially confirmed by coexpression with ICP35cd in *E. coli* (Fig. 4). Inducible, high-level expression of GST-N_o was observed in *E. coli* (strain BL21), but the overexpressed enzyme was largely insoluble. Some GST-N_o was reproducibly isolated in the soluble fraction (Fig. 5, lane 2), and this soluble GST-N_o was affinity purified in batch, using glutathione-agarose beads (Fig. 5, lane 3). We found that a portion of the insoluble material could be resolubilized

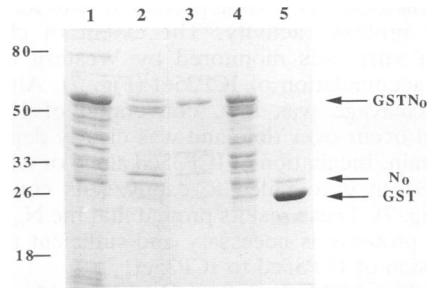

FIG. 5. Affinity purification of GST-N_o and thrombin digestion. The Coomassie blue-stained gel shows fractions from affinity purification of GST-N_o. Lanes: 1, induced culture, total lysate; 2 and 3, comparable amounts of SUP1 and SUP3 (see Materials and Methods) from the same preparation; 4 and 5, comparable amounts of concentrated SUP3 before (lane 4) and after (lane 5) treatment with thrombin.

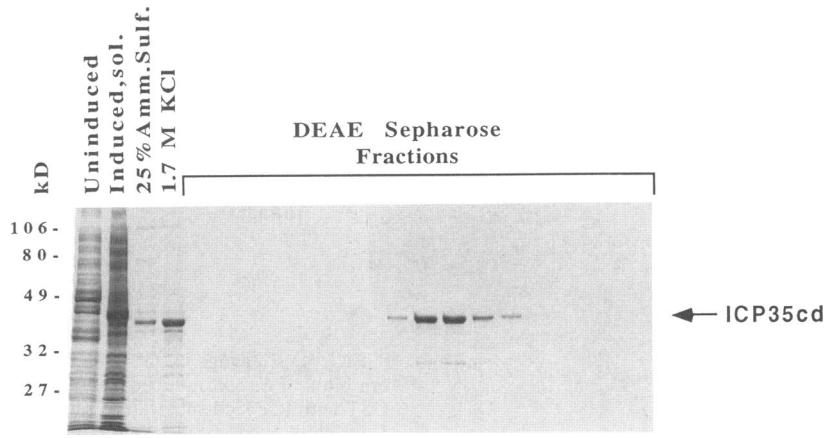

FIG. 6. ICP35cd purification from *E. coli*. The Coomassie blue-stained gel shows ICP35cd substrate purified from an *E. coli* expression clone. Left to right: uninduced cells, induced cells (soluble), 25% ammonium sulfate precipitate, 1.7 M KCl precipitate, DEAE-Sepharose column fractions. The location of ICP35cd is indicated with an arrow. Positions of molecular weight markers are indicated at the left.

by treatment with 4 M urea and could then be affinity purified after a dialysis step (Materials and Methods; Fig. 5, lane 4). A fraction of the affinity-purified material was intact GST-N_o, as judged by Coomassie staining, but the bulk of this soluble material appeared to be smaller species that copurified with intact GST-N_o on glutathione agarose (Fig. 5, lanes 3 and 4). These species were also present in cells prior to lysis of the culture, as determined by Western immunoblotting (data not shown), suggesting that the fusion protein is either unstable or poorly translated in *E. coli*. A thrombin cleavage site in the linker region between GST and N_o (GEX-2T; Pharmacia) was used to demonstrate the integrity of full-length N_o in the fusion protein. The thrombin digestion products of SUP3 contained full-length N_o that was detectable by Coomassie staining and greater, nonstoichiometric amounts of GST (Fig. 5, lane 5). Thus, the smaller species described above that copurified with intact GST-N_o over glutathione-agarose retained an intact GST domain and must, therefore, be truncated within the protease domain. The affinity-purified fusion protein preparation was dialyzed without further treatment and tested for protease activity in vitro.

A substrate for the in vitro assay of HSV-1 protease was prepared by purification of ICP35cd from *E. coli* BL21(DE3) transformed with pT7ICP35A (Fig. 6; Materials and Methods). Purified ICP35cd substrate was incubated with the affinity-purified GST-N_o fusion protein in vitro for up to 20 h to test for protease activity. The extent of cleavage of ICP35cd in vitro was monitored by Western blotting to detect the accumulation of ICP35ef (Fig. 7). Although the extent of cleavage was low, conversion of ICP35cd to ICP35ef did occur over time and was clearly dependent on the N_o domain. Incubation of ICP35cd alone or with affinity-purified GST in vitro failed to catalyze its conversion to ICP35ef (Fig. 7). These results proved that the N_o domain of the HSV-1 protease is necessary and sufficient to catalyze the conversion of ICP35cd to ICP35ef.

Identification of HSV-1 protease cleavage products in HSV-1-infected cells. Up to this point, our initial characterization of UL26 proteins and their cleavage products was carried out in *E. coli*. To confirm the relevance of these results to HSV-1 infection, Western blot and pulse-chase analyses were carried out in HSV-1-infected tissue culture cells with three domain-specific antisera that recognize all of the predicted cleavage products in *E. coli*. These antisera included the N-term rAb, the ICP35 MAb, and a polyclonal rabbit antiserum from rabbits immunized with a synthetic peptide representing the C-terminal 14 amino acids of UL26 (C-term pAb [6]). Total lysates of infected cells on Western blots were reacted with the N-term rAb and produced a profile (Fig. 8) that was predicted from earlier results obtained by using the *E. coli* expression system (Fig. 1). This antiserum reacted specifically with Pra and the two N-terminal products of autoproteolytic cleavage, Prb and N_o (Fig. 8). This result suggests that autoproteolytic cleavage of the HSV-1 protease in infected cells is similar to that observed in *E. coli* and that Prb and N_o are both stable during viral infection. Time course experiments in HSV-1-infected Vero cells revealed that the greatest accumulation of N_o occurred at very late times of infection (18 to 24 h; data not shown). A mouse MAb raised against HSV N_o (Materials and Methods) was used in an immunoprecipitation reaction with lysates of HSV-1- and HSV-2-infected cells that were pulse-labeled with [^{35}S]methionine at 5 h postinfection (Materials and Methods). The major radiolabeled protein specific to HSV-1-infected cells after a 15-min pulse was Pra, and the most abundant products identified during a 4-hour chase were Prb

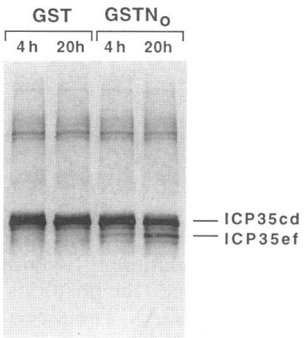

FIG. 7. In vitro *trans* cleavage of ICP35cd. Shown are Western blots of purified ICP35cd incubated with affinity-purified GST or GST-N_o in 50 mM HEPES (pH 7.5)–0.1 mM EDTA–1 mM DTT–50 mM NaCl–100 μg of acetylated BSA per ml at 30°C for 4 or 20 h. Specific cleavage by the HSV-1 protease (GST-N_o) is indicated by the accumulation of ICP35ef.

and No, as expected (Fig. 9). The identity of these species was confirmed by Western blot analysis with the polyclonal N-term rAb (data not shown).

The ICP35 MAb reacted with Pra, Prb, Na, Nb, ICP35cd, and ICP35ef on Western blots of total HSV-1-infected cell extracts (Fig. 8). Thus, the HSV-1-infected cell protein profile revealed by using the ICP35 MAb mimicked the profile in *E. coli* cells which coexpress the protease and ICP35cd (6) (Fig. 1). Na, Nb, ICP35cd, and ICP35ef all failed previously to react with the N-terminal antibody, consistent with the absence in these molecules of sequences upstream of the cleavage site at Ala-247/Ser-248. The ICP35 MAb was also used to examine HSV-1-infected cell proteins in a pulse-chase experiment. The major radiolabeled proteins immunoprecipitated by the ICP35 MAb after a 15-min pulse were Pra and ICP35cd (Fig. 9), as expected. During the subsequent chase interval, the predicted cleavage products, Prb, Na, Nb, and ICP35ef, were observed (Fig. 9). The identity of these species was confirmed by Western blotting with the ICP35 MAb (data not shown).

The C-term pAb antiserum reacted with Pra, Na, and ICP35cd in Western immunoblots of HSV-1-infected cell lysates but not with the corresponding C-terminally cleaved products, Prb, Nb, and ICP35ef, respectively (Fig. 8). This was expected since the epitope recognized by the C-term pAb is removed upon cleavage by the HSV-1 protease. Thus, reactivity of the C-terminal peptide antibody with extracts of HSV-1-infected cells also mimicked that observed with lysates of *E. coli* cells coexpressing HSV-1 protease and ICP35cd (6) (Fig. 1). The 25-amino-acid cleavage product released from the C terminus was not resolved in the gels used for this analysis. This result, together with the results of immunoblots and pulse-chase immunoprecipitation experiments with the other domain-specific antisera, revealed a striking similarity between the pattern of proteolytic processing among proteins in HSV-1-infected cells and in *E. coli* cells which coexpress HSV-1 protease and ICP35cd. They indicate that no other viral proteins are required for the full complement of cleavages by the HSV-1 protease.

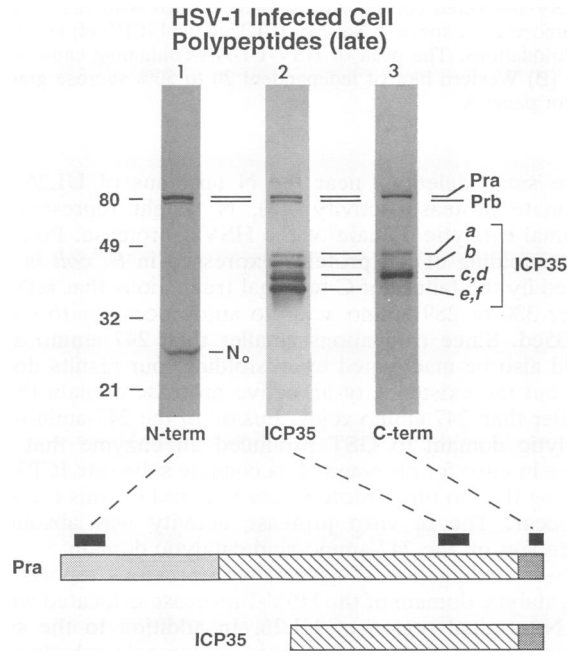

FIG. 8. Western blot of HSV-1-infected cell lysates from KOS-infected BHK-21 cells at 12.5 h postinfection. The location of the epitope for each antiserum is indicated relative to Pra and ICP35cd at the bottom. Lane 1, N-term rAb. Positions of migration of Pra, Prb, and N_o are indicated at the right. Lane 2, ICP35 MAb. Positions of migration of ICP35a and ICP35b (Na and Nb) and of ICP35cd and ICP35ef are indicated at the right. Lane 3, C-term pAb reactive with Pra, Na, and ICP35cd. Positions of molecular weight markers are indicated at left in kilodaltons.

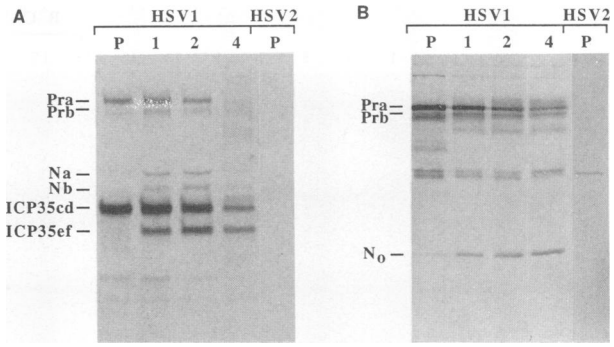

FIG. 9. Pulse-chase in HSV-1-infected cells. BHK-21 cells were infected with HSV-1(Schooler) or HSV-2 (Curtis) for 5 h prior to pulse-labeling for 15 min with [^{35}S]methionine and then immunoprecipitated with ICP35 MAb (A) or HSV N_o MAb (B). Lanes: P, sampled after a 15-min pulse with [^{35}S]methionine; 1, 2, and 4, sampled after 1, 2, and 4 h of chase, respectively, in methionine-rich medium.

HSV-1 protease autoprocessing and ICP35 cleavage products accumulate in B capsids. The association of ICP35ef (3, 8, 15, 20) and VP24 (5) with intermediate B-capsid particles has been described by others (3, 8, 15, 20). We used the three domain-specific antisera to confirm these results and to determine whether other products of HSV protease processing might also be associated with B capsids. To do this, nuclear extracts of HSV-1-infected cells were fractionated by sedimentation in sucrose density gradients, and then gradient fractions were analyzed by Western blotting (Materials and Methods). Sucrose gradient fractions were probed initially with the ICP35 MAb alone. This Western immunoblot showed clearly that Nb is found in B capsids along with ICP35ef, but in much lower amounts (Fig. 10A). The B-capsid peak did not contain significant amounts of viral DNA, which was localized in a capsid peak that sedimented lower in the gradient. Fractions at the top of the gradient that represent soluble nuclear proteins contained comparable amounts of ICP35cd and ICP35ef but no detectable Nb, Pra, Prb, or Na. Only ICP35cd present in fractions from the top of the sucrose gradient reacted positively with the C-term pAb (data not shown). Thus, both processed and unprocessed forms of ICP35 accumulate within the nucleus of HSV-1-infected cells, but only ICP35ef is associated with B-capsid particles. Similarly, B capsids also contain only the fully processed C-terminal fragment of the protease, Nb, but not Pra, Prb, or Na. Others have reported partial amino acid sequence identity between VP21 and the C terminus of UL26 (5). Nb migrates with the size expected for VP21, and our results indicate that VP21 is Nb.

A second set of sucrose density gradient fractions was probed with a mixture of the ICP35 MAb and the N-term rAb to determine whether the N-terminal product(s) of protease autoprocessing could be found in capsid particles along with

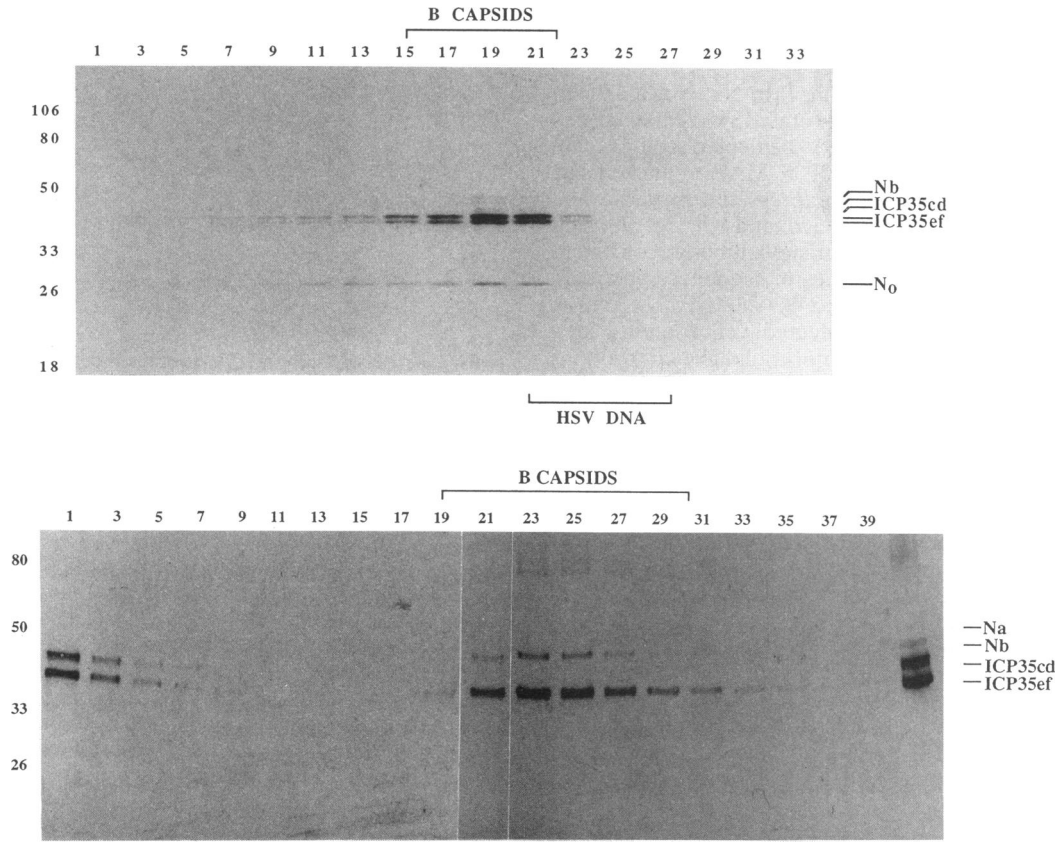

FIG. 10. (A) Western blot of 20 to 50% sucrose gradient fractions of HSV-1-infected cell nuclear extracts probed with ICP35 MAb. Fractions extend from left (20%) to right (50%). Positions of migration of immunoreactive species (Na, Nb, ICP35cd, and ICP35ef) are shown at the right; positions of molecular weight markers are indicated at left in kilodaltons. The peak of HSV-1 DNA-containing capsids was determined by hybridization of gradient fractions (Materials and Methods). (B) Western blot of independent 20 to 50% sucrose gradient fractions probed with ICP35 MAb and N-term rAb, otherwise as described for panel A.

Nb. There was less immunoreactive material in these samples, as only weak reactivity with Nb was detected (Fig. 10B). Nevertheless, the Western analysis shows clearly that ICP35ef and N_o cosedimented in the B-capsid peak (Fig. 10B). The reactivity of N_o in B capsids with the N-term rAb alone was confirmed in a separate Western blot experiment (data not shown). These results prove that N_o is associated with B capsids. This conclusion, taken together with amino acid sequence analysis of VP24 reported by others (5), confirms the identification of VP24 as N_o. Since N_o and Nb, in addition to ICP35ef, were detected in B capsids, it is clear that complete processing by the HSV-1 protease must occur either prior to or immediately upon incorporation of these molecules into B capsids.

DISCUSSION

To map the minimal catalytic domain of the HSV-1 protease, a set of C-terminally truncated protease molecules was coexpressed with ICP35cd in *E. coli*. All of the truncations expressed stable proteins of the predicted sizes in *E. coli*, and the smallest active truncation retained the first 247 amino acids of UL26. This protein is equivalent to N_o, the product of autoproteolytic cleavage of Pra at Ala-247/Ser-248. Truncations retaining 243, 218, or 193 amino acids all failed to cleave ICP35cd, suggesting that residues very near the C terminus of N_o might be required for catalytic activity. Since small deletions near the N terminus of UL26 also eliminate protease activity (13), N_o might represent the minimal catalytic domain of the HSV-1 protease. Potential for misfolding of the proteins expressed in *E. coli* is suggested by the failure of C-terminal truncations that retained either 330 or 289 amino acids to autoprocess or to cleave ICP35cd. Since truncations smaller than 247 amino acids could also be inactivated by misfolding, our results do not rule out the existence of an active protease domain that is smaller than 247 amino acids. Fusion of the 247-amino-acid catalytic domain to GST produced an enzyme that was active in vitro for cleavage of its cognate substrate ICP35cd, proving that no other proteins are required for this cleavage to occur. The in vitro protease activity was absolutely dependent on the 247-amino-acid catalytic domain.

These findings are consistent with previous reports that the catalytic domain of the HSV-1 protease is located within the N-terminal region of UL26. In addition to the small N-terminal deletions noted above, amino acid substitutions at conserved residues upstream of Ala-247 have been shown to eliminate protease activity (13). Also, truncations of UL26 that removed sequences downstream of the ICP35cd initiation codon retained protease activity (13), and more recently, the 247-amino-acid catalytic domain was expressed in mammalian cells and found to direct the cleavage of ICP35cd (14). The N-terminal 247 amino acids of UL26 share sequence homology with the catalytic domains of SCMV and

HCMV proteases (1, 23). Autoprocessing of the SCMV protease at an analogous N-terminal cleavage site releases a catalytic domain that has been shown to retain protease activity when coexpressed with its assembly protein substrate (23). Similar results have also been obtained for the UL80 gene of HCMV (1).

In *E. coli*, the accumulation of N_o, Na, and Nb as a result of autoprocessing was eliminated by the substitution of Ser for Ala-247, which inactivated the N-terminal autoprocessing site. This finding proved that N_o, Na, and Nb arise from autoprocessing of Pra at Ala-247/Ser-248. The accumulation of Prb and Nb was eliminated by substitution of Ser for Ala-610, proving that they arise from autoprocessing at Ala-610/Ser-611. The Ser-247 and Ser-610 mutants both retained protease activity, since they carried out cleavages of ICP35cd and of their respective unaltered autoprocessing sites. In addition, mutation of both P-1 positions simultaneously did not prevent cleavage of ICP35cd, indicating that Pra is an active form of the protease. Liu and Roizman showed recently that mutation of the N-terminal cleavage site eliminated autoprocessing at that site but did not prevent cleavage of ICP35cd in transfected mammalian tissue culture cells (14). As a whole, these results show that autoprocessing of the HSV-1 protease (i.e., release of N_o) is not required to activate the enzyme.

The P-1 mutations both induced a third cleavage near the center of the protease catalytic domain, producing two novel products. Mutagenesis experiments indicated that the smaller product is derived from the larger one by proteolysis at the authentic C-terminal cleavage site. Cleavage within the catalytic domain was greatly reduced upon expression of the wild-type protease in *E. coli*, upon coexpression of mutant proteases with ICP35cd, and in HSV-1-infected cells examined by Western blotting, indicating that it is cryptic in nature. It is unclear whether this cleavage is carried out autoproteolytically by the mutant HSV-1 proteases. The results do suggest, however, that the mutant proteases possess an exposed, labile site that can be cleaved upon expression in *E. coli*. Efficient cleavage near the middle of the wild-type HCMV protease has also been shown to occur upon expression in *E. coli* (1, 22). It was greatly reduced during HCMV infection (1), however, suggesting that the lability of this site is also enhanced in *E. coli*. Thus, the existence of an exposed, labile site within the catalytic domain might be a common feature of herpesvirus proteases expressed in *E. coli*.

Western blot and pulse-chase experiments were carried out with domain-specific antibodies to confirm the identity of proteins in HSV-1-infected cells that correspond to known protease cleavage products. The pattern of proteolysis observed in HSV-1-infected cells was identical to that seen in *E. coli* expressing Pra and ICP35cd, suggesting that no other viral factors are involved in cleavage site selection by the HSV-1 protease. Both a polyclonal antiserum and a MAb raised against bacterially expressed N_o reacted with a protein identical in size to N_o in lysates of HSV-1-infected tissue culture cells. The results of pulse-chase experiments supported the conclusion that N_o, Na, and Nb are all products of protease autoprocessing, and Western blots indicated that Na and Nb are identical to ICP35a and ICP35b, as suggested previously by others (5, 6, 14, 18). These findings, coupled with data from the expression of wild-type (6) and mutant proteases in *E. coli* (Ser-247) or mammalian cells (Pro-247/Arg-248 [14]), strongly suggest that these proteins are derived from the full-length protease and not from ICP35, as suggested by their previous designations. The localization of Nb in HSV-1 B capsids also indicates that Nb is VP21, as others have reported that VP21 shares amino acid sequence with the C terminus of UL26 (5).

The finding that N_o and Nb both accumulate in HSV-1 B capsids along with ICP35ef has implications for models of HSV-1 capsid assembly. Since only the fully processed forms of both protease and ICP35 were found in B capsids, maturational cleavages must all take place either before or immediately upon their assembly into B capsids. The detection of unassembled ICP35cd and ICP35ef at the top of sucrose density gradients is consistent with this conclusion. Also consistent with this conclusion is the fact that ICP35ef purified from B capsids self-assembles in vitro to form toroidal structures reminiscent of a capsid scaffold (16). If this in vitro assembly mimics an essential step in capsid assembly, it indicates that cleavage of ICP35cd could be tolerated prior to the initiation of assembly. The cleavage of ICP35cd might even be required to catalyze the assembly reaction. If this is the case, ICP35cd would not be expected to self-assemble in vitro, but this question has not yet been tested directly. If cleavages occur before assembly, the colocalization of N_o and Nb in B capsids would require that they possess independent mechanisms for localization. Clearly, Nb could be assembled into B capsids independently, since it possesses sequences identical to those of ICP35ef, and could participate in assembly along with ICP35ef. The association of N_o with B capsids, however, would require a stable interaction with ICP35ef or Nb subsequent to their cleavages or with some other capsid component, which suggests that N_o might play a structural role in addition to its role as a catalytic domain during capsid assembly.

The presence of both N_o and Nb in B capsids also suggests an alternative model for assembly, i.e., that the protease catalytic domain is targeted to assembling capsids prior to N-terminal cleavage via the Nb domain of Prb, which would direct its assembly into scaffolds together with ICP35ef. The incorporation of Prb into assembling capsids would then lead to the production of N_o and Nb by autoprocessing in situ when capsid assembly is completed. In this case, complete autoprocessing (N-terminal cleavage) of the protease would be prevented prior to capsid assembly. Given the fact that no other viral factors are required for cleavage, this would suggest the existence of factors that actively inhibit the protease. Alternatively, the catalytic domain of the protease might have a very low affinity for the N-terminal site or exist in an unfavorable conformation prior to capsid assembly. The assembly into B-capsid structures could then trigger a conformational change or cause the release of an inhibiting factor, thereby inducing autoproteolytic cleavage. Alternatively, concentration of the protease and its substrate into a small physical space might serve to enhance the rate of cleavage as assembly takes place. Whatever mechanism is in operation, complete processing would have to occur immediately upon activation of the protease, since only fully processed forms of both the protease and ICP35 are found in assembled B capsids.

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