Autoprocessing of the precursor form of human herpesvirus 6 (HHV-6) proteinase at two sites (termed M and R) is required to generate the mature enzyme. Kinetic constants were determined for the hydrolysis of a series of synthetic peptide substrates by mature HHV-6 proteinase, purified to homogeneity. Truncation or replacement of individual residues in peptides mimicking the R-site sequence, indicated that the minimum length for effective hydrolysis by the viral enzyme was P1-P2-P3-Ala*Ser-P4-Tyr and revealed the importance of the P1 Ala and P4 Tyr residues. Consequently, relevant (P1 or P4) mutations were introduced into the precursor form of the proteinase and the ability of these altered proteins to autoprocess was examined. Introduction of Val in place of the P1 Ala at the M-site essentially abrogated cleavage but mature HHV-6 proteinase was still generated by cleavage at the R-site, indicating that processing of the M-site is not a prerequisite for cleavage of the R-site in the precursor. At the R-site, mutation of the P1 Ala, or of the preceding P3 Tyr residue, prevented processing at the R-site in the precursor so that the mature form of HHV-6 proteinase was not generated. The accumulated data suggest a possible new approach to the design of inhibitors for therapeutic intervention in the life cycle of herpesviruses.

Eight herpesviruses have been implicated to date in the etiology of a number of human diseases. These are subdivided into three families on the basis of their biological properties, host cell range and the organization of their genomes (1). Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) and varicella zoster virus (VZV), the causative agent of chicken pox and shingles, comprise the α-subgroup while Epstein-Barr virus and the recently discovered human herpesvirus 8 (which has been implicated in Kaposi’s sarcoma in human immuno-deficiency virus-positive individuals; Ref. 2) constitute the γ-subfamily. The β-subgroup consists of human cytomegalovirus (HCMV) and human herpesviruses 6 and 7 (HHV-6 and HHV-7). HHV-6 causes the childhood illness exanthem subitum (3) and has also been implicated in the progression of a number of other diseases, including multiple sclerosis (4) and chronic fatigue syndrome (5).

In common with other herpesviruses, HHV-6 encodes its own proteinase, which is essential for capsid maturation, DNA packaging, and the ultimate formation of new virus particles (6). The mature proteinase consists of 230 residues but is synthesized in the form of a precursor, which has an additional 288 residues attached to the C terminus of the mature enzyme (see Ref. 7 and Fig. 1). Autolytic removal of these residues (which themselves constitute a form of the viral assembly protein; Ref. 6) releases the N-terminal proteinase in its mature form; processing takes place at two locations positioned, respectively, at the C terminus of the proteinase (R-site; Fig. 1) and 37 residues from the C terminus of the precursor (M-site; Fig. 1). In the HHV-6 precursor, just as in the counterpart proteins from most other herpesviruses, both of these processing sites consist of ~Ala*Ser~ residues in the ~P1*P1~ positions (7, 8).2 The specificity requirements for cleavage of such peptide bonds have not been examined, however, and as herpesvirus proteinases have been frequently described as attractive targets for the design of antiviral drugs, the present report quantifies the ability of mature HHV-6 proteinase to cleave a systematic series of synthetic peptide substrates and examines the ability of the precursor form to undergo proteolytic processing when mutations are introduced at the R- and M-sites.

MATERIALS AND METHODS

Overlapping PCR Mutagenesis—The gene encoding the full-length proteinase precursor from HHV-6 was generated as described previously (7). Mutations were introduced at appropriate locations using the overlapping PCR technique (9). Pairs of forward and reverse mutagenesis primers (Table I) were designed to introduce the desired mutation and where possible a novel restriction site to facilitate identification of mutant clones. In the Y227A, A491G, and A491V cases (Table I), the mutations were introduced at each site by three successive polymerase chain reactions (PCR), which employed (i) the relevant forward (F) mutagenesis primer and its appropriate reverse (R2 or R4) partner oligonucleotide; (ii) the relevant reverse (R) mutagenesis primer together with its appropriate forward (F4 or F5) partner oligonucleotide; after purification of each of these initial PCR products, they were combined and used as templates in (iii) a subsequent PCR which employed the wild-type flanking oligonucleotides (e.g. R2/F5) as primers (9). For the A230G, A230V, and (RY227A mutants (Table I), substitutions were introduced into the appropriate wild-type gene using a single PCR followed by subcloning. For the mutants A230G and A230V, the (forward) mutagenesis oligonucleotides contained a SnaBI restriction site to facilitate subcloning into the full-length proteinase precursor gene. In only one case, termed (PR)Y227A, was a mutation introduced into a gene that encoded only the mature form of HHV-6 proteinase. In this case, the (reverse) mutagenesis primer was positioned precisely at the region of the gene encoding the C terminus of the mature proteinase and thus contained an in-frame stop codon plus a SalI restriction site for further manipulation.

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1 The abbreviations used are: HSV-1 and -2, human herpes simplex viruses, types 1 and 2, respectively; HHV-6, human herpesvirus 6; HCMV, human cytomegalovirus; VZV, varicella zoster virus; PCR, polymerase chain reaction.
Following verification of its nucleotide sequence, each mutated DNA fragment was subcloned into the expression vector pDS566.His2.Xa described previously (7). Synthetic oligonucleotides were obtained from Pharmacia Biotech (St. Albans, Herts., United Kingdom (U.K.)), Genosys Technologies Ltd. (Cambridge, U.K.), or Life Technologies, Inc. (Paisley, U.K.)

Expression and Immunodetection of Protein Products—Wild-type and mutant constructs were transformed into the Escherichia coli strain M15, pDM1.1. Cells harboring the plasmids were grown at 28 °C until A600 was 0.8, before induction with isopropyl-1-thio-β-D-galactopyranoside (1 mM, final concentration). Samples were taken at various times after induction, and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using separate primary antibodies, which recognized the mature HHV-6 proteinase and the assembly protein region of the precursor (Fig. 1), respectively (7). Alkaline phosphatase-conjugated anti-rabbit IgG antiserum as the secondary antibody allowed the detection of immunoreactive bands by the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate method (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.).

Recombinant Proteinase Purification and Activity—Mature forms of wild-type and Y227A mutant proteinase were produced in the soluble fraction in E. coli and purified to homogeneity by nickel chelate chromatography as described previously (7). Peptide substrates were synthesized on a Milligen 9050 Pepsynthesizer and purified to >95% using reverse-phase HPLC by Dr. B. K. Handa (Roche Research Center, Welwyn Garden City, Herts., U.K.). Accurate concentrations of peptide solutions were determined by amino acid analysis. Peptide cleavage assays were performed (unless otherwise stated) at 21 °C, in 50 mM solutions were determined by amino acid analysis. Peptide cleavage above 21 °C. The convenience of such internally quenched fluorescent substrates in proteinase activity studies has been widely advocated (e.g. see Ref. 10), but it is clear that such usages are not without their limitations. Consequently, for further characterization of HHV-6 proteinase, the cleavage assays were monitored using the more laborious FPLC-based assay and at a temperature of 21 °C.

In order to establish the minimum length of peptide required for cleavage, a systematic series of peptides based on the R-site sequence was examined for their susceptibility to hydrolysis by purified, recombinant HHV-6 proteinase. The first set encompassed the P7-P5 positions to aid solubility (peptide 1, 95% using FPLC-based assay). The equivalent set (peptides 7–10), with two arginine residues introduced into the P4-P5 positions (peptides 3 and 4, respectively) did not alter the values of kcat/Km for both peptides (Table II) indicate that both peptides were readily cleaved. Comparable values for the specificity constants (kcat/Km) for each peptide were calculated when the measurements were made at 21 and 37 °C. In our preliminary study on recombinant HHV-6 proteinase (7), it was reported that the enzyme appeared to show little or no activity at temperatures above 21 °C. This somewhat unexpected finding was derived from using a quenched fluorescent assay system, which employed a synthetic peptide comparable to that of the R-site mimic (Table II) but which was modified at each end with two hydrophobic, internally quenching, fluorescent groups. Assays based on such fluorescence changes are considerably more convenient in practice than the FPLC-based type that was used to derive the kinetic parameters in the present study (Table II). However, it would appear that the introduction of the two bulky reporter groups distorted the processes of interaction between substrate and enzyme considerably, to improve the apparent value of K0.5 by approximately 50-fold (albeit with little influence on kcat) and to prevent effective substrate cleavage above 21 °C. The convenience of such internally quenched fluorescent substrates in proteinase activity studies has been widely advocated (e.g. see Ref. 10), but it is clear that such usages are not without their limitations. Consequently, for further characterization of HHV-6 proteinase, the cleavage reactions were monitored using the more laborious FPLC-based assay and at a temperature of 21 °C.

The sequences of the partner oligonucleotides were: F2, 5’-ATCAGACGTCGACAGCATGTTGA-3’; F4, 5’-TCAGTTTAGACCATGGGCG-3’; F5, 5’-CGCATTCATGTCGGCTG-3’; R2, 5’-GCGCGTGACTTTAAAACATCCATTTTTTTAAAAGCGTGCA-3’; R4, 5’-AATGTTGGAACGACAGCG-3’.

| Mutation site | Direction | Mutagenesis oligonucleotides (5’-3’) | Partner oligonucleotide | Site created |
|---------------|-----------|-------------------------------------|-------------------------|-------------|
| Y227A        | F         | AAATGTAACGCTTAAAGCCTGAGGA           | R4                      | BsrGI       |
|              | R         | TTCACTAGTTTTAAATGGCTGTAATT        | R4                      | BsrGI       |
| (PR) Y227A   | R         | GTCGACTCCAGTTAAATGCGTACATTIT      | R4                      | BsrGI       |
| A491G        | F         | TATTTTAAACGCTGCTTTGGAACCG         | R2                      | BalIII      |
| A491V        | F         | ATTTTTAAACGCTGCTTTGGAACCG         | R2                      | AcII        |
| A230G        | F         | GTAGCTGATATTTAAAAAGGACTGGAACCCCG   | R2                      | BalIII      |
| A230V        | F         | GTAGCTGATATTTAAAAAGGACTGGAACCCCG   | R2                      | ArgI        |

RESULTS AND DISCUSSION

Inspection of the HHV-6 proteinase precursor sequence (7) reveals that in both of the cleavage (R and M) sites, the –P3−R−P5− residues contributing to the scissile peptide bond are –Ala*Ser– (Fig. 1). Synthetic peptides mimicking the R and M cleavage sites were examined for their susceptibility to hydrolysis by recombinant, mature HHV-6 proteinase, purified to homogeneity as described previously (7). The kinetic parameters that were obtained (Table II) indicate that both peptides were readily cleaved. Comparable values for the specificity constants (kcat/Km) for each peptide were calculated when the measurements were made at 21 and 37 °C. In our preliminary study on recombinant HHV-6 proteinase (7), it was reported that the enzyme appeared to show little or no activity at temperatures above 21 °C. This somewhat unexpected finding was derived from using a quenched fluorescent assay system, which employed a synthetic peptide comparable to that of the R-site mimic (in Table II) but which was modified at each end with two hydrophobic, internally quenching, fluorescent groups. Assays based on such fluorescence changes are considerably more convenient in practice than the FPLC-based type that was used to derive the kinetic parameters in the present study (Table II). However, it would appear that the introduction of the two bulky reporter groups distorted the processes of interaction between substrate and enzyme considerably, to improve the apparent value of K0.5 by approximately 50-fold (albeit with little influence on kcat) and to prevent effective substrate cleavage above 21 °C. The convenience of such internally quenched fluorescent substrates in proteinase activity studies has been widely advocated (e.g. see Ref. 10), but it is clear that such usages are not without their limitations. Consequently, for further characterization of HHV-6 proteinase, the cleavage reactions were monitored using the more laborious FPLC-based assay and at a temperature of 21 °C.

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**Fig. 1. Schematic representation of the HHV-6 proteinase precursor.** Autoprocessing at the R- and M-sites (between ~−Ala\(^{201}\)−Ser\(^{231}\)− and ~−Ala\(^{391}\)−Ser\(^{492}\)−, respectively) releases the mature proteinase from the N terminus and generates a (C-terminal) form of the viral assembly protein.

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**Table II**

| Temperature | Cleavage site Peptide | \(K_m\) | \(k_{cat}\) | \(k_{cat}/K_m\) |
|-------------|-----------------------|---------|-----------|----------------|
| °C          |                       |         |           |                |
| 21          | (R) Sc-RRYIKA*SEP-NH₂ | 0.25    | 7         | 28             |
| 37          | (R) Sc-RRYIKA*SEP-NH₂ | 0.4     | 10        | 25             |
| 21          | (M) Sc-RRILNA*SLAPE-NH₂ | 2.0  | 13        | 6              |
| 37          | (M) Sc-RRILNA*SLAPE-NH₂ | 2.8  | 28        | 10             |

**Table III**

| Peptide no. | Peptide sequence | \(K_m\) | \(k_{cat}\) | \(k_{cat}/K_m\) |
|-------------|------------------|---------|-----------|----------------|
|             |                  |         |           |                |
| 1           | Sc-KSTYIKA*SEP弗-VR-NH₂ | 0.6    | 18        | 36             |
| 2           | Sc-STYIKA*SEP弗-VR-NH₂ | 0.7    | 17        | 26             |
| 3           | Sc-TIKA*SEP弗-VR-NH₂ | 2.6    | 69        | 27             |
| 4           | Sc-YIKA*SEP弗-VR-NH₂ | 2.5    | 91        | 26             |
| 5           | Sc-IKA*SEP弗-VR-NH₂ | 6.2    | 11        | 28             |
| 6           | Sc-KA*SEP弗-VR-NH₂ | 0       | 0         | 0              |
| 7           | Sc-RRYIKA*SEP弗-NH₂ | 0.25   | 7         | 28             |
| 8           | Sc-RRYIKA*SEP-NH₂ | 0.6    | 57        | 101            |
| 9           | Sc-RRYIKA*SEP−NH₂ | 1.1    | 23        | 20             |
| 10          | Sc-RRYIKA*SE−NH₂ | 0       | 0         | 0              |

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value, implying that valine is not a favored substituent in P\(_5\)'. The value for the specificity constant for peptide 8 is the highest in the series, even though residues are present in P\(_8\) and P\(_9\). Removal of the P\(_9\)’ substituent to give peptide 9 lowered the value of the specificity constant and the final peptide spanning P\(_9\)−P\(_9\)’ (peptide 10) was not cleaved at all. On this basis, it would appear that the minimal length for efficient hydrolysis of R-site mimics by the proteinase from HHV-6 (a β-subfamily member; see Introduction) would require a peptide spanning P\(_9\)−P\(_9\)’.

**Tides.** Removal of the P\(_9\) substituent resulted in a peptide (peptide 6) that was no longer susceptible to cleavage by mature HHV-6 proteinase, even upon prolonged incubation. On this basis, the C-terminal truncated set of peptides was synthesized to position the two solubilizing arginine residues in the P\(_8\) and P\(_9\) positions. The resultant longest peptide in this series, even upon removal of the Tyr residue in the P\(_4\) position (peptides 4 and 5; Table II), the effect of replacement of this residue by other amino acids was investigated (Table IV). Substitution by Asp, Lys, Nle (which corresponds to lysine without its ε-NH₂ group) or Ile (the equivalent P\(_4\) residue in the M-site sequence; Fig. 1 and the lower peptide in Table II) all resulted in specificity constants that were lowered by at least two orders of magnitude compared with the parent peptide (Table IV). The Tyr residue at P\(_4\) would thus appear to be of crucial importance with regard to R-site cleavage.

In order to investigate this further, this Tyr residue (Tyr\(^{227}\); Fig. 1) was mutated to an Ala within the context of the gene encoding the 528-residue proteinase precursor, and the ability of the mutated proteinase precursor to undergo autoprocessing in E. coli was compared with that of the wild-type gene product (Fig. 2). Samples were removed at different time points and analyzed by Western blotting using two distinct HHV-6 antisera in order to visualize products derived from the proteinase (upper panels) and assembly protein (lower panels), respectively. Processing of the wild-type precursor at both R and M cleavage sites to release the assembly protein and generate the mature form of HHV-6 proteinase was completed between 30 and 60 min (Fig. 2, panel i). However, in the case of the Y227A mutant proteinase precursor, the band at ~65 kDa, which is consistent in size with that predicted for the full-length proteinase precursor (see Fig. 1), persisted throughout the time course (Fig. 2, panel ii). Furthermore, the absence of any band corresponding to mature HHV-6 proteinase (compare upper panels ii and i in Fig. 2) suggests that mutation of Tyr\(^{227}\) to Ala rendered the R-site resistant to cleavage.

In attempts to make this comparison more quantitative and to gain some insight into the significance of Tyr\(^{227}\) in proteinase function, the gene encoding the mature form (i.e. residues 1–230; see Fig. 1) of the Y227A mutant proteinase was subcloned and expressed in E. coli. The resultant, mutant proteinase accumulated in the soluble fraction in E. coli, from where it was purified to homogeneity (as described previously (7) for the
The ability was assessed of this Y227A mutant proteinase to hydrolyze the two synthetic peptides (described in Table II), which mimicked the wild-type R and M cleavage junctions. No cleavage of either substrate was detected, even upon prolonged incubation of each peptide with a large amount of the recombinant, Y227A mutant proteinase. Thus, within the context of the amino acid sequence of the R-site, a tyrosine residue at P4 appears to be necessary in order to achieve effective cleavage of synthetic peptide substrates (Table IV) and the proteinase precursor itself (Fig. 2, panel ii). This suggests that the tyrosine residue must be accessible for recognition by the attacking catalyst. As the mature Y227A mutant proteinase was readily soluble when accumulating to high concentration in E. coli, it is unlikely to have been grossly misfolded as a consequence of the loss of the phenol ring resulting from the Tyr \( \rightarrow \) Ala mutation. The purified, mutant enzyme was inactive, however. The simplest explanation for these observations is that, following cleavage of the extended sequence of the R-site in the proteinase precursor, the tyrosine side chain is important for proper incorporation of this C-terminal region into the correct structure of the newly generated mature proteinase. The human herpesvirus-6 proteinase is not exceptional, however, in having a Tyr residue in this location. Inspection of the R and M cleavage junctions in the proteinase precursors of other herpesviruses reveals that a Tyr residue is always present at the P4 position of the R-site sequence (7) but is never seen at the equivalent position in the M-site.

### Table IV

| P4 substituent | \( K_m \) | \( k_{cat} \) | \( k_{cat}/K_m \) |
|----------------|---------|---------|-----------------|
| Tyrosine       | 0.6     | 57      | 101             |
| Isoleucine     | 4.2     | 1.5     | 0.4             |
| Norleucine     | 3.8     | 1.2     | 0.3             |
| Aspartate      | 1.8     | 0.5     | 0.3             |
| Lysine         |         |         | 0               |

These indicate (i) a deletion in the HHV-6 sequence just before Tyr\(^{227}\), and (ii) a deletion in the HCMV sequence four residues upstream from Gln\(^{116}\) (HCMV numbering), which (iii) is itself replaced by Pro\(^{100}\) in HHV-6 proteinase. These alterations, which occur in loops (16) make modeling exercises imprecise, but it does appear that the residues in HCMV proteinase that line the hydrophobic pocket are likely to be retained in character in the HHV-6 sequence. Thus, it would seem that Tyr\(^{227}\) in HHV-6 proteinase may also be accommodated within a hydrophobic pocket and may well form a hydrogen bond through its phenolic OH group, but the precise nature of such contacts and the relative orientation of the side chains requires definition by determination of a crystal structure for HHV-6 proteinase.
The available structural information from HCMV, VZV, HSV-1, and HSV-2 proteinases (13–18) suggests that the S₁ binding pocket for the P₁ residue of a substrate takes the form of a shallow depression on the binding surface of each of the enzymes. In keeping with this, the ~P₁¹P₁⁻¹ scissile peptide bond at both R and M cleavage sites in the HHV-6 proteinase precursor was found to be between small Ala²³⁰-Ser²³⁰ residues. In order to investigate further the significance of the nature of the P₁ substituent, a series of synthetic peptides was prepared based on the R-site sequence, in which the P₁ Ala residue was replaced. Kinetic parameters were determined for the hydrolysis of each of these by purified, mature wild-type HHV-6 proteinase (Table V). Replacement of the P₁ Ala by a smaller glycine residue (i.e. with no side chain; peptide 11, Table V) still allowed cleavage of the peptide, but the kₐᵡ/kᵦ value was reduced by about half compared with that of the parent peptide (peptide 7, Table V). When the P₁ substituent was a hydrophilic glycine residue, therefore, in place of the natural P₁ Ala residue (peptide 13, Table V), cleavage of the respective peptides was severely reduced and, in the case of the P₁ Val substituent, appeared to be abrogated altogether.

These effects were examined further by introducing Gly or Val in place of the P₁ Ala²³⁰ and Ala²³⁰ residues at the R and M cleavage sites, respectively, in the proteinase precursor (Fig. 1) and comparing the processing events in these mutant proteinase precursors with those observed upon expression of the wild-type precursor gene in E. coli. For the wild-type, M-site cleavage was complete by about 30 min (Fig. 2, panel i) with processing at the R-site in the precursor apparently requiring a little longer (approximately 60 min). For the R-site mutants, processing of the A230G mutant proteinase precursor at the non-mutated M-site appeared to proceed comparably to that of the wild-type precursor (data not shown). However, whereas in the wild-type case, conversion to mature proteinase by cleavage at the R-site was complete by about 60 min (Fig. 2, panel i), the autoconversion reaction was only just beginning after 120 min with the A230G mutant precursor (data not shown). Introduction of a Gly residue, therefore, in place of the natural P₁ Ala substituent appeared to impede but did not prevent autoprocessing. These events were even more apparent in the case of the A230V mutant proteinase precursor. Even after 120 min, no 28-kDa band of mature HHV-6 proteinase could be detected (compare Fig. 2, top panels iii and i), indicating that correct processing at the ~Val²²⁷-Ser²³⁰ mutant R-site had not occurred. Other bands were evident, however (e.g. at ~56 and 52 kDa, respectively, as revealed particularly by the antisera specific for the assembly protein region; see Fig. 2, lower panel iii), which cannot be accounted for by cleavage at either or both of the R and M cleavage sites. These same bands appeared specifically in the case of the Y227A mutant proteinase precursor (Fig. 2, panel ii). During expression of the wild-type precursor, these bands were faintly evident after about 10 min but appeared only transiently and were not detected in the later time points when mature proteinase was accumulating. In contrast, when the gene encoding only the mature form of HHV-6 proteinase was expressed, these protein bands were not detected at all (data not shown). This evidence confirms that the proteins being revealed were not E. coli proteins that chanced to react nonspecifically with the antisera being used, but were indeed derived from the HHV-6 proteinase precursor. As these intermediate proteins persisted only when mutant proteinase precursor genes (e.g. Y227A, A230V) were being expressed and reciprocally, when the mature form of HHV-6 proteinase was NOT being generated, it would seem that these proteins of intermediate size may have been produced by the nonspecific action of E. coli proteinase(s). If active, mature HHV-6 proteinase was being generated simultaneously, then the viral proteinase eventually processed these products and their accumulation was thus prevented. In the cases of the mutant proteinase precursors where mature HHV-6 enzyme was not being generated, then there was no intrinsic ability to process these intermediates and they thus persisted throughout the time course.

Consistent with this interpretation, when the mutations A491G or A491V were introduced at the M-site (Fig. 1), the intermediate proteins were visible only faintly and transiently. Mature HHV-6 proteinase did accumulate, with processing at the (wild-type) R-site being essentially complete in approximately 60 min in the case of the A491G mutant (not shown) and by 120 min for the A491V mutant (Fig. 2, top panel iv). Relative to wild-type, the proteinase precursor band at 65 kDa seemed to persist until later time points (compare top panels i and iv in Fig. 2), suggesting that processing events at the R-site might have been delayed to a minor extent. In contrast, however, the efficiency of processing at the mutated ~Val¹⁹¹-Ser¹⁹² ~M-site in the A491V mutant proteinase precursor was considerably diminished (Fig. 2, lower panel iv). The ~36 kDa form of the assembly protein region (which had not undergone M-site cleavage) appeared to be the predominant product derived from the C terminus of the precursor and had undergone little processing even after 120 min (Fig. 2, compare panels iv and i). From these data, it would appear that processing of the M-site is not a prerequisite for cleavage of the R-site in the proteinase precursor.

These investigations have revealed that, in addition to the P₄ Tyr residue discussed earlier, a P₁ Ala residue is preferable for hydrolysis of peptide substrates (Table V) and for efficiency of autoprocessing of the proteinase precursor. Cleavage of the precursor at the R-site requires that the ~Ala²³⁰-Ser²³⁰ bond is accessible and thus it is unlikely that Ala²³⁰ (and the P₄ residue of Tyr²²⁷) are accommodated in the proteinase precursor in the same orientation as their side chains finally adopt in the mature HHV-6 enzyme. Thus, if this C-terminal region (containing these critical Tyr²²⁷ and Ala²³⁰ residues), which is newly generated as a result of the processing events, can be prevented from folding into its correct juxtaposition with the remainder of the mature proteinase, then this might offer a strategy for the design of inhibitors that would abrogate precursor processing and disrupt the herpes viral life cycle.

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