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Autoprocessing of HSV-1 protease: effect of deletions on autoproteolysis

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Abstract HSV-1 protease is involved in virus maturation. It is autoproteolytic and processed from a larger precursor. We have analysed the autoproteolysis of UL26 ORF and shown by in vitro-coupled transcription and translation that the UL26 encoded protein is processed, leading to the accumulation of its N-terminal domain. The deletion of the residues 245–248 in UL26 ORF abolishes the N-terminal cleavage but not the C-terminal processing. Deletion of the 3′ end of UL26 prevents production of the mature protease. These results strongly suggest that autoproteolysis is achieved in a defined order.

Key words: HSV-1 protease; UL26 open reading frame; Autoproteolysis; In vitro transcription translation

1. Introduction

Herpes viruses have a common assembly pathway. Recent reports have demonstrated the involvement of a protease, the role of which is critical for virus maturation. The same maturation mechanism was also reported for cytomegalo [1,2] and pseudorabies viruses [3] and is suspected to be shared by all members of the herpes virus family [1]. The identification and characterisation of a herpes simplex virus gene product required for encapsidation of viral DNA was first described [4] through the isolation of a temperature-sensitive (*ts 1201*) HSV-1 mutant. This mutant fails to encapsidate viral DNA at the non-permissive temperature. Protein analyses of full and empty capsids showed that full capsids consist of infected cell protein 35 (ICP 35) while empty capsids consist of unprocessed assembly protein precursor [5,6]. HSV-1 genome sequencing [7] has assigned the coding domain of ICP 35 to the UL26 open reading frame. It was demonstrated [8] that UL26 contains two transcriptional units leading to two proteins sharing amino acids. HSV-1 UL26 ORF has been shown [9] to encode a protease responsible for assembly protein precursor maturation. Several groups have concluded that the proteolytic activity of UL26 ORF is located in the N-terminal part and that two cleavages are necessary to produce the mature protease [9–14]. A third cleavage site in the catalytic region of the protease encoded by human cytomegalovirus UL80 ORF has also been identified [2,15].

Attempts to identify the proteolytic cleavage sites have been reported previously using different approaches. For HSV-1, autoprocessing in *Escherichia coli* by expression of a fusion or a non-fusion HSV-1 protease has been described [11,12]. Some mutagenesis studies have also been performed to try to identify the residues implicated in the active site [13,14]. The use of different protease inhibitors has suggested that HSV-1 protease belongs to the serine protease family [13] and more recently Ser^{129} was shown to be part of the active site [16].

In order to characterise the autoprocessing cascade and analyse the amino acid requirements for maturation of UL26 ORF, we have produced the entire protein encoded by UL26, the N-terminal of UL26, and the region corresponding to the first 329 amino acids of UL26. We demonstrate by in vitro transcription and translation that the UL26 encoded protein is processed, leading to the accumulation of its N-terminal domain. The deletion of the 4 residues 245–248 in UL26 allowed us to confirm the necessity of this sequence for the N-terminal cleavage. We also show that the N-terminal cleavage sequence is necessary for production of the mature protease but is dispensable for the C-terminal processing of UL26 ORF. And finally the 329 amino acid protein extending from the N-terminal to 82 amino acids beyond the cleavage site was not able to perform the autoproteolysis necessary to release the mature protease. These results strongly suggest that autoproteolysis is achieved in a defined order.

2. Material and methods

2.1. Plasmid construction

All DNA manipulations were carried out according to standard procedures [17]. Restriction enzymes were from New England Biolabs, amplification reagents were from Perkin-Elmer Cetus. The coding sequence of the HSV-1 assembly protein precursor (1.9 kb) and of the HSV-1 protease (0.74 kb) were obtained by amplification of pGX142 (a gift of Dr. V. Preston, MRC Virology Unit, Glasgow, UK) using PCR. pGX142 contains the entire UL26 DNA sequence. Oligonucleotides corresponding to the 5′ and 3′ ends of both the protease precursor and the protease, with extensions containing restriction sites for cloning, were synthesised on a 381A Applied Biosystem DNA synthesiser. Primer sequences used for PCR amplification were (5′ GGGAATTCAGGAATATAGATCTTATGGAGCCGATGCCCCG 3′) and (5′ TTAAAGCTTAGATCTTCATCAGCGGGCCCCCATCATCTG 3′) for HSVA construction. For HSVB, the same forward primer was used but the reverse primer was (5′ TTAAAGCTTAGATCTTCATCACGCCTGGAGGTAGGTGTG 3′). PCR reactions were performed for 30 cycles of 3 min at 95°C, 2 min at 68°C and 1 min at 72°C. Before amplification, primers were placed for 10 min at 100°C and then 5 min on ice to avoid primer annealing [18]. The resulting amplification products were gel-purified, digested with *Eco*R1 and *Hin*dIII and subcloned in pBS (Stratagene) linearised with the same endonucleases. The resulting recombinant plasmids were pBSHSVA encoding amino acids 1–635 and pBSHSVB encoding amino acids 1–247 of the protease. Sequence analysis of the two plasmids was performed on a 373A Applied Biosystem DNA sequencer with the Taq Dyedeoxy Terminator Cycle reagents (Applied Biosystems). The amplification conditions were adjusted to accomodate the GC-rich content of the DNA and were as follows: 1 min at 96°C, 15 s at 65°C and 4 min at 60°C for 25 cycles.

For in vitro transcription and translation, the *Bgl*II fragments from pBS recombinant plasmids were inserted in *Bam*HI-digested pSPT18 vector (Boehringer-Mannheim) giving pSPTHSVA and pSPTHSVB. A mutant HSVA construct was produced by replacing the *Hpa*I–*Apo*I fragment in pSPTHSVA by 2 complementary oligonucleotides leading

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Abbrevations: ICP35, infected cell proteins 35; HSV-1, Herpes simplex virus type 1; ORF, open reading frame; CMV, cytomegalovirus.

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to pSPTHSVAΔLQAS. These 2 oligonucleotides are identical to the original fragment but without nucleotides corresponding to amino acids 245–248 which encode the putative cleavage site LQAS (Fig. 1). Plasmid pSPTHSVG encoding residues 1–329 was obtained by removing the *Bam*HI–*Hin*dIII fragment. The resulting constructs pSPTHSVAΔLQAS and pSPTHSVG were sequenced using the same conditions as the original construct.

2.2. In vitro transcription and translation

In vitro transcription and translation were performed simultaneously by using the TNT-coupled reticulocyte lysate system (Promega). Briefly, 5 μg of circular plasmid pSPTHSVA, pSPTHSVB, pSPTHSVG or pSPTHSVAΔLQAS were incubated with 25 μl rabbit reticulocyte lysate according to the manufacturer's protocol in the presence of SP6 polymerase and 40 μCi [^{35}S]methionine at 1000 Ci/mmol (Dupont-NEN). A negative control without DNA template, or a positive control (supplied by Promega), were performed in parallel. In all assays, the final volume was 50 μl and the incubation was for 2 h at 30°C. For analysis of the processing of the translated proteins, aliquots of 12.5 μl were removed at different times during the reaction. In some experiments, the transcription/translation reaction mix was further incubated at 37°C up to 18 h. After incubation, products of translation were resolved on a 12.5% denaturant polyacrylamide gel [19] and analysed by autoradiography.

3. Results and discussion

3.1. In vitro processing of HSV-1 protease

HSV-1 UL26 ORF was obtained by amplification of pGX142 and subsequently subcloned into pSPT18 in order to transcribe and translate the ORF. Two different constructs, pSPTHSVA and pSPTHSVB, were made. pSPTHSVA contains the entire UL26 ORF while pSPTHSVB encodes the N-terminal part of UL26 up to amino acid 247 after which a termination codon was inserted (Fig. 1). A similar construct coding for residues 1–247 has been used previously in transfection and infection experiments [14].

Protein products present in the translation mix were analysed by gel-electrophoresis at 30 min intervals. The size of the translation products of pSPTHSVA coding for the entire UL26 ORF varied with time. After 30 min, the first protein product of apparent MW 66 kDa was synthesised (Fig. 2A, lane 4). A limited number of lower molecular weight proteins appeared with time. Five labelled proteins were detected: 2 migrated to around 66 kDa, 2 to 45 kDa and 1 to 30 kDa (Fig. 2A, lanes 3–6). These labelled proteins were all specific to the transcription and translation of UL26 as they did not appear in either the negative or the positive control samples even after prolonged incubation at 37°C. These five proteins correspond to the calculated molecular weights of full-length and proteolytic products of UL26 ORF, assuming cleavage occurs between residues 247–248 and between residues 610–611 resulting in proteins of 66,470, 63,881, 39,869, 37,280 and 26,619 Da. When the translation mix was incubated at 37°C for more than 2 h, the 66 kDa protein disappeared accompanied by a corresponding increase in the smaller proteins, suggesting that these proteins represent the final proteolytic products (Fig. 2B, lane 1).

In vitro transcription/translation of pSPTHSVB, which encodes amino acids 1–247, resulted in the synthesis of a protein with a relative gel mobility inferior to 30 kDa within 30 min which accumulated over the time period (Fig. 2A, lanes 7–10). The relative mobility of this protein is in good agreement with the calculated molecular weight (26 619 Da) for amino acids 1–247 of UL26 ORF. Upon further incubation no additional bands appeared but there was an accumulation of the 30 kDa protein (Fig. 2A, lane 10). The translation product of pSPTHSVB co-migrates exactly with the shortest protein obtained from pSPTHSVA coding for the complete UL26 ORF, demonstrating that the 30 kDa autoproteolytic product of UL26 ORF corresponds to the N-terminal 247 amino acids of the precursor protein (Fig. 2A, lanes 6 and 8). This 30 kDa protein appears as a doublet in most experiments. Doublet bands have been reported and have been characterised individually under the name of ICP35c, ICP35d, ICP35e and ICP35f [5]. The origin of these doublets is still not clear. It was postulated that they arose from initiation at Met^{1} and Met^{10} [8,9]. More recently, the region responsible for the doublet formation was located between residues 307 and 417 by the use of different cDNA constructs and observing the behaviour of the expressed proteins by gel-electrophoresis. However, there was no evidence of a protein doublet formed from the 1–247 construct [14]. Under our conditions, a faint band appears under the major 30 kDa protein either after the processing of the 635 residue protein or when the 30 kDa protein is synthetised directly from pSPTHSVB (Fig. 2A, lanes 8–10, and Fig. 2B, lane 2). The two bands appear to be related as they increase in parallel. We cannot explain the origin of this additional band. It could result from some specific or non-specific proteolysis or be a migration peculiarity of HSV-1 protease. Prolonged incubation of the translational reaction mixture of pSPTHSVB does not result in any additional processed proteins (Fig. 2B, lane 2), as has been shown for human cytomegalovirus UL80 ORF expressed in *E. coli* [2]. The third cleavage site in UL80, leading to 2 fragments of 13 and 16 kDa, appears to be specific to cytomegalovirus protease as no form shorter than 247 residues has ever been reported for HSV-1 protease.

Effects of protease inhibitors on the processing of UL26 ORF have been studied after inhibition of translation by cycloheximide [13]. In our experiments, we did not stop translation in order to visualise the products formed during translation and processing of UL26 ORF. Most of the reported translation experiments were performed with UL26 ORF and with UL26.5

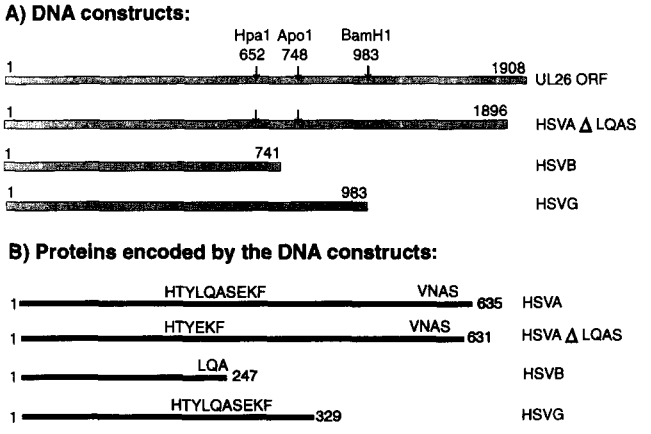

Fig. 1. Schematic maps of the constructions. (A) DNA constructs for HSVA, HSVB and HSVG. Numbers refer to the nucleotide sequence, 1 being the first A of the initiating methionine. HSVAΔLQAS results in the replacement of the *Hpa*I–*Apo*I fragment by 2 synthetic oligonucleotides. (B) Predicted coding domains of the 4 constructs. Numbering refers to amino acid, 1 being the initiating methionine. The difference in the coding domain between HSVA and HSVAΔLQAS is indicated. HSVB ends at Ala^{247} and HSVG encodes amino acids 1–329. The C-terminal cleavage site, V608-N609-A610-S611, is indicated.

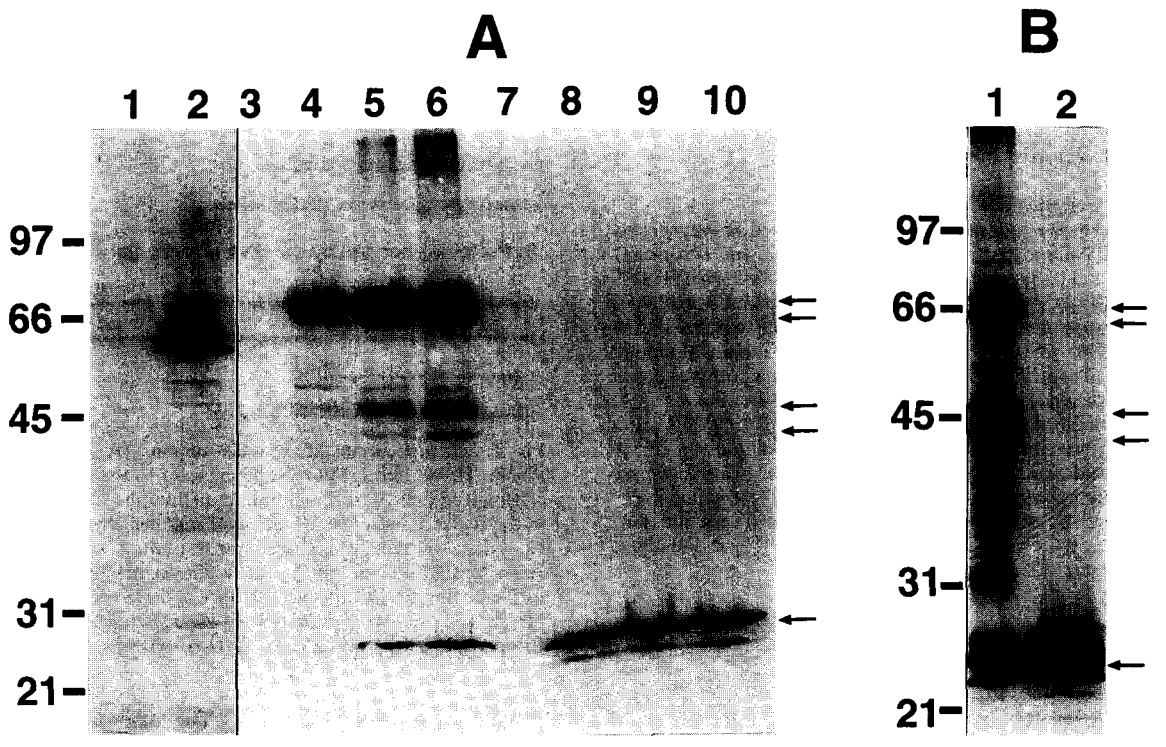

Fig. 2. In vitro transcription and translation of pSPTHSVA and pSPTHSVB. (A) Lanes: 1, negative control; 2, positive control after 2 h of incubation at 30°C; 3–6, proteins synthesised after transcription/translation of pSPTHSVA analysed at time 0, 30, 60, 90 min; 7–10, translational products of pSPTHSVB at time 0, 30, 60 and 90 min. (B) Lanes 1 and 2, result of transcription/translation of, respectively, pSPTHSVA and pSPTHSVB after an incubation of 22 h at 37°C. The molecular weights are indicated in kDa. Proteins resulting from expression and processing of UL26 ORF are indicated by an arrow.

ORF which encodes residues 307–635 [8]. Translation of the shortest HSV-1 protease corresponding to amino acids 1–247 has not been reported previously.

The same type of construct was studied for the HCMV by expression in *E. coli* by radio-pulse-labelling and by coupled transcription/translation in bacterial S30 extracts [2]. From that study, an autoproteolytic cleavage was clearly demonstrated when experiments were performed by radiolabelling in total *E. coli* cells compared to bacterial S30 extracts. For the simian cytomegalovirus [1], trials of transcription/translation of the full length protease precursor in a rabbit reticulocyte system did not result in autoproteolysis. The conclusion was that maturation of the protease required an activation step which can occur in vivo but not in vitro. In our experiments, UL26 ORF is fully processed in vitro in a time-dependent manner. Our experiments demonstrate that the first cleavage occurs at the C-terminus. Following the release of active protease, both cleavages occur in parallel, and this is demonstrated by the presence of the 2 bands around 45 kDa. Our in vitro expression system permits autoproteolytic processing and can be used to analyse deletions or modifications that affect these events.

3.2. Deletion of the N-terminal cleavage site

Different approaches have been used to identify both cleavage sites in HSV-1 protease. Proteolytic fragments of UL26 expressed in *E. coli* were identified by N-terminal peptide sequencing [11] and site-directed mutagenesis of the putative N-terminal cleavage site (Ala^{247}Arg–Ser^{248}Pro) resulted in the absence of proteolysis [14]. We approached the question by removing the entire sequence Leu^{245}-Gln^{246}-Ala^{247}-Ser^{248} and analysing the results of the deletion by in vitro transcription and translation. Experiments were carried out in parallel for pSPTHSVA and pSPTHSVAΔLQAS using the same conditions. When translation products were analysed immediately after the reaction, both constructs produced the same labelled proteins (Fig. 3A, lane 2 and 7). To investigate the autoproteolysis of the mutated construct, the same experiment was repeated but the translational mixes were incubated for different times at 30°C in parallel with a positive control which was treated in the same manner. During incubation, the unmutated clone underwent autoprocessing but in the mutated clone only the 60 kDa form was formed (Fig. 3A, lanes 1–10). The C-terminal cleavage occured but no shorter forms were detected. This result is in good agreement with the result found following substitution of Ala^{247}–Ser^{248} by Arg–Pro [14] where C-terminal cleavage was observed. This means that the protease is still active but cannot be completely processed. The fact that the protease is active is not surprising since the catalytic domain is untouched. Site-directed mutagenesis of Glu^{114} and Glu^{115} produced an inactive protease [14], and HSV-1 protease labelling allowed the identification of Ser^{129} as part of the active site [16]. From our experiments, we can conclude that LQAS is the N-terminal cleavage site and is necessary for the processing of UL26 into its mature form encompassed by residues 1–247. However, this sequence is not necessary for the C-terminal cleavage to occur.

3.3. Deletion of the C-terminal domain

In an attempt to determine if the presence of only the N-terminal cleavage site of the protease was sufficient for matura-

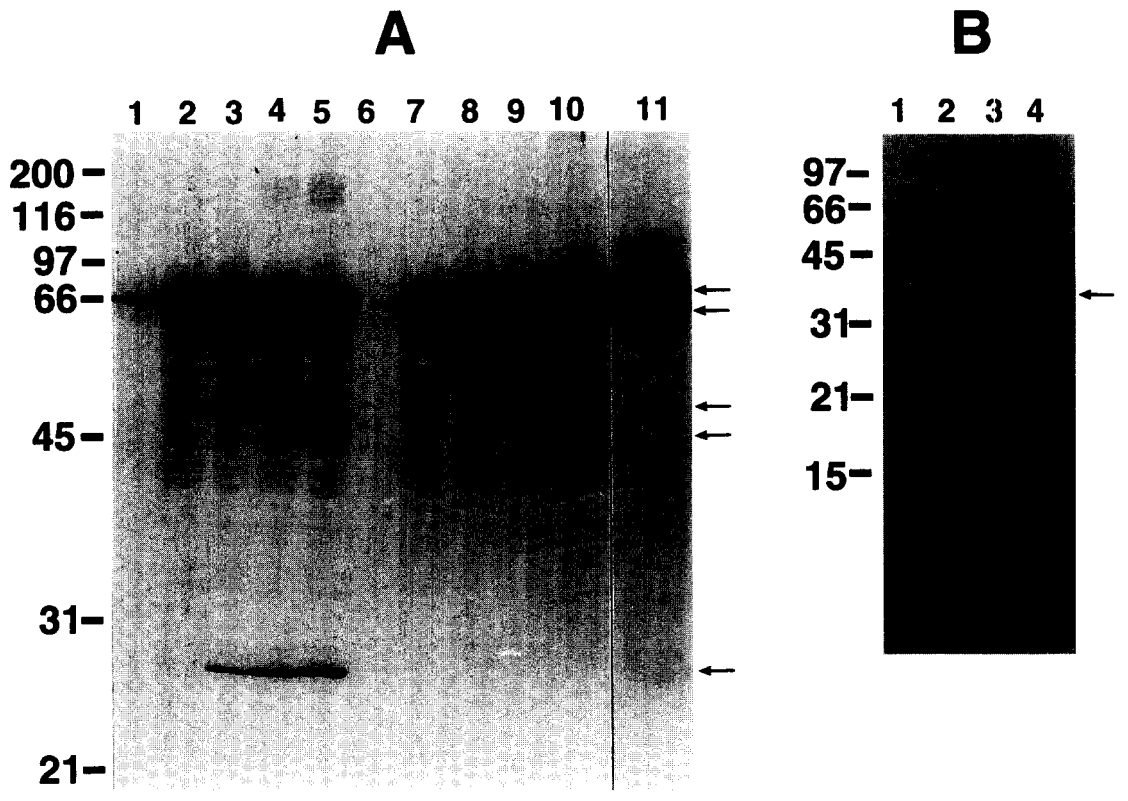

Fig. 3. In vitro transcription/translation of pSPTHSVA, pSPTHSVAΔLQAS and pSPTHSVG. (A) pSPTHSVA and pSPTHSVAΔLQAS. Lanes 1–5, translation products of pSPTHSVA after 0, 30, 60, 90 and 120 min of reaction. Lanes 6–11, translation products of pSPTHSVAΔLQAS, respectively, at time 0, 30 , 60 , 90, 120 min and after 16 h (this last incubation was performed at 37°C after the 2 h of transcription/translation at 30°C). Arrows indicate the different proteolysis products. (B) In vitro transcription and translation of pSPTHSVG. Lanes 1–4, translation products of pSPTHSVG after 0, 30, 60, 90 and 120 min of reaction.

tion of the protease, we investigated the behaviour of the first 329 amino acids. For this experiment, plasmid pSPTHSVG was constructed (Fig. 1). Transcription and translation of pSPTHSVG were carried out using the same conditions as the three other constructs and the results are shown in Fig. 3B. Upon incubation from 0 to 2 h, a single protein with a relative gel mobility superior to 31 kDa is synthesised. This corresponds to the expected molecular weight for HSVG which, upon calculation, should be 35,035 Da. One might expect autoproteolysis to occur at the intact LQAS site to produce mature HSV-1 protease but, unexpectedly, no protein co-migrating with HSVB is detected even after over-exposure of the gel. The absence of processing could be related to the substrate specificity of the protease. This is unlikely to be the case as the construct contains 82 residues after the cleavage sequence LQAS. Studies using synthetic substrate peptides have shown cleavage of a P9–P5′ sequence [20]. Maturation of the precursor appears to require an initial cleavage at the C-terminal site followed by the the N-terminal cleavage. This is in agreement with the result obtained with the N-terminal deleted construction where processing at the C-terminal site still occurred. The maturation appears to be well orchestrated, following a defined succession of events. Our results with the 4 constructs suggest that cleavage at the C-terminal end of the precursor is necessary for release of the mature protease in the expression system used. These results, however, do not preclude the possibility that the 247–329 tail in HSVG may shield the N-terminal cleavage site. Point mutations in the C-terminal cleavage site, for example at position 610, would rule out this alternative interpretation and would demonstrate the requirement of C-terminal cleavage for release of mature HSV-1 protease. For the cytomegalovirus, the terminology maturational (M) site and release (R) site has been suggested [1]. Whether an activation step is necessary, as postulated [1], or whether some post-translational events are necessary, as suggested [20], remain to be demonstrated. By peptide substrate mimics, it was clearly shown that the M and R sites were not cleaved with the same efficiency [15]. This difference could be a way of regulating the production of the mature protease during the virus life cycle. HSV-1 protease is an important potential target for drug design against herpes viruses. An understanding of its precise mode of action in vivo and its regulation are necessary in order to design potent and effective inhibitors of the virus assembly.

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References

[1] Welch, A.R., Woods, A.S., McNally, L.M., Cotter, R.J., and Gibson, W. (1991) Proc. Natl. Acad. Sci. USA 88, 10792–10796.

[2] Baum, E.Z., Bebernitz, G.A., Hulmes, J.D., Muzithras, V.P., Jones, T.R. and Gluzman, Y. (1993) J. Virol. 67, 497–506.
[3] De Wind, N., Wagenaar, F., Pol, J., Kimman, T. and Berns, A. (1992) J. Virol. 66, 7096–7103.
[4] Preston, V.G., Coates, J.A.V. and Rixon, F.J. (1983) J. Virol. 45, 1056–1064.
[5] Braun, D.K., Roizman, B. and Perreira, L. (1984) J. Virol. 49, 142–153.
[6] Rixon, F.J., Cross, A.N., Addison, C. and PReston, V.G. (1988) J. Gen. Virol. 69, 2879–2891.
[7] McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. and Taylor, P. (1988) J. Gen. Virol. 69, 1531–1574.
[8] Liu, F. and Roizman, B. (1991) J. Virol. 65, 206–212.
[9] Liu, F. and Roizman, B. (1991) J. Virol. 65, 5149–5156.
[10] Preston, V.G., Rixon, F.J., McDouglall, I.M., McGregor, M. and Al Kobaisi, M.F. (1992) Virology 186, 87–98.
[11] DiIianni, C.L., Drier, D.A., Deckman, I.C., McCann III, P.J., Liu, F., Roizman, B., Colonno, R.J. and Cordingley, M.G (1993) J. Biol. Chem. 268, 2048–2051.
[12] Deckman, I.C., Hagen, M. and McCann III, P. (1992) J. Virol. 66, 7362–7367.
[13] Liu, F. and Roizman, B (1992) Proc. Natl. Acad. Sci. USA 89, 2076–2080.
[14] Liu, F. and Roizman, B (1993) J. Virol. 67, 1300–1309.
[15] Burck, P.J., Berg, D.H., Luk, T.P., Sassmannshausen, L.M., Wakulchick, M., Smith, D.P., Hsiung, H.M., Becker, G.W., Gibson, W. and Villarreal, E.C. (1994) J. Virol. 68, 2937–2946.
[16] DiIanni, C. L., Stevens, J.T., Bolgar, M., O'Boyle II, D.R., Weinheimer, S.P. and Colonno, R.J. (1994) J. Biol. Chem. 269, 12672– 12676.
[17] Sambrook, J.L., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] KUreishi, A. and BRyan, L.E. (1992) Nucleic Acids Res. 20, 1155.
[19] LAemmli, U.K. (1970) Nature 227, 680–685.
[20] DiIanni, C.L., Mapelli, C., Drier, D.A., Tsao, J., Natarajan, S., Riexinger, D., Festin, S.M., Bolgar, M., Yamanaka, G., Weinheimer, S.P., Meyers, C.A., Colonno, R.J. and Cordingley, M.G. (1993) J. Biol. Chem. 268, 25449–25454.