

## Characterization of the proteinase specified by varicella-zoster virus gene 33

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**Varicella-zoster virus (VZV) genes 33 and 33.5 are predicted to encode the VZV proteinase and its substrate (the assembly protein) respectively. These genes were expressed in insect cells using recombinant baculovirus and it was confirmed that gene 33 encodes a proteinase capable of auto-proteolytic processing at two positions. When VZV gene 33.5 was co-expressed with the VZV proteinase, processing of the VZV33.5 gene product was observed. A polyclonal antiserum to the VZV assembly protein domain highlighted a set of proteins in VZV infected HEL cells identical to those identified in insect cells expressing VZV genes 33 and 33.5. To facilitate further characterization of the VZV proteinase the enzyme was purified by affinity chromatography from an *E. coli* expression system and *in vitro* activity was observed.**

Varicella-zoster virus (VZV) is a member of the *Herpesviridae*, a diverse family of double-stranded DNA viruses known to encode a range of structural and non-structural proteins. Among these are the proteinase and assembly proteins, both of which play essential roles in capsid assembly and maturation (Preston *et al.*, 1983; Gao *et al.*, 1994). They have been characterized from herpes simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) (Gibson *et al.*, 1994; Donaghy & Jupp, 1995) and the three-dimensional structure of the HCMV proteinase has recently been solved (Chen *et al.*, 1996; Qiu *et al.*, 1996; Shieh *et al.*, 1996; Tong *et al.*, 1996).

Sequence homology suggests that VZV genes 33 and 33.5 encode the VZV proteinase and assembly protein respectively (Welch *et al.*, 1991; Gibson *et al.*, 1994). The nomenclature used follows that suggested by Gibson *et al.* (1994) (Fig. 1*a*).

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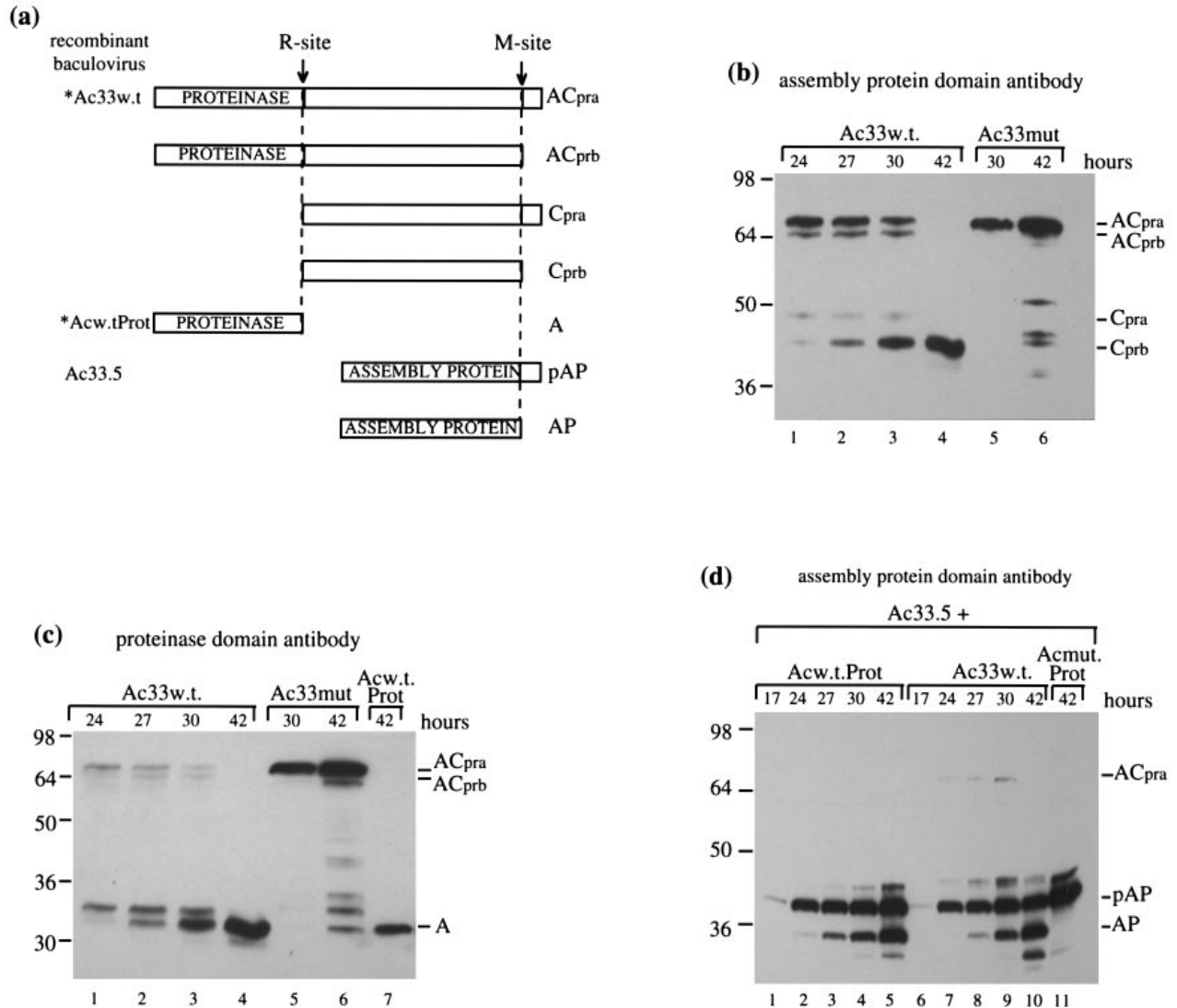
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To facilitate a more detailed analysis of the VZV proteinase, various fragments of VZV gene 33 have been introduced into plasmids for expression in heterologous systems. A plasmid containing the *Kpn*I D fragment of the VZV genome was supplied by A. J. Davison (MRC Virology Unit, Glasgow, UK). The VZV33 ORF (nucleotides 62138–60324; Davison & Scott, 1986) was amplified by PCR, using the *Kpn*I D fragment as a template, with oligonucleotide primers designed to anneal and generate *Nco*I sites (underlined) at the VZV33 initiation codon (bold text) (5' CGT GGG GTG TTT ACC ATG GCT GCT GAA GCT 3') (nucleotides 62153–62124) and 3' to the stop codon (5' CCA AAC ACG CCT TCC ATG GAT GTA ATA CTT 3') (nucleotides 60280–60309). Sub-cloning of the amplified PCR product into the *Nco*I site of the T7 expression vector pRSETB (Invitrogen) generated the construct p33w.t.

A construct expressing the proteinase domain(A) (amino acid residues 1–236 of AC<sub>pra</sub>) was generated using a primer (5' C GAA GGG GAA TTA AAT ATT AC 3') (nucleotides 62064–62044) that annealed 5' of the unique *Spe*I site (62007) in VZV gene 33, and a second primer which was designed to introduce a stop codon (bold type) and an *Eco*RI site (underlined) after the codon (61433) for Ala-236 (5' C AAG TC G AAT TCC GGT **CTA** AGC CTG AAG ATA AAC GTG ACC CA 3') (nucleotides 61412–61453). The PCR fragment was cloned into p33w.t. restricted with *Spe*I/*Eco*RI (the *Eco*RI site is located in the polylinker of pRSETB, 3' to the stop codon of VZV33) generating the construct pw.t.Prot.

The VZV assembly protein precursor (pAP) is thought to be encoded by the VZV33.5 ORF, which is contained within and in-frame with the 3' half of VZV gene 33. The VZV33.5 ORF was restricted from p33w.t. as a *Dra*I/*Eco*RI fragment, and subcloned into pRSETA (Invitrogen) restricted with *Pvu*II/*Eco*RI, generating the plasmid p33.5. The *Dra*I/*Eco*RI fragment encompasses the entire VZV33.5 ORF plus 11 codons upstream of the Met-305 start codon of VZV33.5.

Site-directed mutagenesis has indicated that a conserved histidine residue in domain-2 is a component of the catalytic apparatus of HSV and HCMV proteinases (Gibson *et al.*, 1995). The corresponding histidine residue in VZV, identified at position 52 by amino acid sequence alignment of the herpesvirus proteinase homologues, was mutated to an Ala residue (His-52 → Ala) by mispriming PCR (Kadowaki *et al.*,



**Fig. 1.** Western blot analysis of various VZV proteins expressed by recombinant baculoviruses. (a) Schematic representation of the proteolytic processing mediated by the VZV proteinase. Autoproteolysis of AC<sub>pra</sub> at the release (R)- and maturation (M)-sites produces products AC<sub>prb</sub>, C<sub>pra</sub>, C<sub>prb</sub> and A. Cleavage of pAP by the proteinase at the M-site generates the mature assembly protein (AP). Each recombinant baculovirus is shown alongside the protein expressed (those marked with an asterisk were also made as inactive mutants). (b, c) Sf9 cells were infected with baculovirus recombinants Ac33w.t. (lanes 1–4) or Ac33mut. (lanes 5 and 6). The infected cells were harvested at the indicated times and analysed by Western blotting using antiserum to (b) the assembly protein domain and (c) the proteinase domain. In addition, panel (c) lane 7 shows Sf9 cells infected with the baculovirus recombinant Acw.t.Prot. (d) Western blot analysis (using the assembly protein domain antiserum) of cells co-infected with: Ac33.5 and Acw.t.Prot. (lanes 1–5), Ac33.5 and Ac33w.t. (lanes 6–10), and Ac33.5 and Acmut.Prot. (lane 11). The various protein products (shown schematically in a) are indicated on the right side of each panel and the molecular masses of proteins (kDa) are denoted on the left side.

1989). This replacement in the proteinase region of p33w.t. and pw.t.Prot generated the clones p33mut. and pmut.Prot respectively.

The VZV inserts from these five *E. coli* constructs were excised using *Bgl*III and *Hind*III, sub-cloned into the baculovirus transfer vector pBlueBacIII (Invitrogen) and used to generate recombinant baculovirus (Ac33w.t.; Ac33mut.; Acw.t.Prot.; Acmut.Prot.; Ac33.5) following the manufacturer's protocol (Invitrogen).

Antisera were raised (separately) in rabbits against (a) mature VZV proteinase and (b) the whole assembly protein, each expressed in the form of a fusion protein with GST using the method described previously (Overton *et al.*, 1992). These were used to visualize expression of the various VZV constructs by Western blotting.

Expression of the full-length VZV33 ORF was studied by infecting *Spodoptera frugiperda* cells (Sf9) with the recombinant baculovirus Ac33w.t. and harvesting the cells at various times.

The samples generated were analysed by SDS-PAGE and immunoblotting (Fig. 1*b, c*, left side). At 24, 27 and 30 h, the largest protein (apparent molecular mass  $\sim 68$  kDa) detected with both the assembly protein and the proteinase domain antisera was coincident in size with that expected for the proteinase precursor ( $AC_{pra}$ ). Both antisera also revealed a protein band approximately 3 kDa smaller than  $AC_{pra}$  (Fig. 1*b, c*) at these times. This is consistent with cleavage of  $AC_{pra}$  at the maturation site only to generate  $AC_{prb}$  (Fig. 1*a*). Processing at the release-site alone accounts for the product  $C_{pra}$  which reacted with the antiserum to the assembly protein but not the antiserum to VZV proteinase (compare Fig. 1*b* and 1*c*). The fully processed product ( $C_{prb}$ ), revealed by the antiserum to the assembly protein only, accumulated over these early times and was the only immunoreactive protein at 42 h (Fig. 1*b*, left side). The other fully processed product, the proteinase (A), also accumulated with time and was the only protein detected by the proteinase antiserum at 42 h (Fig. 1*c*, left side). In insect cells infected with the recombinant baculovirus Acw.t.Prot (encoding only the proteinase domain) a protein with an apparent molecular mass of  $\sim 31$  kDa accumulated (Fig. 1*c*, lane 7). This was coincident in size with the final proteinase product that accumulated from processing of the precursor protein (Fig. 1*c*, lanes 1–4). This indicates that processing was occurring at the expected sites.

In cells infected with the Ac33mut. baculovirus (encoding the His-52  $\rightarrow$  Ala mutation in the proteinase), only the full-length proteinase precursor  $AC_{pra}$  was detected at 30 h by immunostaining with both antisera. At 42 h, some (non-specific) degradation was evident but most of the protein was still present in the  $\sim 68$  kDa precursor form of  $AC_{pra}$  (compare lanes 6 and 4 in Fig. 1*b* and 1*c*).

In order to demonstrate specifically that the proteinase (A) was capable of acting *in trans* on a suitable substrate, co-infection time-courses were performed using the recombinant baculovirus constructs and whole cell samples were analysed at each time-point by immunoblotting using the antiserum to the assembly protein.

Cells co-infected with Ac33.5 and Acw.t.Prot showed an accumulation of two major bands, a 40 kDa protein, corresponding in size to that of the unprocessed assembly protein precursor (pAP) and a 37 kDa moiety (AP) (Fig. 1*d*, lanes 1–5). The apparent difference in molecular mass between the precursor and product (approximately 3 kDa) is consistent with cleavage having occurred at the maturation site (Fig. 1*a*).

A co-infection time-course with Ac33.5 and Ac33w.t. gave similar results (Fig. 1*d*, lanes 6–10). The only apparent difference between the two sets of samples was the presence of a small amount of proteinase precursor  $AC_{pra}$  in the time-course involving that recombinant (Fig. 1*d*, lanes 7–9).  $AC_{prb}$  was also observed in these samples upon longer exposure of the ECL developed Western blot (data not shown). The products  $C_{pra}$  and  $C_{prb}$  were difficult to identify because of their close proximity to the more abundant assembly protein.

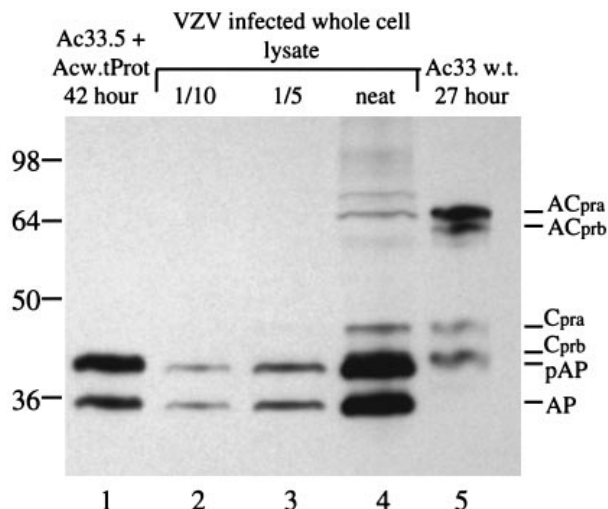


Fig. 2. Western blot analysis of VZV infected HEL cells. Whole cell extracts of VZV infected HEL cells were analysed at dilutions of 1:10 (lane 2), 1:5 (lane 3) and undiluted (lane 4). Extracts of Sf9 cells infected with Ac33w.t. (lane 5) and co-infected with Ac33.5 and Acw.t.Prot (lane 1) were analysed alongside the HEL cell samples. The antiserum to the assembly protein domain was used. The proteins identified (refer to Fig. 1*a* for nomenclature of the proteins) are labelled on the right and the positions of the molecular mass markers (kDa) are indicated on the left.

When the assembly protein precursor was expressed with the proteinase (A) encoding the His-52  $\rightarrow$  Ala mutation, no processing was observed (Fig. 1*d*, lane 11).

The seven polypeptides detected in insect cells are represented schematically in Fig. 1*a*). The size of the products derived by processing of  $AC_{pra}$  and pAP is consistent with cleavage at the predicted release and maturation sites and the pattern of cleavage is almost identical to that seen for the HSV-1 proteinase (Deckman *et al.*, 1992).

Each of the baculovirus recombinants was engineered to express its respective VZV protein in authentic form, equivalent to those produced in cells infected with VZV itself. HEL cells were infected with VZV (using infected whole cells as an inoculum) and harvested after 3–4 days when the cultures showed advanced cytopathic effect. Samples of insect cells expressing the various components of VZV33 and VZV infected HEL cells were compared by Western blot analysis using the antiserum to the assembly protein. The insect cell samples chosen for the comparison contained each of the polypeptides derived from Ac33 and Ac33.5, although the released proteinase (A) was not highlighted by the antiserum to the assembly protein (Fig. 2, lanes 1 and 5). Three dilutions of the VZV infected cell lysate were analysed (Fig. 2, lanes 2–4). The more abundant AP and pAP are seen at each dilution loaded (Fig. 2, lanes 2–4). The two forms of the assembly protein show identical migration profiles to the polypeptides that accumulated in insect cells which had been co-infected with the Acw.t.Prot and Ac33.5 baculovirus recombinants (Fig. 2, lanes 1–4).  $AC_{pra}$  and the various products derived from it (Fig. 2, lane 4) were less abundant in the VZV infected cell

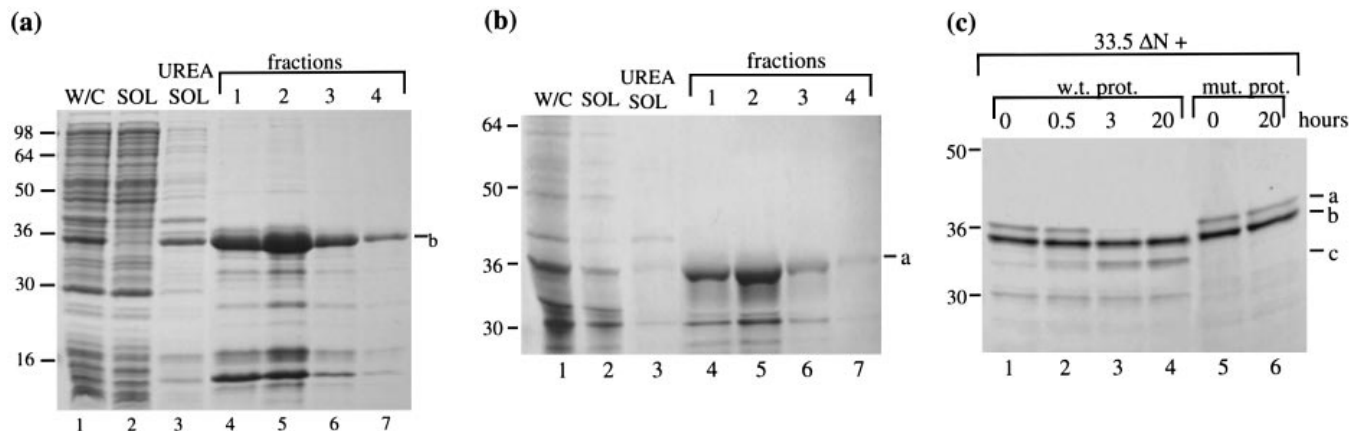


Fig. 3. (a, b) Affinity purification of 6-His-VZV proteinase and the 6-His-33.5 $\Delta$ N substrate from *E. coli*. The Coomassie blue stained gels show extracts and fractions eluted from the column during the purification of the respective proteins. Lanes: 1, whole cell lysate of induced cells; 2, soluble extract derived from the induced cells; 3, insoluble material from the initial lysis solubilized in 7 M urea. 6-His-proteinase was purified from the urea solubilized extract (a, lane 3) by nickel-chelate chromatography. Fractions collected are in (a), lanes 4–7. 6-His-33.5 $\Delta$ N was purified from the soluble extract of *E. coli* following lysis of the cells (b, lane 2). The fractions from the nickel-chelate chromatography are shown in (b), lanes 4–7. The purified proteins are labelled (6-His-proteinase, band b; 6-His-33.5 $\Delta$ N, band a). (c) Processing of the truncated assembly protein precursor (6His-33.5 $\Delta$ N) by 6-His-VZV proteinase monitored *in vitro*. Refolded, purified 6-His-proteinase (band b) was incubated with the purified 6-His-33.5 $\Delta$ N protein substrate for 0, 0.5, 3 and 20 h (lanes 1–4). The 6-His-33.5 $\Delta$ N substrate was incubated separately with an equivalent preparation of 6-His-proteinase carrying the His-52  $\rightarrow$  Ala mutation (lanes 5 and 6). The cleavage product (lanes 1–4), is denoted as band c and the positions of the molecular mass markers (kDa) are indicated on the left side of the panel.

lysate and AC<sub>prb</sub> was not detectable. The molecular masses of the proteins expressed in insect cells infected with Ac33w.t. corresponded with those in the VZV infected cells (Fig. 2, lanes 4 and 5). C<sub>prb</sub> was difficult to identify in the VZV infected cell samples because of its close proximity to the more abundant pAP band.

The similarity in the processing profiles observed in the infected insect cells and in VZV infected cells confirms that the cleavage events occurring in the recombinant expression system accurately reflect the events taking place in VZV infected cells. By these means, the presence of an active proteinase in the amino-terminal region of AC<sub>pra</sub> had been established.

*E. coli* strain BL21(DE3) pLys-S harbouring the expression plasmid pw.t.Prot [which encodes the proteinase (A) with a 6-histidine tag at its amino terminus] was induced with IPTG in order to produce recombinant VZV proteinase. Cells expressing the fusion protein were lysed with a French press in buffer consisting of 50 mM Tris-HCl, pH 8.0, and 500 mM NaCl. The insoluble material from the cell extract was solubilized in 7 M urea, and the 6-His-proteinase was bound to Ni-NTA resin (Qiagen) in buffer containing 7 M urea and eluted using buffer containing 500 mM imidazole and 7 M urea (Fig. 3a, lanes 1–7). Following purification, the proteinase was refolded by dialysis against buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 10% glycerol and 100 mM NaCl.

A potential substrate for the proteinase was expressed in *E. coli* strain BL21(DE3) pLys-S by sub-cloning the *EcoRV/EcoRI*

fragment from p33w.t. into pRSETB restricted with *PvuII/EcoRI*, generating the clone p33.5 $\Delta$ N. This construct expresses the assembly protein precursor (pAP) but with an N-terminal deletion of 91 amino acids. The putative substrate was also expressed with a 6-His N-terminal tag, and was purified from the soluble fraction of *E. coli* lysates using nickel chelate chromatography (Fig. 3b, lanes 1–7).

The preparations of refolded proteinase and truncated assembly protein precursor were mixed and incubated in buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA, 100 mM NaCl, 10% glycerol at 21 °C. Samples of the reaction mix taken at various times were analysed on polyacrylamide gels and stained with Coomassie blue (Fig. 3c). After 3 h, cleavage of the substrate (band a) by the proteinase (band b) was detected by the production of a smaller species (band c in Fig. 3c, lanes 1–4). The reduction in size was approximately 3 kDa, as expected for cleavage at the maturation site. When the protein substrate was incubated with an equivalent preparation of refolded proteinase containing the His-52  $\rightarrow$  Ala mutation, no processing was observed throughout the time-course (Fig. 3c, lanes 5 and 6). These data confirm that the VZV33 ORF encodes an active proteinase and as seen with its counterparts (Baum *et al.*, 1993; Liu & Roizman, 1993; Weinheimer *et al.*, 1993; Donaghy & Jupp, 1995) the VZV proteinase was able to process pAP supplied in *trans*. This activity was lost when the conserved His-52 residue was mutated to an alanine, confirming that the proteinase moiety of VZV33 is located at the amino terminus, and that the His-52 residue is likely to be a component of the active site.

The antiserum to the assembly protein revealed the presence of five major polypeptides in VZV infected cells. Comparison with the recombinant proteins expressed in insect cells enabled the identification of each of these proteins as being the various products of the VZV33 and VZV33.5 ORFs, confirming that VZV33.5 is the nested gene encoding the assembly protein precursor (pAP). The product AC<sub>prb</sub> was not detected in VZV infected HEL cells (Fig. 2, lane 4), suggesting an order of processing AC<sub>pra</sub> → C<sub>pra</sub> → C<sub>prb</sub>, whereas in Sf9 cells AC<sub>prb</sub> is produced (Fig. 2, lane 5) indicating two possible processing pathways: AC<sub>pra</sub> → C<sub>pra</sub> → C<sub>prb</sub> and AC<sub>pra</sub> → AC<sub>prb</sub> → C<sub>prb</sub>. Pulse-chase radiolabelling experiments may clarify any differences in the order of cleavage in the two systems.

The four proteins C<sub>pra</sub>, C<sub>prb</sub>, pAP and AP (Fig. 2, lane 4) are likely to be the equivalent of the ICP35 assembly protein family of HSV-1. Previous studies with VZV infected cells identified a group of four proteins (the p32/p36 complex) that were also identified as being the homologues of the HSV-1 assembly proteins (Friedrichs & Grose, 1986; Harper *et al.*, 1995). The similarities shared between the p32/p36 complex and the group of four proteins described here suggests that they are the same family of VZV proteins.

The observed activity of the VZV proteinase towards the truncated assembly protein substrate *in vitro* opens the way for further biochemical characterization of the VZV enzyme.

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