

Processing and intracellular localization of the herpes simplex virus type 1 proteinase

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The herpes simplex virus type 1 (HSV-1) capsid protein VP24 (encoded by UL26) was expressed as a GST-fusion protein and used to prepare a group of monoclonal antibodies. These were used to characterize the protein in capsids and virus infected cells and demonstrated that it exists as two polypeptide species. The nature of the relationship between these two species was investigated and found to be associated with disulphide bonding. Under non-reducing conditions a species corresponding to dimers of VP24 was identified in preparations of B capsids, the site of action of the proteinase. Biochemical subcellular fractionation studies suggested that only cleaved forms of UL26 and UL26.5 gene products could be detected in the nucleus of the infected cell at early times post-infection.

Herpesvirus particle assembly occurs in the nucleus of the infected cell (Darlington & Moss, 1968; Schwartz & Roizman, 1969), and involves the obligate proteolytic processing of a scaffolding protein (Gao *et al.*, 1994; Preston *et al.*, 1994). The proteinase activity of herpes simplex virus type 1 (HSV-1) is encoded by the UL26 open reading frame which produces a 635 amino acid (aa) protein with autocatalytic protease activity. Cleavage of this precursor polypeptide occurs between alanine and serine residues at positions 247–248 and 610–611 to generate the capsid proteins VP24 and VP21, and a C-terminal 25 aa fragment (McGeoch *et al.*, 1988; Liu & Roizman, 1991*a*; Davison *et al.*, 1992; Dilanni *et al.*, 1993*a,b*). The major substrate of the proteinase is encoded by a collinear gene designated UL26.5 (Liu & Roizman, 1991*b*, 1993), which specifies the scaffolding protein VP22a. This polypeptide is also cleaved 25 amino acids from its C terminus.

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Although the activity of the proteinase was originally thought to be slow in comparison with those encoded by other viruses (Deckman *et al.*, 1992; Dilanni *et al.*, 1993*a, b*) more recent reports have identified certain reagents, such as glycerol, which are capable of activating the enzyme (Hall & Darke, 1995; Yamanaka *et al.*, 1995). Of particular interest is a recent paper by Darke *et al.* (1996) which demonstrated that the active form of the human cytomegalovirus (hCMV) proteinase is a dimer and that the enzyme exists in a monomer:dimer equilibrium. The results suggest that reagents such as glycerol are capable of increasing the proportion of dimers and hence enhancing the activity of the proteinase.

Much of the work which has been carried out on the UL26 gene product has made use of antibodies targeted to the C-terminal domain of the protein. Detailed characterization of the N-terminal domain has been hampered because of the lack of versatile antibody reagents which recognize VP24. In this report we describe the creation and characterization of a number of monoclonal antibodies which are specific for VP24, and the characterization of the proteinase and its substrate in infected cells and capsids. We also present data which suggest that the activated form of the HSV-1 proteinase may be a dimer.

Ten anti-VP24 monoclonal antibody producing hybridoma cell lines were raised against a GST–VP24 fusion protein that was produced using standard methodologies. Deletion analysis indicated that the epitopes recognized by all of the antibodies mapped to the N-terminal 75 amino acids of VP24.

In Fig. 1(*a*), lanes 3–4, anti-VP24 antibody AJS 7 was used to probe a Western blot of uninfected/HSV-1 infected BHK cells. No reaction was seen with extracts of uninfected cells, whereas a doublet of around 75 kDa (corresponding to full-length and C-terminal cleaved UL26 gene product) was seen in the lane containing HSV-1 infected cells along with two other bands of 28 and 24 kDa (corresponding to forms of VP24). AJS 7 was also reacted with a Western blot of preparations of A, B and C capsids [prepared essentially according to the method of Newcomb & Brown (1991)] under reducing and non-reducing conditions (Fig. 1*b*, lanes 1–3 and 4–6). Full-length and C-terminal cleaved forms of the UL26 gene product were not

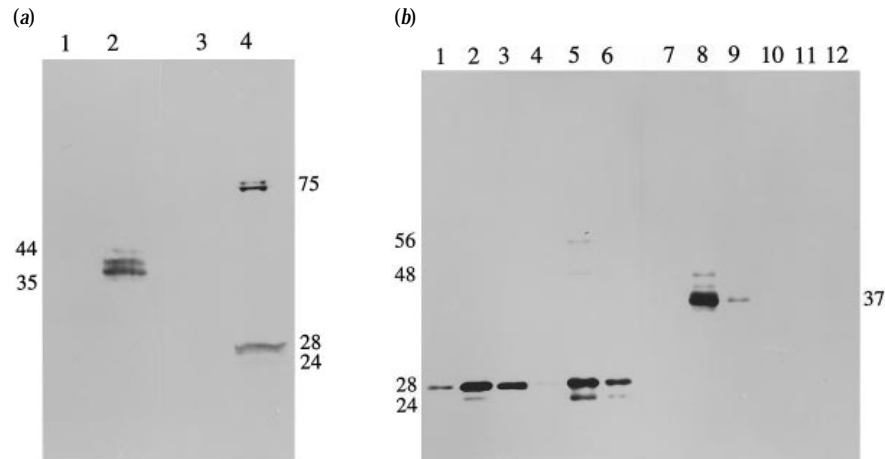


Fig. 1. (a) Reactions of anti-VP24 and anti-VP22a antibodies with mock infected and HSV-1 infected BHK cells. Cells were harvested 18 h after infection and analysed by SDS-PAGE on a 10% gel followed by Western blotting. The blot was reacted with either CY45 (anti-VP22a, lanes 1 and 2) or AJS 7 (anti-VP24, lanes 3 and 4) and the reactive species detected as outlined in Fig. 2. Lanes 1 and 3, mock infected BHK cells. Lanes 2 and 4, HSV-1 infected BHK cells. The positions of species described in the text are identified by molecular mass markers (kDa). (b) Reactions of anti-VP24 and anti-VP22a antibodies with preparations of A, B and C capsids on Western blot. Capsids were prepared as described in Methods. Equal volumes of the preparations were analysed by SDS-PAGE on a 10% gel followed by Western blotting. (Reduced samples were boiled in loading buffer in the presence of 10 mM DTT as normal and unreduced samples were boiled in its absence.) Lanes 1, 4, 7 and 10, A capsids. Lanes 2, 5, 8 and 11, B capsids. Lanes 3, 6, 9 and 12, C capsids. Lanes 1–3 and 7–9, reducing conditions. Lanes 4–6 and 10–12, non-reducing conditions. Lanes 1–6 reacted with AJS 7 (anti-VP24). Lanes 7–12 reacted with CY45 (anti-VP22a). The positions of species described in the text are identified by molecular mass markers (kDa).

detectable. Under reducing conditions B capsids obviously contained the two forms of VP24, with the higher molecular mass form being predominant (as in infected cells). Only the higher molecular mass form could be detected under reducing conditions in A and C capsids, though this may have been due to the lower molecular mass form being present in extremely small amounts. Under non-reducing conditions the lower molecular mass form of VP24 increased relative to the higher molecular mass form in B and C capsids, suggesting that the former may be related to the latter by an intra-molecular disulphide bond. In the case of A capsids, the 28 kDa form decreases in intensity under non-reducing conditions. Of greater interest, however, is the appearance of two additional species in the lane containing B capsids under non-reducing conditions. At molecular masses of 56 and 48 kDa these may correspond to dimers of both forms of VP24.

CY45 and CY46 are monoclonal antibodies which recognize VP22a (Yang, 1994). These reagents were used to examine the forms of UL26.5 gene products which might be present in the samples which were described above. CY45 recognized a family of proteins between 35 and 44 kDa in molecular mass on Western blots of HSV-1 infected BHK cell extracts (Fig. 1*a*, lane 2), in agreement with previously published data (Braun *et al.*, 1984). The results obtained with the A, B and C capsids would seem to support the view that VP22a is indeed a minor component of the HSV-1 particle (see Fig. 3*b*, lanes 7–12). Under reducing conditions B capsids contained all of the different forms of VP22a (although cleaved forms were much more abundant than uncleaved forms),

whereas only a single processed 37 kDa form was detectable in A and C capsids, possibly due to low level contamination with B capsids. Under non-reducing conditions none of the forms of VP22a were detectable in any of the capsid types. This could be due to an alteration in the conformation of the epitope recognized by the antibody, or VP22a being sequestered in large complexes which were too big to enter the separating gel. In both cases it is likely that the reason is related to disulphide bonds. An aliquot of each of these capsid types was analysed by SDS-PAGE/Coomassie staining to check their purity, which was found to be consistent with previously published results (e.g. Davison *et al.*, 1992).

Extracts of HSV-1 infected cells were prepared at various times after infection. Aliquots of each sample were then run on SDS-PAGE, Western blotted and reacted with either AJS 7 or CY46 to detect UL26 or UL26.5 gene products.

Fig. 2(*a*) shows that full-length UL26 gene product was detectable at 4 h post-infection with VP24 appearing at 6 h post-infection. Progression from the 8 to the 24 h sample saw a reduction in the intensity of the full-length and C-terminal cleaved UL26 gene product, and an increase in intensity of the VP24 band. CY45 (Fig. 2*b*) revealed that forms of VP22a were weakly detectable at 6 h post-infection, and became increasingly more intense at subsequent time-points. It should be noted that, when detectable, all forms of VP22a were present, although lower molecular mass forms increased in relative intensity at later time-points.

It was decided to take the analysis one stage further and characterize the forms of UL26 and UL26.5 gene products

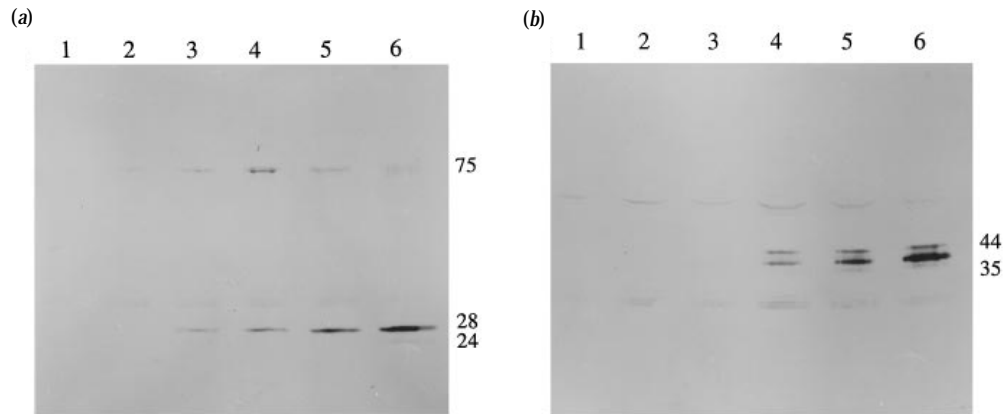


Fig. 2. Reaction of anti-VP24 and anti-VP22a antibodies with a time-course of virus infected cells on Western blot. BHK cells (1×10^6) were infected at an m.o.i. of 5 with HSV-1 and harvested post-infection at the times indicated below. The samples were then analysed on duplicate 10% SDS-PAGE gels followed by Western blotting. The Western blots were reacted with (a) AJS 7 (anti-VP24) or (b) CY45 (anti-VP22a). Lane 1, 2 h. Lane 2, 4 h. Lane 3, 6 h. Lane 4, 8 h. Lane 5, 10 h. Lane 6, 24 h. The positions of species described in the text are identified by molecular mass markers (kDa) shown to the right of the figure.

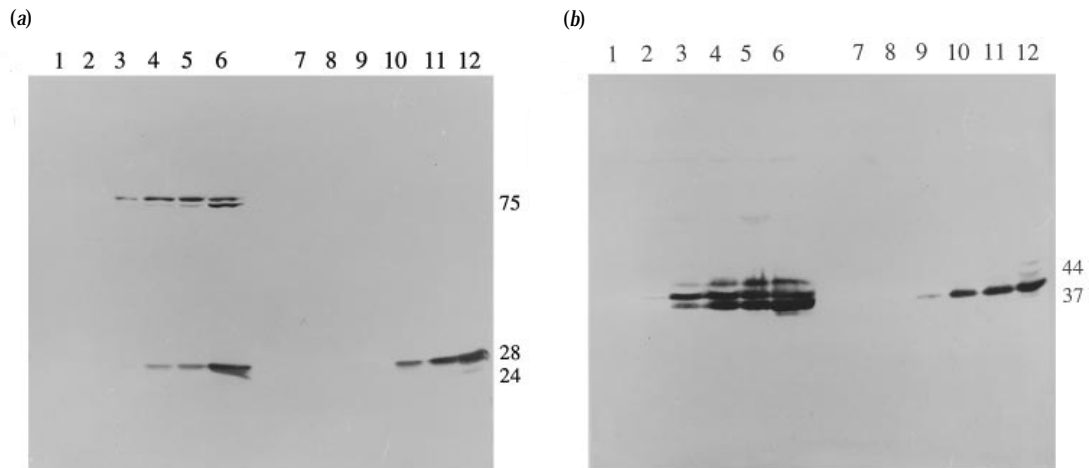


Fig. 3. Analysis of nuclear and cytoplasmic fractions of virus infected cells with anti-VP24 and anti-VP22a antibodies. BHK cells (1×10^6) were infected at an m.o.i. of 5 with HSV-1 and harvested post-infection at the times indicated below. Nuclear and cytoplasmic fractions were then prepared as described in Methods. The samples were analysed on duplicate 10% SDS-PAGE gels which were then Western blotted and reacted with (a) AJS 7 or (b) CY45. Lanes 1–6, cytoplasmic fractions. Lanes 7–12, nuclear fractions. Lanes 1 and 7, 2 h. Lanes 2 and 8, 4 h. Lanes 3 and 9, 6 h. Lanes 4 and 10, 8 h. Lanes 5 and 11, 10 h. Lanes 6 and 12, 24 h. The positions of species described in the text are identified by molecular mass markers (kDa) shown to the right of the figure.

present in nuclear and cytoplasmic fractions. These were prepared by resuspending cell pellets in 1% Triton X-100 in PBS, followed by incubation on ice. The samples were then centrifuged at 2000g for 5 min. Supernatants were collected as cytoplasmic fractions, whilst pellets were washed twice in Triton X-100/PBS and used as nuclear fractions. Cytoplasmic fractions of infected BHK cells reacted with AJS 7 (Fig. 3a) revealed the presence of very small amounts of 75 kDa full-length UL26 gene product at 4 h post-infection (not visible in the figure). At 6 h post-infection a weak band of 28 kDa corresponding to VP24 appeared, in addition to very small amounts of C-terminal cleaved UL26 gene product. The intensities of these two bands increased at time-points 8, 10

and 24 h post-infection, whilst the amount of the full-length gene product remained constant. When nuclear fractions of the same time-points were analysed a striking difference was apparent. At 6 h post-infection a very weak band corresponding to VP24 appeared, the intensity of which increased at time-points 8, 10 and 24 h. At no time were full-length or C-terminal cleaved forms of the UL26 gene product observed in the nuclear fraction. As in the original experiment (Fig. 3a), the lower molecular mass form of VP24 was only evident at 24 h post-infection in both the nuclear and cytoplasmic fractions.

When aliquots of the same fractions were analysed using CY45 (anti-VP22a), differences were again observed (Fig. 3b). At 4 h a single (40 kDa) form of VP22a (presumably uncleaved)

was observed in the cytoplasmic fraction. At 6 h post-infection two more bands appeared, one 44 kDa and one 37 kDa. These bands increased in intensity at time-points 8, 10 and 24 h post-infection. At 24 h a fourth band, of a lower molecular mass, was also evident. The first evidence of VP22a in the nucleus was at 6 h post-infection, when a weak band corresponding to a cleaved form of VP22a appeared (37 kDa). This band continued to increase in intensity at the 8, 10 and 24 h time-points, with weak bands corresponding to the other forms of the protein also present in the last fraction. The presence of these other species could well be attributable to the structural deterioration of subcellular compartments, as a result of the cytopathic effect of the virus, resulting in contamination of the nuclear fraction.

In this report VP24 has been shown to exist in at least two different forms in infected cells and capsids, probably due to disulphide bond formation. In addition, species corresponding to dimers of VP24 have been detected in B capsids, the site of action of the proteinase. In a recent article Darke *et al.* (1996) used an *in vitro* system to demonstrate that the active form of the hCMV proteinase is a dimer, and proposed that dimerization might provide an appropriate temporal trigger for proteolytic activity during the assembly of capsids. Analysis of the hCMV protease dimer in this paper showed no evidence of disulphide linkages. However, two recent papers by Baum *et al.* (1996*a, b*) have demonstrated the formation of disulphide bonds within hCMV proteinase in the presence of oxidizing compounds. This event resulted in the inactivation of the proteinase and led the authors to speculate that disulphide bond formation might be a plausible mechanism for control of proteolysis during the virus life-cycle. We could only detect the presence of the VP24 dimer under non-reducing conditions, suggesting that disulphide linkages may have an important role to play – at least in the case of HSV-1 (which has three candidate cysteine residues at amino acid positions 65, 88 and 152). On the basis of this data a model may be proposed in which disulphide bond interchange is involved in the formation of the proteinase dimer, and hence activation of the proteinase. Such an event would probably only occur to a significant degree when multiple copies of the proteinase were in close proximity in the nucleus of the infected cell (i.e. components of the scaffold within the assembling capsid shell). Whilst this manuscript was in preparation, Robertson *et al.* (1996) proposed that the intragenic complementation which they observed with two mutant HSV-1 proteinases might be due to the formation of a functionally active heterodimer.

This work was submitted in partial fulfilment (A.J.S) for the degree of PhD within the Department of Microbiology, University of Leeds, supported by a Frank Parkinson scholarship. The production of monoclonal antibodies and the cloning of the DNA sequence encoding VP24 was carried out in this department. We are grateful to Dr R. A. Killington for assistance in the preliminary stages of monoclonal antibody

production. This work was supported in part by grants from the MRC and the Wellcome Trust.

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Received 6 September 1996; Accepted 14 November 1996