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Recombinant full-length human cytomegalovirus protease has lower activity than recombinant processed protease domain in purified enzyme and cell-based assays

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Abstract

Herpesviruses encode a protease that is essential for virus replication. The protease undergoes cleavage to a processed form during capsid maturation. A recombinant 75 kDa form of the protease from human cytomegalovirus was purified and compared with the recombinant 29 kDa processed form. Modification with an active site titrant suggested that most of each recombinant protease preparation was active (66 and 86%, respectively). Protease activity was compared using a low-molecular weight peptide substrate and the native substrate, capsid assembly protein. In addition, a cell-based assay for both enzymes was developed in which the target sequence of the protease has been fused inframe into the herpes simplex virus VP16 molecule. Cleavage of the fusion protein by the protease releases the carboxyl terminal transactivation domain, resulting in a decrease in the ability of the fusion molecule to transactivate a target promoter linked to a reporter gene in mammalian cells. Results suggest that the 75 kDa form of the enzyme is significantly less active than the 29 kDa form by all criteria.

Keywords: Cytomegalovirus protease; Herpesviruses; Drug target

1. Introduction

Herpesviruses are a cause of significant medical complications especially in immunocompromised hosts such as transplant, AIDS and chemotherapy patients. The herpesviruses are divided into three subfamilies, the α-, β- and γ-herpesviruses, represented by herpes simplex viruses, cytomegaloviruses and Epstein–Barr virus, respectively. Recently, a virally encoded serine protease (Liu and Roizman, 1991; Preston et al., 1983; Welch et al., 1991) was discovered that is essential for herpesvirus capsid assembly and infectious virus production (for review, see Holwerda, 1997; Homa and Brown, 1997). The protease activity is in an amino terminal domain of a large open reading frame (ORF) designated UL26 in herpes simplex virus and UL80 in human cytomegalovirus. The full-length protease pro-

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cesses itself at the consensus sequence, (V,L,I)-X-A↓S, found at the release (R)-site near the middle of the encoded protein and the maturation (M)-site near the carboxyl terminus. It also cleaves the M-site of capsid assembly protein (CAP), a viral protein translated from the carboxyl terminal region of the large ORF. The proteases of some members of the herpesvirus family also cleave the protease domain itself at an inactivation (I)-site although this cleavage does not inactivate the protease (Hall and Gibson, 1997; Holwerda et al., 1994). The protease is conserved among the different herpesvirus groups and has been shown to have an active site serine (DiIanni et al., 1994; Hoog et al., 1997). Deletions of the protease catalytic domain or mutations of the target sequences have been generated in recombinant HSV and show that the protease is essential for virus replication in cell culture and virulence in vivo (Gao et al., 1994; Matusick-Kumar et al., 1994, 1995a,b). While the recombinant 29 kDa serine protease domain is currently the exclusive protease target for development of antiviral agents (Abood et al., 1997; Baum et al., 1996a,b; Chu et al., 1996; Flynn et al., 1997a,b; Jarvest et al., 1996; Pinto et al., 1996; Siegel et al., 1998), there is evidence that the full-length protease is the physiologically relevant form responsible for capsid maturation (Robertson et al., 1996).

To characterize this biologically relevant larger form of the protease, the isolation of recombinant full-length (75 kDa) hCMV protease and an initial in vitro comparison of its activity to that of the 29 kDa protease domain, including cleavage of capsid assembly protein has been performed. An assay was also developed in mammalian cells in which the target sequence of the protease (M-site) was fused inframe in a strong transactivator, the herpes simplex virus VP16 molecule. The position of the fusion is such that cleavage by the assemblin protease would release the transactivation domain of VP16 rendering the molecule unable to transactivate the *Escherichia coli lac Z* gene product that is under control of a VP16-responsive promoter. The results, using different substrates in both enzyme and mammalian cell-based assays, indicate that the 75 kDa form of the protease is significantly less active than the processed 29 kDa form.

2. Methods

2.1. Plasmids

pMON3375 was modified to create the VP16 molecule with the protease target sequence fused inframe using standard molecular biology techniques. This plasmid has the VP16 molecule under control of the mouse mammary tumor virus long terminal repeat (MMTV LTR) and has the SV40 virus polyadenylation signal 3′ of the coding region (Hippenmeyer and Highkin, 1993). Briefly, the HCMV M-site sequence replaced the *Sac*II–*Sal*I site near the 3′ end of the VP16 coding region. An extra *Sal*I site near the 5′ end of the coding region was first mutated by partial digestion and insertion of a multilinker. The HCMV UL80 maturation site was obtained using PCR of plasmid pMON22209 (from B. Holwerda) that contains the entire HCMV UL80 ORF. PCR primers were 5′-ATCCGCGGGAAAG-GTAGCTGAGCGC-3′ in the forward direction and 5′-ATATCGACAGCGACGCGGTAGC-GAG-3′ in the reverse direction. The reaction resulted in a product that has *Sac*II and *Sal*I sites at the 5′ and 3′ ends, respectively. The fragment was cloned into pCRII (Invitrogen, San Diego, CA) and sequenced using dideoxy nucleotides. The fragment was isolated by *Sac*II and *Sal*I digestion and ligated into the 3′ *Sac*II–*Sal*I site of linker-modified pMON3375. Digestion of the modified pMON3375 with *Sac*II and *Sal*I releases a 19 bp fragment. The 5′ *Sal*I site was reconstituted by swapping the *Kpn*I fragment from unmodified pMON3375. The resulting plasmid with the maturation site insertion was designated pMON15847. An additional plasmid with a single amino acid mutation in the HCMV UL80 maturation site was constructed in the same manner except that the substrate for the PCR step was pMON22237, which has the valine to glycine mutation at the P3 position.

The HCMV 29 Kd single-chain protease gene was modified to substitute valine to alanine at amino acid 141 and alanine to proline at amino acid 144 by site-directed mutagenesis (Promega, Madison, WI). The open reading frame was isolated from pMON27700 (S. Boyce, Pharmacia) by

digestion with *Nco*I and *Hin*dIII. The ends were made blunt by treating with Klenow polymerase. Plasmid pMON1440 (T. Warren, Pharmacia), which has the HCMV major immediate early promoter (− 760– + 3, relative to the initiation of transcription) followed by a unique *Bam*HI site and the SV40 virus polyadenylation signal, was digested with *Bam*HI and treated with Klenow polymerase. Ligation of the HCMV protease gene into pMON1440 resulted in pMON15848.

The HCMV UL80/80.5 open reading frame was obtained as a *Xba*I/*Hin*dIII fragment and the ends were made blunt using Klenow polymerase. The fragment was ligated into *Bam*HI digested pMON1440 that had also been made blunt as above. The resulting plasmid, pMON15855, has the open reading frame under control of the HCMV major immediate early promoter. The UL80/80.5 open reading frame has a His-6 tag at the amino terminus. In addition, several other changes were incorporated by site-directed mutagenesis to inhibit autocleavage. These include V141A and A144P (I-site), a serine to proline mutation at the R-site (P1′), an alanine to threonine at the R-site (P1 position) and an valine to alanine at the M-site (P3 position). In one construct, the active site serine (amino acid 132) was also mutated to alanine.

The gene encoding the HCMV major capsid protein (UL86) was isolated from MH17 (from W. Gibson, Johns Hopkins) by *Bgl*II digestion. The 4.1 kbp fragment was ligated into the *Bam*HI site of pMON1440 to yield pMON15854.

2.2. Purification of the 29 and 75 kDa proteases and CAP

The 29 kDa protease and the hCMV capsid assembly protein were expressed in *E. coli* and purified as previously described (Holwerda et al., 1994). The 75 kDa protease was also expressed in *E. coli*. *E. coli* cells were sonicated in 5 volumes (v/w) lysis buffer (100 mM sodium phosphate buffer, pH 6.3, with 300 mM NaCl and 20 mM imidazole) and refractile bodies recovered by centrifugation (20 min $5000 \times g$). Refractile bodies were washed twice by suspending in lysis buffer and centrifuging at $2000 \times g$ and then solubilized by the addition of lysis buffer containing 6 M urea. The resulting solution was applied to a nickel-charged nitrilotriacetic acid–agarose column equilibrated with lysis buffer containing 6 M urea. After washing the column with 4 M urea in 5 mM Tris buffer, pH 8.0, His-6 tagged proteins were eluted with a 0–100 mM linear imidazole gradient in 4 M urea, 5 mM Tris buffer, pH 8.0. Fractions were analyzed for protein (Bradford, 1976) and by gel electrophoresis in the presence of sodium dodecyl sulfate under reducing conditions using PhastSystem reagents and instrumentation (Pharmacia). The 75 kDa protease migrated as a 94-kDa band on 10–15% gradient or 20% homogenous gels stained with Coomassie blue, and was contaminated with a number of lower molecular weight species. Fractions containing 75 kDa protease were then applied to an anion-exchange HPLC column (DEAE 5 PW, TosoHaas) equilibrated with 4 M urea, 5 mM Tris buffer, pH 8.0, and eluted with a linear gradient of NaCl in this same buffer. The 75 kDa protease eluted at 40–50 mM NaCl as a $>90\%$ pure species as judged by gel electrophoresis. The protease was then refolded to an active form by adding solid dithiothreitol to a final concentration of 100 mM and dialyzing against lysis buffer containing 2 M urea, followed by dialysis against lysis buffer containing 0.1 mM dithiothreitol. Although initially $>90\%$ pure, incubation at room temperature overnight resulted in about 50% cleavage at the M-site, in spite of the V to A mutation at the P3 residue. To stabilize the preparation, convenient portions were rapidly frozen on dry ice and stored at -80 °C.

2.3. Protease assays

A chromogenic assay was used to routinely measure the activity of the 29 and 75 kDa proteases. Stock solutions of the substrate, succinyl–Ala–Gly–Val–Val–Asn–Ala–*p*-nitroanilide (custom synthesis, American Peptide Company) were prepared in DMSO. For K_m and k_{cat} determinations, nine parts protease (0.5–1.5 μM) in assay buffer (10 mM sodium phosphate, pH 7.4, 150 mM sodium acetate, 0.1% CHAPS,

10 mM dithiothreitol, 20% glycerol) was mixed with one part substrate in DMSO to give final substrate concentrations of 0–2 mM. The absorbance increase of 160 μl reaction mixtures in a 96-well plate was monitored at room temperature and at 405, 650 nm reference, using a plate reader (Vmax, Molecular Devices). Activity in AU min^{-1} was plotted against substrate concentration, V_{max} and K_m values determined by non-linear least squares analysis (Leatherbarow, R.J. (1998) GraFit Version 4.0, Erithacus Software Ltd., Staines, UK). A standard curve of *p*-nitroaniline in this same buffer yielded the factor 0.0047 AU μM^{-1}, allowing conversion of absorbance to μM product concentrations. Protein concentrations were determined by absorbance at 280 nm, using extinction coefficients calculated from amino acid composition (Gill and von Hippel, 1989).

CAP cleavage reactions were performed in 8 mM HEPES buffer, pH 7.4, with 0.08% Tween 20 and 10 mM dithiothreitol. Reactions were terminated either by adding an equal volume of 2 × sample buffer for electrophoresis samples, or by adding an equal volume of 1% acetic acid, followed by 2 volumes 20% acetonitrile, 0.1% trifluoroacetic acid in water for samples to be analyzed by HPLC. For gel electrophoresis, PhastSystem reagents and apparatus (Pharmacia) were used as described above. For HPLC analysis, a Phenomenex 'Jupiter' C-18, 250 × 4.6 mm, 5 m, 300 × column at 1 ml min^{-1} and room temperature was used. Solvents were 1 g l^{-1} trifluoroacetic acid in water (A) and 0.85 g l^{-1} trifluoroacetic acid in acetonitrile (B). Cleaved and uncleaved CAP were separated using a 21 min linear gradient from 10 to 52% solvent B in solvent A, and the peaks quantified by integration.

2.4. Inhibition of the 75 kDa protease by diisopropyl fluorophosphate

Enzyme in chromogenic assay buffer (see above) was inhibited by the addition of diisopropyl fluorophosphate (Sigma) in propylene glycol. Activity was measured in the chromogenic assay, the natural logarithm of residual activity plotted versus time, and rate of inactivation (k_{obs}) calculated from the slope of this plot. Rates appeared to be linear with inhibitor concentration in the range 0–10 mM, and the second order rate constant was estimated as the slope of the plot of k_{obs} versus inhibitor concentration.

Control and diisopropyl fluorophosphate-modified protease was digested with trypsin and the active-site peptide characterized by reversed-phase HPLC and mass spectrometry as described previously (Holwerda et al., 1994).

2.5. Cells

Vero cells were obtained from American Type Culture Collection (Rockville, MD) and were maintained in DMEM (Life Technologies, Gaithersberg, MD) containing 5% fetal bovine serum (Life Technologies). Cells were transfected with plasmid DNA using LipofectAMINE (Life Technologies) and OptiMEM (Life Technologies) as a source of serum free media. DNA concentrations used in the transfections were equalized by addition of pGEM-4Z. For 96-well plate assays, the wells were seeded with 3.5×10^4 cells per well. The following day, DNA mixtures were set up such that each well received 0.1 ug of reporter plasmid, 0.01 ug of VP16/M-site plasmid and, typically, 0.005 ug of protease expression plasmid in 100 ul OptiMEM/LipofectAMINE. DNA was applied to the wells for 6 h and then replaced with growth media for 48 h. For Western blots, 5×10^5 Vero cells were seeded in 30 mm dishes one day prior to transfection. Transfections utilized various combinations consisting of 5 ug of VP16, VP16/M-site or VP16/mutant M-site expression plasmid; 5 ug of the 29 or 75 kDa protease expression plasmid or 5 ug of the UL86 expression plasmid. Total DNA input was normalized to 15 ug total using pGEM-4z.

2.6. β-Galactosidase assays

Cells were washed once with Dulbecco's phosphate-buffered saline (Life Technologies) and lysed by addition of 1 × Reporter Lysis Buffer (Promega, Madison, WI) at room temperature for 15–30 min. The cells were then frozen for later

assay or used directly. Typically, 1 volume of the lysed cell extract was incubated with one volume of 2 × galactosidase reaction buffer (Promega) that contains ONPG (*o*-nitrophenyl-*b*-galactopyranoside) in a 96-well plate. Incubation times and temperatures vary as noted in figure legends. The reactions were stopped by 3 volumes of 1 M CAPS, pH 11. The optical density was determined using a spectrophotometer at 405–410 nm.

2.7. Western blots

Cells were lysed in 2 × sample buffer (Novex, San Diego, CA) supplemented with 10% (v/v) beta-mercaptoethanol and boiled 5 min. Cell extracts were applied to 16% polyacrylamide gel electrophoresis using Tris–glycine buffer. Proteins were transferred to PVDF membranes and probed with the designated primary antisera and secondary antisera conjugated with alkaline phosphatase (Promega) using the Promega ProtoBlot Western AP system. Molecular weight markers were SeeBlue (Novex). Rabbit antiserum against HSV VP16 (Triezenberg et al., 1988) was kindly provided by S. Triezenberg (Michigan State). Affinity purified rabbit antisera against the HCMV UL80 protease was supplied by B. Holwerda (Pharmacia). Rabbit antipeptide antiserum that recognizes the carboxy terminal region of VP16 was raised against amino acids 471–490 (Hippenmeyer and Highkin, 1993).

3. Results

3.1. Activity of the 75 kDa protease

Native full-length protease as been shown to undergo facile self-processing when expressed in recombinant host cells (for review, see Holwerda, 1997). Purification of recombinant full-length cytomegalovirus protease was facilitated by inhibiting cleavage at the release and maturation sites by site-directed mutagenesis, performing all purification steps under denaturing conditions, and refolding the protease under conditions non-optimal for activity (pH 6.3 and in the absence of glycerol).

To compare the activity of this refolded 75 kDa protease with the activity of the recombinant 29 kDa protease domain that expresses in soluble form in *E. coli*, it was important to establish the active concentrations of both preparations with an active site titrant. When the 75 kDa protease was completely inactivated by overnight incubation with 10 mM diisopropyl fluorophosphate, subsequent tryptic peptide analysis and mass spectrometry showed that 66% of the peptide containing the active site serine had been modified with a single residue of this inhibitor (data not shown). No other incorporation of inhibitor was observed. A similar complete inactivation of the 29 kDa protease with diisopropyl fluorophosphate resulted in 86% modification of the active site peptide. Subsequent activity determinations were corrected to reflect these active enzyme percentages.

Three separate measures (Table 1) suggested that the full-length 75 kDa protease had a lower activity than the 29 kDa protease domain. First, the protease cleaved a chromogenic *p*-nitroanilide peptide substrate based on the native M-site sequence 11 times more slowly than the 29 kDa enzyme, when k_{cat}/K_m values were compared. For the 29 kDa protease, the estimated K_m value (1.9 mM) was near the highest substrate concentration that could be used because of solubility (2 mM). For the 75 kDa protease, a completely linear relationship between activity and substrate over the range 0–2 mM was consistent with a K_m significantly greater than 2 mM. Since the individ-

Table 1
In vitro activity comparisons of 29 and 75 kDa hCMV proteases

Parameter	29 kDa	75 kDa
k_{cat}/K_m (min^{-1} mM^{-1})[a]	5.7	0.52
Inactivation rate (M^{-1} s^{-1})[b]	0.27	0.011
k_{cat} (min^{-1})[c]	19	3

[a] Measured with a *p*-nitroanalide substrate as described in Section 2.

[b] Second order rate constant for inactivation with diisopropyl fluorophosphate (see Section 2).

[c] Maximum cleavage rate measured with capsid assembly protein at 2–8 μM as described in Section 2.

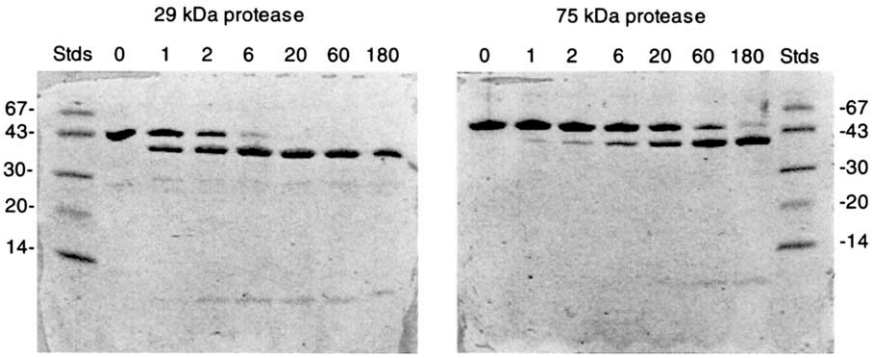

Fig. 1. Cleavage of HCMV capsid assembly protein by the 29 (left) and 75 kDa (right) forms of the HCMV protease. Incubation for the indicated times in minutes were as described in Section 2. Reaction mixtures included 5.3 μg ml^{-1} 29 kDa protease (0.18 μM) or 13.7 μg ml^{-1} 75 kDa protease (0.18 μM) and 398 μg ml^{-1} (10 μM) capsid assembly protein. Uncleaved 39 kDa capsid assembly protein migrates just above the 43 kDa standard and is cleaved into 33 and 6 kDa products. The 6 kDa product is visible as a faint band migrating below the 14 kDa standard.

ual kinetic constants could not be determined, only k_{cat}/K_m is reported in Table 1. It is suggested from these data, however, that at least part of the difference in activity between the two enzymes is accounted for by a decreased K_m for substrate of the 29 kDa compared to the 75 kDa form. The activity of the 75 kDa enzyme was not the result of contaminating *E. coli* proteins, since an identically purified and refolded preparation of 75 kDa protease in which the active site Ser-132 was changed to Ala (S132A) showed no activity (data not shown).

Second, the 75 kDa protease was inactivated less rapidly by diisopropyl fluorophosphate than the 29 kDa protease. The 25-fold slower rate of inactivation provided evidence that the activity of this preparation was not due to a small amount of contaminating 29 kDa protease, which might have resulted through self-cleavage. Any 29 kDa protease in the 75 kDa protease preparation should have been rapidly inactivated.

Finally, Fig. 1 shows a time course of CAP cleavage catalyzed by similar amounts of 29 or 75 kDa protease. It is evident that the 75 kDa protease catalyzes this reaction significantly slower. Quantifying the rate of cleavage by HPLC separation of reaction mixtures suggested that the K_m for this substrate was significantly less than 2 μM for both enzymes (the reaction velocity appeared constant in the substrate range 2–8 μM) and that the k_{cat} for the 75 kDa enzyme was about sixfold lower than the k_{cat} for the 29 kDa enzyme (Table 1). Others have reported a k_{cat} of 13 min^{-1} and a K_m of 3 μM for the 29 kDa enzyme under similar assay conditions (Pinko et al., 1995).

While it is difficult to unequivocally establish that the lower activity of the 75 kDa protease is not due to some defect in its structure resulting from the refolding process, the following observations suggest proper refolding and substrate recognition. First, no serine residues were modified by diisopropyl fluorophosphate other than the active site serine. Specific modification with diisopropyl fluorophosphate requires that the catalytic serine residue be properly positioned in the active site of the refolded protease. Second, comparing the relative rates of modification by diisopropyl fluorophosphate (25-fold), the rates of low molecular weight substrate cleavage (11-fold), and the rates of capsid assembly protein cleavage (sixfold), one observes that the difference between the 75 and 29 kDa enzyme preparations becomes progressively less as the modifier or substrate becomes more physiologically relevant (Table 1). This strongly suggests the proper folding of additional regions of the 75 kDa protease responsible for recognition of peptide sequences and the native substrate, capsid assembly protein. If these

substrate recognition regions had been improperly folded, a similar or greater difference in activity between the two proteases might have been expected for the physiological substrate. Finally, it should be remembered that the activity measurements given in Table 1 have been corrected for active site concentration as measured by reactivity with diisopropyl fluorophosphate, and thus represent a comparison between active as opposed to total protein in these preparations.

3.2. Transactivator cleavage assay—general scheme and VP16 fusion protein construction

To evaluate herpesvirus protease activity in mammalian cells, a transient assay was devised (Fig. 2a). Cells are transfected with a reporter plasmid that expresses *lac Z* under control of the VP16 responsive IE175 promoter from HSV-1. Cotransfection of a plasmid expressing a modified VP16, which has the HCMV M-site fused in-frame, results in transactivation of the reporter

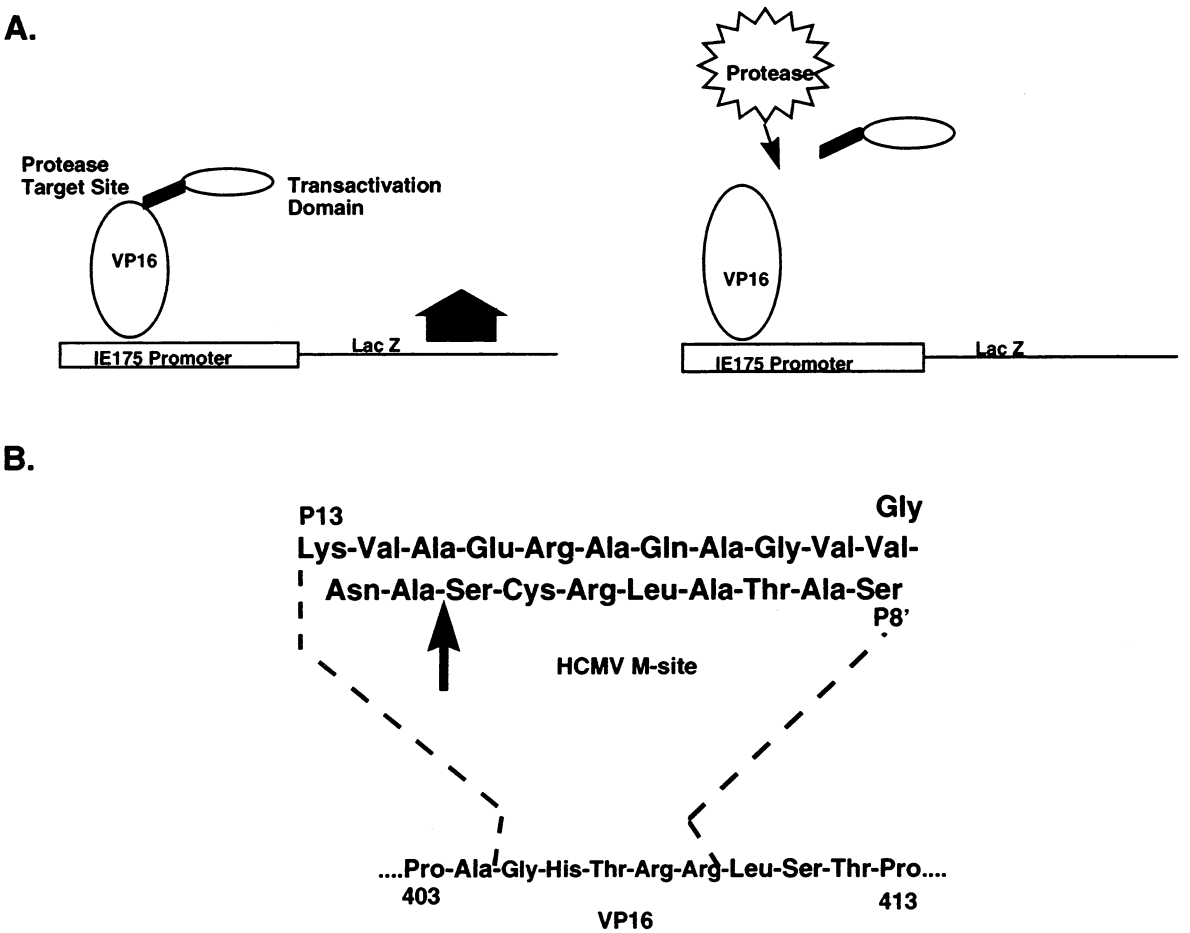

Fig. 2. Assay scheme and VP16/M-site fusion molecule. (A) Schematic of assay. The VP16/M-site fusion molecule is shown as two domains separated by the M-site protease target. Coexpression of the protease results in cleavage of the fusion transactivator leading to a decrease in β-galactosidase activity. It is not known whether the cleaved VP16/M-site molecule still binds the promoter as shown. (B) The fusion region of VP16/M-site is shown starting with amino acid 403 of the wild type VP16. Amino acids Gly–His–Thr–Arg–Arg were removed during insertion of the M-site sequences that begin with amino acid Lys and end with Ser. The vertical arrow indicates the cleavage site between Ala and Ser. The VP16/M-site mutant molecule has a Val to Gly substitution at position P3 as shown. The sequence extends beyond amino acid 413 to the natural termination at amino acid 490.

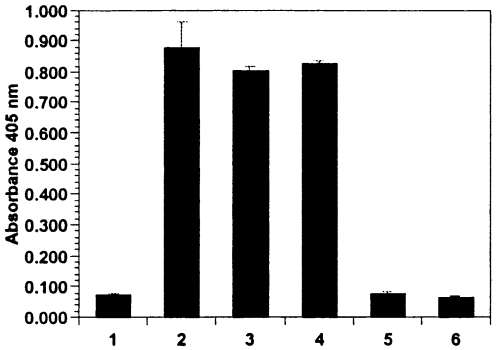

Fig. 3. HCMV 29 kDa protease expression decreases β-galactosidase activity. Cells were transfected with equivalent amounts of DNA containing 5 ug of IE175-β-gal (lane 1), IE175-β-gal with 0.5 ug of wild type VP16 (lane 2) or VP16/M-site plasmid (lane 3) and IE175-β-gal, VP16 and 0.5 ug of HCMV 29 kDa protease expression plasmid (lane 4), IE175-β-gal, VP16/M-site and 0.5 ug of HCMV 29 kDa protease expression plasmid (lane 5). IE175-β-gal with HCMV 29 kDa protease expression plasmid is shown as a control to show that the protease expression plasmid did not affect the reporter plasmid background (lane 6). Extracts were prepared 48 h post-transfection. Fifty ul was assayed from each dish for β-galactosidase activity. Each transfection was done in triplicate. Error bars represent the S.D.

plasmid. A triple transfection containing a third plasmid that expresses the HCMV protease results in cleavage of the modified VP16 transactivator such that it can no longer transactivate the reporter plasmid.

Two fusion proteins were generated using the herpes simplex virus VP16 transactivator and the HCMV UL80 maturation site sequences (Fig. 2b). The VP16/M-site fusion replaces amino acids 405–409 of wild type VP16 with the M-site sequence (lysine at P13 to serine at P8′). The VP16/mutant M-site fusion is identical to VP16/M-site, except that it has a valine to glycine mutation at position P3 of the M-site.

3.3. Transactivation activity of VP16 fusion protein and inhibition by expression of HCMV protease

Vero cells were transfected with different combinations of IE175-β-gal expression vector, the wild type VP16 expression vector, the VP16/M-site fusion and the HCMV protease vector and β-galactosidase activity measured 24 h later (Fig. 3). Transient expression of IE175-β-gal results in a small amount of β-galactosidase activity (Fig. 3, lane 1). This activity was stimulated by coexpression of either wild type VP16 (lane 2) or the VP16/M-site fusion (lane 3). Both transactivator expression plasmids stimulated β-galactosidase expression to the same degree indicating that the insertion of the HCMV M-site sequences was not deleterious to the transactivation ability of the VP16. Triple transfection of IE175-β-gal, the wild type VP16 and the HCMV 29 kDa protease plasmids (lane 4) resulted in β-galactosidase induction to the same extent as when the protease expression plasmid was omitted. There are no HCMV protease consensus cleavage sites in the β-galactosidase protein or the wild type VP16 protein. Coexpression of all three plasmids demonstrated that no cryptic or unidentified sites existed. Triple plasmid transfection of IE175-β-gal, the VP16/M-site fusion and the 29 kDa protease (lane 5) resulted in a decrease in the amount of β-galactosidase activity down to the level of IE175 alone. These results suggest that the 29 kDa HCMV protease is cleaving the VP16 fusion molecule at the HCMV M-site resulting in release of the strong transactivator domain of VP16.

3.4. Inhibition of β-galactosidase activity is dependent on the amount of protease plasmid

If expression of the protease is responsible for the reduction in β-galactosidase activity, then that effect should be titrated by varying the amount of protease expression plasmid in the cells. Cells were transfected as above but with differing amounts of HCMV 29 kDa protease plasmid. The amount of β-galactosidase activity in the cell extracts increases as the amount of protease expression plasmid decreases (Fig. 4). This is consistent with the effect seen in the experiment of Fig. 3 being mediated by the protease.

3.5. Inhibition of β-galactosidase activity is diminished by a maturation site amino acid substitution

If the decrease in β-galactosidase activity in the assay system is due to cleavage of the VP16 fusion

protein at the maturation site, then substitution of amino acids in this region should decrease the effect of the protease. The consensus cleavage sites of the herpesvirus proteases are highly conserved within the different groups of herpesviruses (Welch et al., 1991; Loutsch et al., 1994). Most α- and β-herpesviruses have a conserved valine at the P3 position of the maturation site (Welch et al., 1991; DiIanni et al., 1993). The effect of changing this valine to a glycine is shown in Fig. 5. While the ability of the mutated fusion protein to transactivate the target was not affected, addition of the 29 kDa protease expression plasmid to the transfection reduced β-galactosidase activity only 25–50% in different trials. These results are consistent with reports that changes in the P3 position decrease the ability of the protease to cleave the normal substrate (Welch et al., 1993; Sardana et al., 1994; B. Holwerda, personal communication).

Physical evidence of VP16 fusion cleavage was achieved in mammalian cells by overexpressing the different ORFs using the HCMV major immediate early promoter and detecting the products by Western blotting. Coexpression of the 29 kDa HCMV protease resulted in a decrease in the apparent MW of VP16/M-site ($M_r = 66{,}000$) to about 50 kDa (Fig. 6A and B, lane 6). This is consistent with cleavage at the HCMV maturation

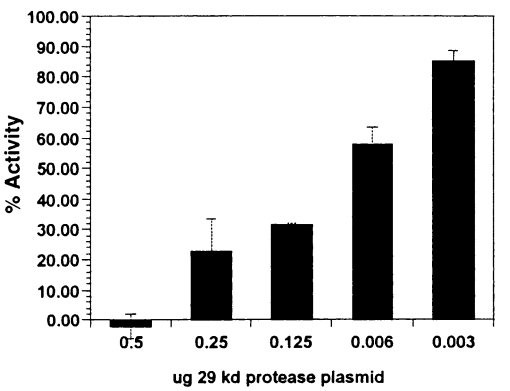

Fig. 4. Titration of the 29 kDa protease plasmid alters the β-galactosidase activity. Transfections were performed as detailed in Fig. 3 except that the amount of 29 kDa HCMV protease plasmid was altered as shown. Total DNA was normalized by addition of pGEM-4z DNA. Activity is expressed as calculated in Table 2.

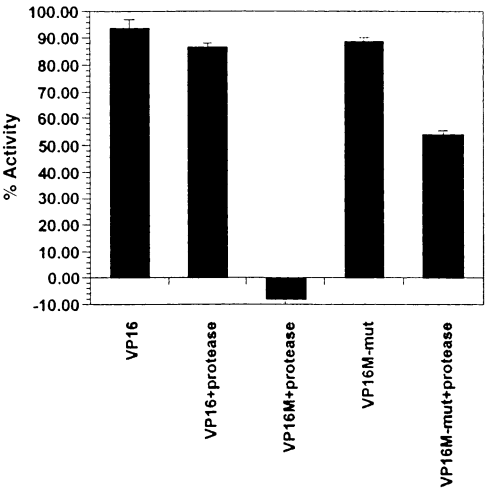

Fig. 5. A mutation in the P3 position of the M-site affects the β-galactosidase activity. Cells were transfected as in the legend to Fig. 3 with IE175-β-gal with the various additions shown. 0.5 ug of VP16/mutant M-site plasmid DNA (VP16-m-mut) was transfected. β-Galactosidase activity is calculated as shown Table 2.

site resulting in release of the last 88 amino acids. Wild type VP16 ($M_r = 65{,}000$) was not cleaved by the protease (Fig. 6A and B, lane 5). Coexpression of the 29 kDa protease and the VP16/mutant M-site resulted in partial cleavage of the transactivator. A small amount of uncleaved transactivator can be seen using antisera specific to the carboxyl terminal amino acids of VP16 (Fig. 6A lane 7). The cleaved transactivator appears the same as the VP16/M-site when visualized using antisera to the whole VP16 molecule (compare Fig. 6B, lanes 6 and 7). These conditions using the stronger promoters do not reflect the stoichiometries used in the β-galactosidase experiments because of the need to visualize the products. Therefore, no attempt has been made to correlate the amount of VP16/M-site cleavage in the β-galactosidase assays with the Western blot results.

3.6. Effects of full-length protease on the cleavage reaction

The full-length HCMV UL80/80.5 open reading frame was expressed to determine its ability to

cleave the VP16/M-site substrate. The version of the protease expressed has a His-6 tag at the amino terminus and mutations at the R- and M-site to prevent autocleavage as well as the mutations at the I-site. Previous studies have shown that cleavage of the R-site is not necessary for protease activity (Liu and Roizman, 1993; Welch et al., 1993; Matusick-Kumar et al., 1995a). Transfecting an equivalent amount (0.005 ug per well) of UL80/80.5 resulted in no decrease in β-galactosidase activity compared to the 29 kDa protease (Table 2). Increasing the amount of UL80/80.5 resulted in a small decrease in β-galactosidase activity to 84% of control at about three times the amount of DNA. The dramatic decrease in activity seen with higher amounts of DNA was due to the mass of plasmid transfected since a non-protease expressing control plasmid, pGEM-4z, had the same effect. Therefore, the full-length protease is not equivalent to the 29 kDa protease in this assay. It appears to have some activity but cannot be titrated to high enough levels to see consistent activity above background without causing non-specific effects. This is consistent with the present report in which the full-length protease has 6–11-fold lower activity (Table 1). This result was confirmed in a Western blot showing little or no cleavage of the VP16/M-site substrate with the 75 kDa protease (Fig. 7A, lane 3). However, there appears to be a small amount of autocleavage of the 75 kDa protease at its own M-site showing that the protease is active, despite a M-site mutation. As previously mentioned, cleavage was also observed to take place at the M-site in purified 75 kDa protease preparations (Fig. 7B, lane 3). Addition of supplemental 29 kDa protease results in complete cleavage of the VP16/M-site substrate (Fig. 7A, lane5) and the 75 kDa protease M-site (Fig. 7B, lane 5). Western blotting of the 29 and 75 kDa proteases suggests that at equal DNA transfection reactions, the expression of the 29 kDa protease is stronger than the 75 kDa protease. This may explain some of the loss of activity of the 75 kDa species under these conditions or reflect faster turnover of the 75 kDa form, however, titration with increasing amounts of UL80/80.5 did not result in cleavage of the VP16/M-site substrate within the range of the assay.

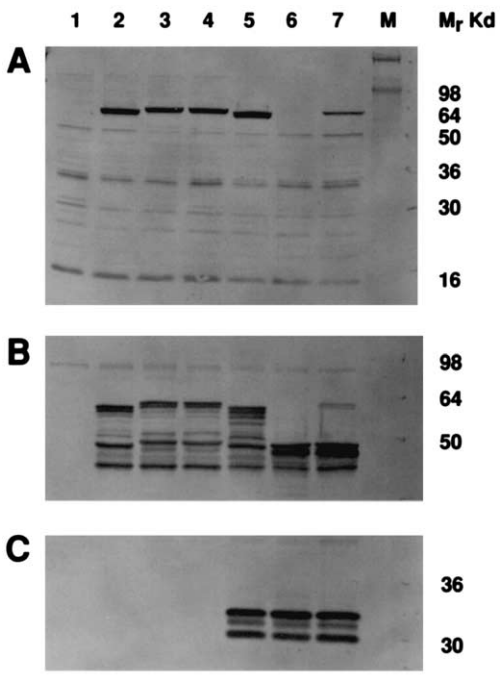

Fig. 6. Western blot of transfected cells. Blots probed with anti-peptide antisera against the carboxyl terminal amino acids of VP16 (panel A); probed with antisera generated against the entire VP16 molecule (panel B) or probed with anti-protease antisera (panel C). Lane 1, mock transfected cells; lane 2, transfected with the IE175-β-gal and wild type VP16; lane 3, transfected with IE175-β-gal with VP16/M-site; lane 4, transfected with VP16/mutant M-site; lanes 5–7 are lanes 2–4 with 29 kDa protease expression plasmid. M was loaded with SeeBlue molecular weight markers.

3.7. Effects of the major capsid protein on protease activity

Biological data concerning the action of the herpesvirus proteases suggests that cleavages occur just prior to or during the formation of capsids (Gao et al., 1994). Therefore, cleavage must not take place in the cytoplasm during synthesis in a normal infection. It has also been shown the carboxyl terminal amino acids of the full-length open reading frame interacts with the major capsid protein in herpes simplex virus and cytomegalovirus assembly (Desai and Person, 1996; Hong et al., 1996; Kennard et al., 1995; Matusick-

Table 2
Activity of 75 kDa protease on VP16/M-site cleavage as measured by β-galactosidase activity

% β-Galactosidase activity[a]			
ug per well transfected	pGEM-4z control	75 kDa protease	29 kDa protease
0.005	90.0	105.2	7.15
0.017	95.0	83.8	NT
0.33	0	0	NT
0.5	0	0	NT

Data from two independent determinations done in triplicate. NT, not tested.

[a] Calculated as β-galactosidase activity in the presence of (protease, VP16/M-site and IE175-β-gal plasmids) minus (the activity of the IE175-β-gal plasmid) divided by the activity of in the presence of (VP16/M-site and IE175-β-gal plasmids) minus the (IE175-β-gal plasmid) times 100.

Kumar et al., 1995b; Thomsen et al., 1995; Wood et al., 1997). Therefore, one possibility is that the interaction with the major capsid protein may result in an inactive protease until assembly occurs in the nucleus. Alternatively, since the full-length protease is less active than the 29 kDa form, interaction with the major capsid protein may stimulate activity in the context of the developing capsid. To test this possibility, either form of the protease was transfected into cells in the presence or absence of a plasmid expressing the major capsid protein of HCMV. The results show that there was no inhibition of the 29 kDa form of the enzyme by coexpression of UL86 ORF at equimolar or 10-fold higher concentrations (data not shown). These results were confirmed by Western blotting in which cotransfection of the UL86 expression vector did not enhance the 75 kDa protease activity on the VP16/M-site molecule (Fig. 7, lane 6). Nor did transfection of the UL86 expression plasmid inhibit the cleavage of the VP16/M-site molecule by the 29 kDa protease (Fig. 7, lane 7).

4. Discussion

The cytomegalovirus genome has an open reading frame, UL80, whose products are essential for virus replication in that they supply structural information and possess protease activity required for capsid assembly and maturation. Owing to its ease of expression and purification, the 29 kDa recombinant protease domain is currently the exclusive protease target for development of antiviral agents (Abood et al., 1997; Baum et al., 1996a,b; Chu et al., 1996; Flynn et al., 1997a,b; Jarvest et al., 1996; Pinto et al., 1996; Siegel et al., 1998). In virally infected cells, however, the protease is only produced as a fusion with the capsid assembly protein and must remain fused to the assembly protein domain to be packaged into the immature nuclear capsids. The assembly protein domain has nuclear localization sequences (Plafker and Gibson, 1998) and sequences that promote self-assembly and interaction with the major capsid protein (Pelletier et al., 1997; Wood et al., 1997). Thus, cleavage at the R-site prior to packaging would leave the protease stranded in the cytoplasm. Transcomplimentation studies with mutant HSV-1 viruses have shown that while cleavage at the R-site is required for viral replication, this cleavage occurs after capsid assembly, and the released protease domain does not need to be active, implying a function other than catalytic (Matusick-Kumar et al., 1995a; Robertson et al., 1996). Thus, the proteolytic activity essential for capsid maturation appears to be provided by the full-length product of the protease-assembly protein open reading frame.

This report is the first to describe the purification and in vitro activity of the full-length product of the hCMV UL80 open reading frame. Purification was facilitated by incorporation of a poly-His sequence at the N-terminus and by mutation of the I, R, and M cleavage sites. The effect of these

modifications on activity was not investigated in the present study. However, recombinant poly-His and native hCMV protease domains are reported to have similar activities (Holwerda et al., 1994). Additionally, the X-ray crystal structure of the poly-His protease (Shieh et al., 1996) shows no obvious differences compared to protease lacking the N-terminal poly-His sequence and the first 7–8 amino acids of the native sequence are disordered in all the structures reported (Chen et al., 1996; Qiu et al., 1996; Tong et al., 1996). These observations suggest little effect of the N-terminal poly-His sequence on structure or activity. Various cleavage site mutations also appear to have little effect on activity. Mutation of the I site has been shown to have no effect on activity of the protease domain (O'Boyle et al., 1995). It has also been observed in transfected cell assays that a wide variety of deletions or mutations of the R- or M-site in the full-length protease failed to affect its ability to cleave substrates containing native R- or M-sites (Godefroy and Guenet, 1995; Jones et al., 1994; Liu and Roizman, 1993; Weinheimer et al., 1993; Welch et al., 1993).

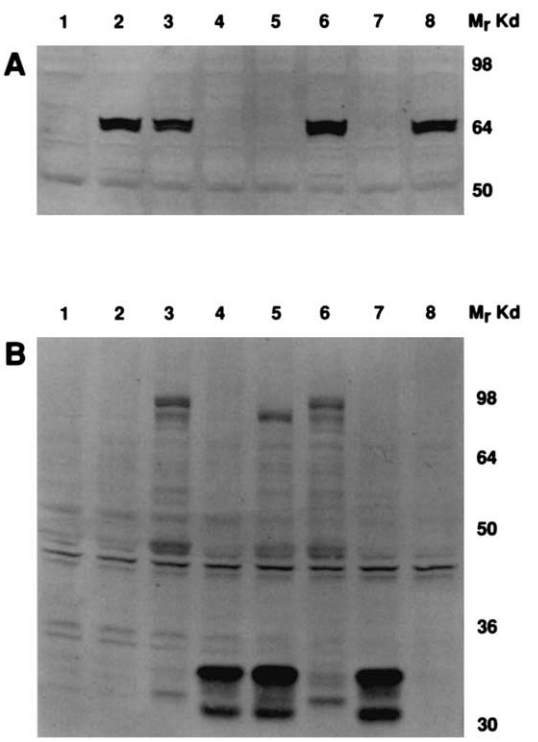

Fig. 7. Western blot analyzing 75 kDa protease activity. Cell extracts were transferred to PDVF paper and probed with antisera generated against the carboxy terminal amino acids of VP16 (A) or the protease (B). Lane 1, mock infected; lane 2, VP16/M-site; lane 3, 75 kDa protease and VP16/M-site; lane 4, 29 kDa protease and VP16/M-site; lane 5, 75 kDa protease, 29 kDa protease and VP16/M-site; lane 6, same as lane 3 but with UL86; lane 7, same as lane 4 with UL86; lane 8, UL86 with VP16/M-site.

Compared to the 29 kDa protease, the purified full-length 75 kDa hCMV protease was shown to have significantly lower activity in the present study, whether assayed using a synthetic paranitroanilide peptide substrate, hCMV capsid assembly protein, M-site cleavage in mammalian cells or as judged by its slower inactivation by diisopropylfluorophosphate. Because the 29 kDa protease was expressed in soluble form and the 75 kDa protease required refolding, activity comparisons were made based on active enzyme concentrations established by extent of modification with an active site titrant. Proper folding of the active 75 kDa protease was also suggested by the progressively smaller difference in activity between the 75 and 29 kDa enzyme preparations as the modifier or substrate became more complex and physiologically relevant (Table 1).

The 25-fold slower active site acylation and inactivation of the 75 kDa protease with diisopropyl fluorophosphate compared to the 29 kDa protease may have important implications for the design of antiviral protease inhibitors. Low molecular weight inhibitors that appear to acylate the active site serine of the 29 kDa protease in vitro have been identified by several laboratories (Pinto et al., 1996; Jarvest et al., 1996; Abood et al., 1997; Flynn et al., 1997a). The present results suggest that the 75 kDa protease may be less efficiently inhibited by these types of inhibitors than suggested by their reactivity with the 29 kDa form.

A lower activity of the full-length protease in which the protease domain is extended by the native 46 kDa C-terminal assembly protein domain is consistent with other observations. Darke et al. (1994) observed that the activity of the HSV-1 protease domain extended 59 amino acids beyond the R-site, increased 21-fold upon re-

moval of this extension. Others have observed that addition of a purification tag at the C-terminus completely inhibited activity of the 29 kDa hCMV protease (Smith et al., 1994). The activity of the protease has been shown to increase dramatically upon dimerization (Darke et al., 1996; Margosiak et al., 1996). The X-ray structure of the protease domain dimer suggests that the two assembly protein domains would extend from the same side of the oligomer (Chen et al., 1996; Qiu et al., 1996; Shieh et al., 1996; Tong et al., 1996) and could interact with each other, possibly influencing dimerization and activity.

As mentioned above, little is known about the coordinated regulation of the protease activity within the infected cell. Therefore, a transient protease assay system was engineered similar to the system reported by Lawler and Snyder (1999) to determine the effects of the natural mammalian cell environment on the activity of the protease in a quantitative fashion. In this assay system, the full-length protease did not have any detectable proteolytic activity toward the VP16/M-site target despite the fact that it could autocleave at its own M-site. One possibility is that the full-length protease is too slow, relative to the 29 kDa protease domain, to be accurately quantitated in this assay. Another possibility is that the engineered M-site in the VP16 molecule is not optimum for cleavage by the full-length protease. This implies that the two forms of the protease are sensitive to the structural information surrounding the cleavage site to different degrees. Lastly, there may be localization differences between the short and long forms relative to the VP16/M-site transactivator that preclude efficient cleavage by the full-length protease. If the 75 kDa protease preferentially resided in the cytoplasm, it might suggest that the nuclear localized VP16 substrate might not be as accessible for cleavage. Therefore, it would appear that the 29 kDa protease had better activity. However, it has been well documented that the full-length ORF protein is localized to the nucleus while the protease domain appears to be approximately equally localized to the nucleus and cytoplasm (Matusick-Kumar et al., 1995a; Robertson et al., 1996; Wood et al., 1997; Plafker and Gibson, 1998). This does not preclude the possibility that the full-length protease proceeds to form higher order structures in the nucleus that may limit accessibility to the VP16/M-site substrate. Cotransfection of the HCMV major capsid protein did not have any positive effect on the cleavage of the VP16/M-site fusion by the full-length protease. Previous results with the yeast two-hybrid system and glutathione *S*-transferase fusions showed that the major capsid protein interacts with the carboxyl terminal region of the capsid assembly protein and the full-length protease reading frame but not the smaller 29 kDa protease domain (Desai and Person, 1996; Hong et al., 1996; Kennard et al., 1995; Matusick-Kumar et al., 1995b; Wood et al., 1997). Whether the full-length protease is interacting with the major capsid protein in the mammalian cell under the conditions in the present report is difficult to ascertain. However, the major capsid protein does not appear to stimulate the activity of the full-length protease to the level of the 29 kDa protease domain.

The transactivator cleavage assay results indicate that the HCMV protease domain can cleave its M-site target even when that target is embedded in a heterologous protein. The size of the target sequences required for the cleavage with respect to the P and P′ sites have not been determined in this context and may reflect on the differences in efficiency of cleavage between the 29 kDa and full-length protease. However, the lower efficiency of cleavage by the full-length protease in this report is consistent with in vitro data and suggests that cellular factors do not enhance that activity. Because this is a virus-free system, we cannot rule out the possibility that virus infection induces a cellular protein that regulates the activity of the protease. Cotransfection of additional HCMV genes may reveal important regulatory interactions. In addition, a single amino acid change in the M-site conserved sequence results in a decrease in cleavage in the fusion protein, again consistent with previous results (Sardana et al., 1994). The R-site sequences or I-site sequences may also be good substrates in the fusion with VP16. Interestingly, Lawler and Snyder used a smaller M-site linker in the context of their GAL4 transactivator cleavage assay consisting of

GVVNA↓SCRL (Lawler and Snyder, 1999) in conjunction with the simian cytomegalovirus full-length protease. However, since the protease construct was able to autocleave down to the smaller domain, it is not clear whether the large form or the short form of the protease was responsible for the cleavage of the transactivator substrate. Due to differences in quantitation and the transactivator used in the two transactivator cleavage systems, it is difficult to determine whether one linker is cleaved more efficiently in the mammalian cell.

By several criteria our results show that the full-length HCMV protease is not as efficient as the shorter protease domain. While this may have important implications for inhibitor design, how this difference in activities is regulated in herpesvirus replication has yet to be elucidated.

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