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The Protease and the Assembly Protein of Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8)

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A genomic clone encoding the protease (Pr) and the assembly protein (AP) of Kaposi's sarcoma-associated herpesvirus (KSHV) (also called human herpesvirus 8) has been isolated and sequenced. As with other herpesviruses, the Pr and AP coding regions are present within a single long open reading frame. The mature KSHV Pr and AP polypeptides are predicted to contain 230 and 283 residues, respectively. The amino acid sequence of KSHV Pr has 56% identity with that of herpesvirus saimiri, the most similar virus by phylogenetic comparison. Pr is expressed in infected human cells as a late viral gene product, as suggested by RNA analysis of KSHV-infected BCBL-1 cells. Expression of the Pr domain in *Escherichia coli* yields an enzymatically active species, as determined by cleavage of synthetic peptide substrates, while an active-site mutant of this same domain yields minimal proteolytic activity. Sequence comparisons with human cytomegalovirus (HCMV) Pr permitted the identification of the catalytic residues, Ser114, His46, and His134, based on the known structure of the HCMV enzyme. The amino acid sequences of the release site of KSHV Pr (Tyr-Leu-Lys-Ala*Ser-Leu-Ile-Pro) and the maturation site (Arg-Leu-Glu-Ala*Ser-Ser-Arg-Ser) show that the extended substrate binding pocket differs from that of other members of the family. The conservation of amino acids known to be involved in the dimer interface region of HCMV Pr suggests that KSHV Pr assembles in a similar fashion. These features of the viral protease provide opportunities to develop specific inhibitors of its enzymatic activity.

Kaposi's sarcoma (KS), once considered a rare tumor largely confined to elderly Mediterranean and African men, has recently reemerged as the most common neoplasm of patients with AIDS: 15 to 25% of such patients will develop this tumor in the course of their human immunodeficiency virus (HIV) infection. While HIV infection is an important risk factor in KS development (20, 22), epidemiologic studies indicate that it is not sufficient to explain the etiology of the disease (5, 6). For example, KS is far more prevalent in AIDS patients who acquire HIV infection by sexual routes than in those who contract HIV by percutaneous inoculation or vertical transmission. These findings suggest that a second, sexually transmitted cofactor may be required for KS development. Recently, DNA sequences of a novel human herpesvirus (KS-associated herpesvirus [KSHV], also called human herpesvirus 8 [HHV-8]) have been identified in KS tumors (9). A growing body of evidence suggests an important role for this virus in KS pathogenesis: (i) infection precedes tumorigenesis and is associated with a striking increase in the risk of subsequent KS development (43, 68); (ii) the distribution of infection among HIV-positive patients parallels known KS risk (24, 37); (iii) all forms of KS, whether HIV-positive or HIV-negative, are strongly associated with KSHV infection (2, 9, 11, 33, 44, 57, 61); and (iv) infection is targeted to the endothelial (spindle) cells, thought to be central to KS pathogenesis (7). These findings raise the important possibility that prevention or suppression of KSHV infection could reduce the risk of KS development. Here we report the cloning, expression, and initial biochemical characterization of a KSHV gene product with the potential to be a target for specific antiviral therapy.

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Herpesviruses mature through a common assembly pathway, which is best studied using herpes simplex virus (HSV). Viral capsids assemble in the nucleus late in infection. Immature capsids lack DNA but contain an abundant internal polypeptide, the assembly protein (AP), that is not found in the mature, DNA-containing particles (25, 27, 34, 48, 55). This protein is known to interact, through its carboxy-terminal domain, with the major capsid protein (32, 69). This interaction is required for nuclear transport of the major capsid protein (47) and has also been proposed to act as a scaffold to facilitate the assembly of the capsid shell. Following immature capsid assembly, AP undergoes proteolytic processing at the so-called maturation site (M-site) near its carboxy terminus, which removes the last 25 amino acids (26). This proteolysis is mediated by a virally encoded serine protease (Pr) (40, 52, 67). Herpesvirus Pr molecules show clear homology, ranging from 90% amino acid sequence identity among closely related viruses to 30% identity between distantly related viruses. These sequences are unrelated to the two major classes of presently characterized cellular serine Pr's, the chymotrypsin and subtilisin families (49). The viral enzymes display similar substrate specificities, preferring an Ala residue at P1, Tyr at P4, and Ser at P1′ (see reference 58 for nomenclature). Cleavage of AP by the viral Pr is essential for viral growth; HSV mutants bearing an inactive Pr accumulate capsids lacking viral DNA (17, 23, 50, 51, 59, 63). Presumably, cleavage of AP is required to allow its release from the capsid and permit the packaging of newly replicated viral DNA.

The coding strategy for the Pr and AP is similar in all well-characterized herpesvirus family members. The Pr and AP coding sequences are found in a single large open reading frame (ORF) (Fig. 1) encoding a polyprotein (Pr/AP) whose amino- and carboxy-terminal domains represent Pr and AP, respectively. Active Pr excises itself from Pr/AP by cleavage at

the so-called release site (R-site) and can then cleave AP at the M-site. Most AP, however, is not generated by Pr/AP cleavage; rather, a separate mRNA initiated within the ORF directs AP translation from an internal AUG codon. As expected for virion structural components, Pr/AP and AP transcripts are expressed as late viral genes in the lytic cycle and presumably are not expressed during latent infection.

Recent success with protease inhibitors in antiviral therapy for HIV provides a precedent for a similar strategy for anti-KSHV therapeutics (13). Defining the protease target of KSHV and its natural substrates is an essential step in achieving this goal. In this report, we present results of the cloning and purification of the KSHV Pr as well as a preliminary investigation of its enzymatic activity. In addition, evidence for the in vivo expression of Pr and Pr/AP as late viral gene products is presented, along with an analysis of their relationship to other herpesvirus Pr and precursor Pr/AP gene products.

MATERIALS AND METHODS

Sequencing studies. A partial KS genome had been previously subcloned from a pulmonary KS tumor as 10- to 15-kb overlapping segments in λ phage (23). DNA was prepared from a clone known to encode the thymidine kinase (TK) gene from which a 5.8-kb *Bam*HI-*Hin*dIII DNA fragment was subcloned into Bluescript II KS (Stratagene). A *Hin*dIII-*Sac*I digest resulted in four KS-specific DNA fragments of 1.9, 1.5, 1.1, and 0.8 kb. These fragments were subcloned into Bluescript and sequenced with T3 and T7 primers. The 1.5- and 0.8-kb fragments contained DNA sequences that encoded a protein that aligned with herpesvirus saimiri (HVS) Pr sequences. The nucleotide sequences of these fragments were determined by sequencing both strands of the DNA. Synthetic oligonucleotide primers were used to generate overlapping sequences of the entire insert. The primers were synthesized on an Applied Biosystems 391 DNA synthesizer. Automated DNA sequencing was carried out on an Applied Biosystems 377 Prism sequencer, and manual DNA sequencing was carried out under standard conditions.

Construction of expression vectors. A linear DNA fragment containing the KSHV Pr domain was amplified from the plasmid pBS1.5 (see Fig. 1) by PCR. The 5′ primer for this amplification was 5′-GG GGG TCC GGA CAG GGC CTG TAC GTC GGA-3′, and the 3′ primer was 5′-GG GGG AAG CTT CTA GGC CTT TAA ATA CAC CGG-3′.

In order to create a bacterial expression vector for KSHV Pr, the product of the PCR was digested with the restriction endonucleases *Bam*HI and *Hin*dIII prior to ligation into similarly treated pQE30 DNA (Qiagen). This construct ($pHis_6$-KSP), designed to overexpress the wild-type Pr with the amino acid sequence Met-Arg-Gly-Ser-His_6-Gly-Ser replacing the initiating Met residue (His_6-Pr), allows affinity purification of the His-tagged enzyme by metal chelate chromatography. This plasmid was propagated in *Escherichia coli* XL1-Blue (Stratagene) to alleviate DNA degradation and rearrangement.

The change from Ser114, encoded by TCT, to Ala (GCC) was performed by PCR-mediated site-directed mutagenesis of the Pr ORF (3). The DNA oligonucleotides 5′-CTC CCG GGG CTG GCC TTA TCG TCC ATA C-3′ and 5′-TAT GGA CGA TAA GGC CAG CCC CGG GAG-3′, corresponding to the mutated upper and lower strands, respectively, were used to introduce the substitutions into the Pr ORF (the mismatched bases are underlined). These PCR products were gel purified and subjected to a second round of PCR in order to construct the full-length coding sequence of His_6-Pr(S114A). The presence of the correct substitution was verified by DNA sequence analysis subsequent to insertion into the *Bam*HI and *Hin*dIII restriction sites of pQE30 to construct the plasmid $pHis_6$-KSP(S114A).

Bacterial growth and protein expression. The above-mentioned plasmids were separately transformed into *E. coli* X-90 to afford high-level expression of recombinant protease gene products (21). Bacterial cultures were grown at 37°C in Luria-Bertani medium to an optical density at 600 nm of 0.8, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.2 mM. Cultures were then grown for 3 h to allow protein expression and were harvested by centrifugation.

Protein purification. Cell pellets were resuspended in sonication buffer (50 mM Tris-HCl [pH 8.0], 0.5 M KCl, 10% [vol/vol] glycerol, 1 mM β-mercaptoethanol) and sonicated on ice. Lysates were then pelleted at 10,000 × *g*, and the insoluble matter was resuspended in denaturing buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM β-mercaptoethanol) containing 6 M deionized urea, by slowly stirring on ice for 30 min. The resuspended pellet was then centrifuged as described above, and the supernatant was incubated with 3 to 4 ml of Ni-nitrilotriacetic acid agarose (Qiagen) which had been equilibrated in denaturing buffer-urea by slow stirring on ice for 1 h. This slurry was packed into a column reservoir and subjected to a 1-liter reverse urea gradient, from 6 to 0 M, in denaturing buffer. The refolded, Ni-bound proteins were then washed in column buffer (50 mM Tris-HCl [pH 8.0], 500 mM KCl, 1 mM β-mercaptoethanol, 10% glycerol) containing 0.01% (vol/vol) Tween 20 and 10 mM imidazole before the column was equilibrated in the absence of Tween 20. Bound protein was then eluted with a 10 mM to 0.5 M imidazole gradient in column buffer. Protein-containing fractions were pooled and dialyzed against storage buffer (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 10% glycerol, 0.1 mM dithiothreitol) before being aliquoted and frozen at −80°C. Protein concentrations were estimated by using a calculated extinction coefficient at 280 nm of 0.9 $ml \cdot mg^{-1} \cdot cm^{-1}$.

Antibody production and immunoblot analysis. Polyclonal antiserum against purified KSHV His_6-Pr was raised in rabbits (Animal Pharm Services, Inc., Healdsburg, Calif.). For immunoblot analysis, protein samples were run on a 10% polyacrylamide–sodium dodecyl sulfate (SDS) gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was treated with TBST (Tris-buffered saline with 0.1% [vol/vol] Triton X-100) containing 5% (wt/vol) nonfat dry milk, washed with TBST containing 1% milk, and probed with a 1:250 dilution of the anti-Pr (α-KSP) polyclonal serum in the same buffer. After being washed as described above, a 1:5,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated serum (Pierce) was then applied in TBST–1% milk. Antibody-bound protein bands were detected by enhanced chemiluminescence (Amersham).

Synthesis and chemical characterization of the KSHV M-site synthetic substrate. The KSHV M-site substrate, Mca-γAbu-Asn-Arg-Leu-Glu-Ala-Ser-Ser-Arg-Ser-Ser-Lys(Dnp)-NH_2, was synthesized by using standard amino acids, 7-methoxycoumarin-4-acetyl (Mca), gamma amino butyric acid (γAbu), and 2,4-dinitrophenyl (Dnp)-Lys. 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids and Fmoc-amide resin were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). The Fmoc-Lys (Dnp)-OH was prepared by the method of Nagase et al. (46). Peptide synthesis was performed on a semiautomated Labortec SP4000 synthesizer. All couplings were mediated by dicyclohexylcarbodiimide and 2 equivalents of 1-hydroxybenzotriazole. Colorimetric analyses (36) of Fmoc deblocking and acylation steps were routinely performed. 7-Methoxycoumarin 4-acetic acid was coupled as the succinimide ester. Following completion of solid-phase assembly, the peptide was cleaved from the resin and simultaneously deprotected with reagent K (38). The product was purified by preparative reverse-phase high-performance liquid chromatography on a Rainin Dynamax octyldecyl silane column. Pure fractions were collected and lyophilized. The product was characterized as follows by electrospray ionization mass spectrometry: (M+H) = 1701 (observed) (theory, 1701). The amino acid composition was consistent with theory.

Fluorometric enzyme assays. Pr assays were performed with two fluorogenic peptide substrates. The first contained the KSHV M-site, Mca-γAbu-Asn-Arg-Leu-Glu-Ala*Ser-Ser-Arg-Ser-Ser-Lys-(Dnp)-COOH. The second, for comparative purposes, contained its related M-site from human cytomegalovirus (HCMV), NH_2-DABCYL-Arg-Gly-Val-Val-Asn-Ala*Ser-Ser-Arg-Leu-Ala-EDANS-COOH (Bachem, King of Prussia, Pa.), where DABCYL represents 4-(4′-dimethylaminophenylazo)benzoic acid and EDANS signifies (2′-aminoethylamino)-naphthalene-1-sulfonic acid. Reaction progress curves were monitored optically by a Perkin-Elmer LS-5B luminescence spectrophotometer interfaced with a Macintosh SE-30 computer. His_6-Pr and His_6-Pr(S114A) were diluted roughly 10-fold from storage buffer into assay buffer (50 mM potassium phosphate [pH 7.0], 150 mM $NaCl_2$, 25% [vol/vol] glycerol, 1 mM β-mercaptoethanol), equilibrated to 37°C for 5 min in thermostated quartz cuvettes, and manually mixed with substrate. The final KSHV Pr concentrations were 430 nM, and the substrate concentration range was kept below 25 μM for the HCMV M-site and 5 μM for the KSHV oligopeptide, due to solubility and inner-filter concerns. Time-evolved fluorescence enhancement was monitored by exciting the KSHV M-site peptide at 325 nm and detecting emission at 393 nm; the HCMV substrate samples were excited at 355 nm, and their emissions were detected at 495 nm. Trypsin and its D102N variant (14) were diluted into the same assay buffer to final concentrations of 1 and 430 nM, respectively, and treated identically to the KSHV enzymes.

Kinetics. The increase in fluorescence intensity during substrate cleavage was recorded as a function of time. The initial velocity, measured as fluorescence intensity per unit time, was calculated from the slope during the linear phase of cleavage by using Kaleiodograph operating software. The inner-filter effect was negligible in the range of substrate concentrations used, as measured by the linearity of fluorescence with respect to substrate concentration. On the basis of standard compound fluorescence extinction data, initial velocities were converted from fluorescence units to product concentration, and these data were fitted to the Michaelis-Menten equation to determine specificity constants, k_{cat}/K_m (where k_{cat} is the catalytic constant and K_m is the Michaelis constant), for each enzyme-substrate combination.

Northern blot analysis. BCBL-1 cells (54) were treated with phosphoroformic acid (PFA) at a concentration of 500 μM for at least 3 days prior to induction. Untreated and PFA-treated cells were then split into identical flasks, at 3×10^5 cells/ml, and left unstimulated or induced with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) at a concentration of 20 ng/ml. After 48 h, total RNA was harvested with RNAzol B as recommended by the manufacturer (Tel-Test, Friendswood, Tex.). To enrich the polyadenylated RNA fraction, 300-μg RNA samples, uninduced and induced with TPA and with and without PFA treatment, were affinity purified by the Oligotex mRNA purification system (Qiagen). RNA was separated on a 1% agarose–17% formaldehyde gel, transferred to a Hy-

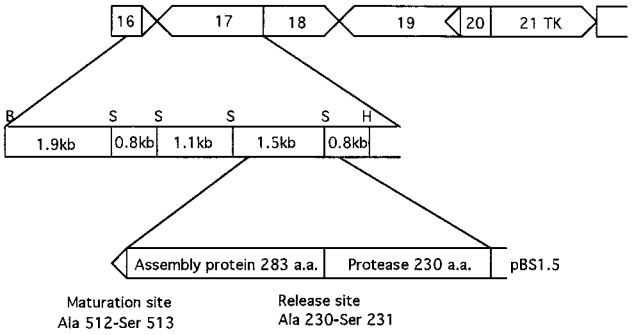

FIG. 1. Schematic of partial genome of KSHV and location of ORF 17. Arrowheads denote the directions of transcription. Abbreviations: B, *Bam*HI; S, *Sac*I; H, *Hin*dIII; a.a., amino acids.

bond-N nylon membrane in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 h, and UV cross-linked. For the double-stranded Pr/AP probe, the 1.5-kb insert from pBS1.5 was gel purified and labelled using the rediPrime random prime kit (Amersham). For the antisense single-stranded probes, either the plasmid containing the 1.5-kb *Sac*I fragment or the plasmid containing the 0.8-kb *Sac*I fragment was digested with *Eco*RI and phenol extracted and RNA was made with T3 RNA polymerase in the presence of radiolabelled UTP, followed by DNase treatment. Hybridization was performed as before (54), and the blot was exposed to Kodak XAR5 film.

Nucleotide sequence accession number. The DNA sequence of the KSHV Pr/AP has been submitted to GenBank (accession no. AF010430).

RESULTS

Pr/AP coding region of KSHV. The closest known relative of KSHV is HVS, a T-lymphotropic herpesvirus which infects monkeys (1). Previous work has shown that the HVS and KSHV genomes are of similar size and display substantial regions of colinearity in the organization of ORFs. ORF 17 of HVS encodes the viral Pr and resides near the TK gene encoded by ORF 21. In earlier sequencing of phage λ clones from a KS-derived genomic library, we identified the KSHV TK gene within a 13-kb clone (Fig. 1) (70). The high degree of colinearity between KSHV and HVS genomes in this region (45) suggested that the gene encoding the KSHV Pr/AP would likely reside nearby. Subsequent mapping and DNA sequencing of adjacent fragments from this region identified a KSHV ORF encoding a protein with striking homology to the HVS Pr. This presumptive KSHV Pr region resides at the 5′ end of a larger coding region whose 3′ portion displays evident homology to the AP gene of HVS, further suggesting that this ORF is indeed the gene coding for the Pr/AP function of KSHV.

The DNA sequence of this ORF reveals an Ala-Ser dipeptide at a position corresponding to the R-site in other herpesviruses. Thus, KSHV Pr is predicted to be a 230-amino-acid polypeptide when released from the precursor Pr/AP (Fig. 1). Figure 2 shows the alignment of the putative KSHV Pr sequence with those of other herpesviruses. The highest sequence identity (56% overall) was observed with HVS Pr. The seven conserved domains (CDs) previously found in other herpesvirus Pr's were also identified in the KSHV sequence (41, 66). These domains span Leu5 to Ser15 (CD5), Leu40 to Thr55 (CD2), Gly64 to Ser73 (CD4), Trp109 to His119 (CD3), Val135 to Tyr150 (CD1), Phe161 to Val164 (CD6), and Leu196 to Ile222 (CD7). Previous studies of HCMV Pr have identified Ser132, His63 (18, 28, 31, 66), and His157 (10, 53, 60, 65) as members of the catalytic triad. These amino acid residues are absolutely conserved among all herpesvirus Pr's, and the corresponding amino acids in the KSHV enzyme are Ser114, His46, and His134, respectively. The R-site residues Tyr-Leu-Lys-Ala*Ser-Leu-Ile-Pro of KSHV are identical at the P4 and P1 positions of all family members, with P1′ being Ser in all cases except one, infectious laryngotracheitis virus. The other recognition sites vary significantly in their flanking positions. The oxyanion stabilization signature sequence containing Gly-Arg-Arg-Xaa-Gly-Thr was identified between residues 136 to 150 in KSHV Pr; this sequence is absolutely conserved in all 14 known amino acid sequences of herpesvirus Pr's (66). This region in KSHV is identical to that in HVS. These findings suggest that the KSHV gene product has the sequence features of a functional protease.

The DNA sequence of the AP domain of Pr/AP predicts a 309-amino-acid protein. Unlike that of Pr, the amino acid sequence of KSHV AP is only weakly homologous to that of HVS. The predicted M-site is composed of Arg-Leu-Glu-Ala*Ser-Ser-Arg-Ser (Fig. 2). M-site residues of herpesvirus APs show more variety than R-site sequences; except for P1 and P1′, none of the other residues of the site is conserved. Cleavage of AP at the M-site by Pr would release the 283-amino-acid, mature AP from the 26-amino-acid carboxy-terminal fragment.

Purification of KSHV protease and specific immunoreactivity. The portion of the KSHV Pr/AP ORF corresponding to the viral Pr was subcloned into an *E. coli* expression vector transcriptionally regulated by an IPTG-inducible promoter (see Materials and Methods). An amino-terminal His_6 tag was incorporated into the Pr ORF to facilitate purification. A variant was also constructed in which Ser114 was converted to Ala (S114A). Following overexpression of His_6-Pr and His_6-Pr(S114A), these proteins were subjected to metal-chelate affinity chromatography under denaturing conditions, refolded, and eluted. Purified protein, as well as samples from throughout expression and purification, was analyzed by SDS-polyacrylamide gel electrophoresis and deemed greater than 90% pure (Fig. 3). A protein yield of roughly 10 mg from 1-liter bacterial cultures was obtained.

Expression of KSHV Pr and the S114A variant in *E. coli* yielded bands which migrated near 31 kDa, corresponding to the full-length translated Pr domain, terminating at the putative R-site alanine residue (Fig. 3). This apparent size, slightly larger than the expected 27 kDa, is most likely due to incorporation of the His_6 tag. Two faster-migrating proteolytic fragments that may have resulted from autoproteolysis were also observed. The S114A preparation is significantly more homogeneous, perhaps due to lower levels of autoproteolysis (Fig. 3A, lanes 6 and 7). In order to verify that the extra bands are not contaminating *E. coli* polypeptides, the products were examined by immunoblotting with an antibody raised to the recombinant, full-length Pr. A control bacterial extract was analyzed on the same immunoblot as the purified His_6-Pr material and gave rise to no significant, specific detection of the same bands in the purified fraction of His_6-Pr (Fig. 3B). These results indicate the purity of the protein fractions and the specificity of the antibody, α-KSP, for KSHV Pr. As a further control, a parallel immunoblot was assayed with a commercially available monoclonal antibody (Qiagen) directed against the amino-terminal Arg-Gly-Ser-His_4 epitope of His_6-Pr (data not shown). This experiment yielded essentially the same set of bands as that with α-KSP. The additional protein species thus contain the amino-terminal His_6 tag and have arisen due to either proteolysis during expression and/or purification by an endogenous bacterial enzyme or autodigestion of KSHV His_6-Pr at as-yet-uncharacterized sites.

Kinetic properties of purified KSHV Pr domain variants. To determine whether the recombinant Pr was catalytically active,

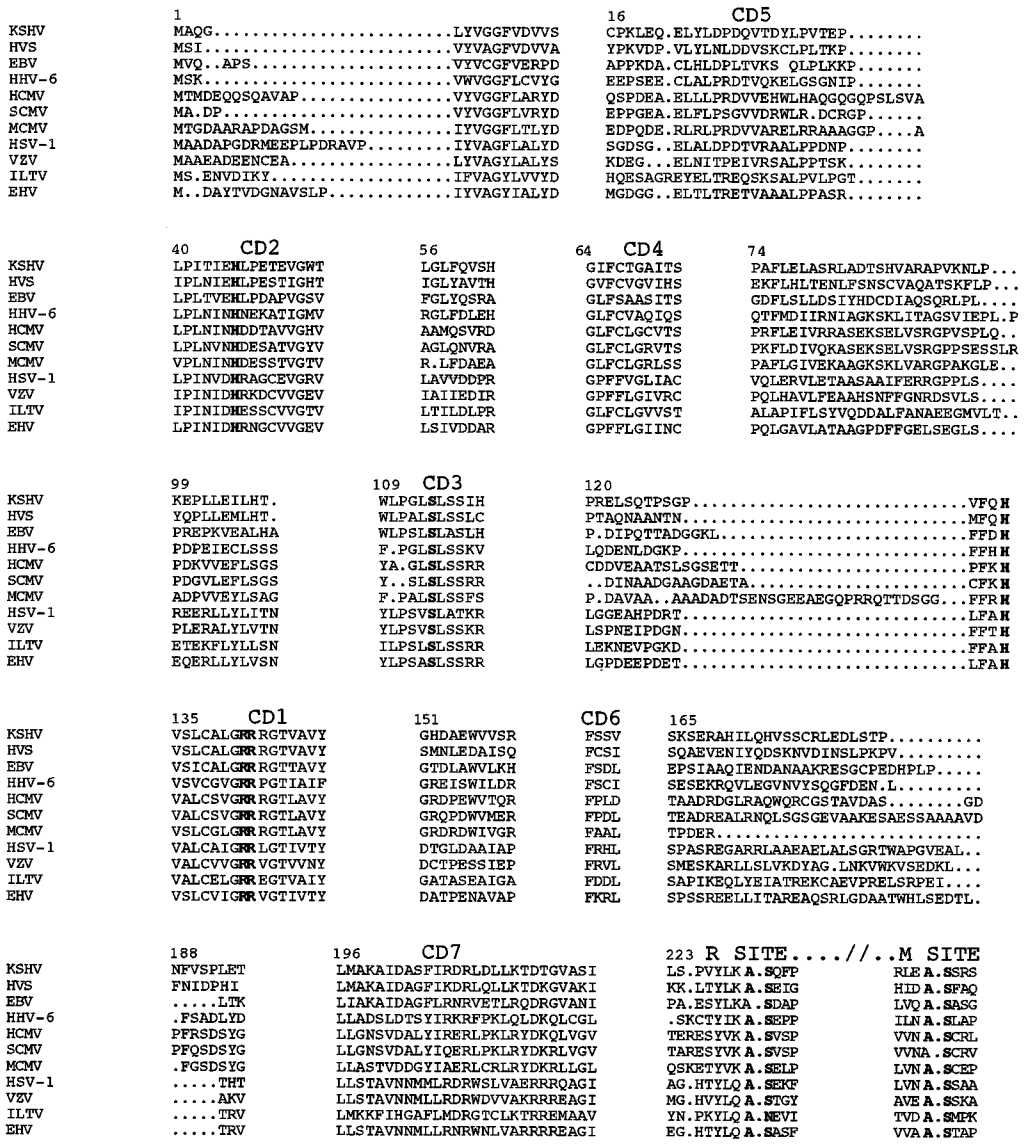

FIG. 2. Alignment of herpesvirus Pr's and AP maturation sites. The nucleotide sequence for each viral Pr was retrieved from GenBank and used to deduce the amino acid sequences shown. The order of the proteases is in accordance with the level of similarity to KSHV protease. Conserved active-site histidines and serine, the arginines implicated in oxyanion stabilization, and the cleavage site alanines and serines are shown in boldface type. The AP is located between the R-site and M-site (11). Conserved domains CD1 to CD7 are marked and numbered according to the established numbering system (37, 61). The arabic numbers above the top lines are the KSHV amino acid sequence numbers. Abbreviations: EBV, Epstein-Barr virus; SCMV, simian cytomegalovirus; MSMV, murine cytomegalovirus; HSV-1, herpes simplex virus type 1; VZV, varicella-zoster virus; ITLV, infectious laryngotracheitis virus; EHV, equine herpes-virus.

the abilities of the His_6-Pr and His_6-Pr(S114A) proteins to cleave synthetic peptide substrates were examined. The substrates, containing either the KSHV or HCMV M-site sequences flanked by a fluorogenic donor-acceptor pair, allow continuous monitoring of peptide hydrolysis due to fluorescence enhancement arising from donor-acceptor separation (30). Since the substrate specificity among the herpesvirus family of Pr's is not strict, we expected KSHV Pr to recognize the HCMV M-site substrate. Both substrates contain internal Arg residues, allowing for cleavage by trypsin, an extremely well-characterized serine protease. We can thus obtain preliminary information regarding the KSHV enzyme's substrate specificity, as well as perform a comparative analysis of its catalytic efficiency versus those of selected trypsin variants of differing activity.

There is indeed a difference in proteolysis catalyzed by KSHV Pr and the active-site variant. Both the KSHV and HCMV substrates demonstrate minimal hydrolysis by the S114A variant over 1 h, perhaps due to some residual proteolytic activity (for which kinetic parameters were not able to be resolved in the present assay). On the other hand, while native KSHV Pr has a low absolute velocity, the k_{cat}/K_m values indicated for this enzyme are comparable to those for its related herpesviral maturational Pr's (Table 1). Such values have shown significant enhancement under various conditions for the HSV Pr (29) and will likely do so for KSHV Pr in further biochemical studies as well. In addition, our k_{cat}/K_m values could be higher upon the considerations of dimerization and the fact that we may be slightly below this assembly threshold. Darke et al., by careful enzymatic analysis, demonstrated ap-

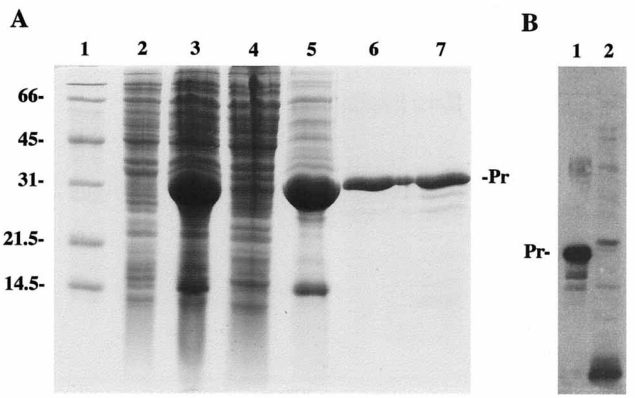

FIG. 3. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of KSHV Pr expression and purification. (A) Coomassie blue-stained, 12.5% polyacrylamide–SDS gel. Lane 1 contains molecular size standards, with their masses (in kilodaltons) annotated to the left. Lane 2 contains a bacterial lysate harvested prior to IPTG induction. Lane 3 contains a lysate collected at the time of protein harvest. Lane 4 contains the soluble fraction after sonication of the lysate. Lane 5 contains urea-solubilized inclusion bodies. Lane 6 contains purified KSHV His_6-Pr(S114A). Lane 7 contains KSHV His_6-Pr. (B) Immunoblot of purified KSHV His_6-Pr (lane 1) and pQE30-transformed, IPTG-induced bacterial lysate (lane 2). The migration of Pr, noted between the two panels, differs due to different electrophoresis conditions.

parent HCMV Pr K_d values very near our experimental KSHV Pr concentration (16), and Cole observed slightly higher values in sedimentation studies (12). In any case, there is specific cleavage of a KSHV substrate by the purified KSHV Pr as well as apparent cross-species reactivity with a related HCMV site.

Due to its low velocity, the proteolytic activity of this newly identified Pr was compared with those of trypsin and trypsin D102N (15). Trypsin has an extremely high rate of catalytic turnover and has served as a paradigm for the understanding of site-specific enzyme-directed peptide hydrolysis. Trypsin D102N, in which the active-site Asp residue has been replaced with Asn, has a catalytic efficiency orders of magnitude lower than that of trypsin, depending on solution conditions (in our assay, roughly 10,000-fold lower). We reasoned that these two proteases would provide a sound basis for comparison with the KSHV Pr, which lacks an Asp within its catalytic triad. Indeed, 1 nM trypsin hydrolyzes both substrates at a much higher rate than 430 nM KSHV Pr, indicating that trypsin is substantially more active than the KSHV enzyme (Table 1). On the other hand, trypsin D102N and KSHV Pr have similar kinetic profiles at identical enzyme concentrations (Table 1). Thus, the lack of an active-site Asp, with conservation of the other two catalytic triad members, His and Ser, results in similar rates of peptide hydrolysis by two unrelated serine proteases, whether engineered, as in trypsin, or naturally occurring, as in KSHV Pr.

TABLE 1. k_{cat}/K_m values for herpesviral Pr's and trypsins

Enzyme	k_{cat}/K_m (M^{-1} min^{-1})		
	KSHV M-site	HCMV M-site	Related herpesvirus processing site(s)[f]
KSHV Pr[a]	165	590	
KSHV Pr S114A	ND[b]	ND	
Trypsin D102N[a]	457	2,162	
Trypsin[a]	3.7×10^6	2.3×10^6	
HSV-1 Pr			<100–2,200,[c] 27–27,000[d]
HCMV Pr[e]			110–4,000
HHV-6 Pr[e]			3,000–17,000

[a] Values determined in this study.
[b] ND, not detectable in this assay.
[c] Values from reference 18.
[d] Values from reference 29.
[e] Values from reference 64.
[f] See individual references for exact substrates used.

In vivo transcription of KSHV AP and Pr/AP mRNAs during the viral life cycle. To examine the transcription of the KSHV Pr/AP and AP genes in lytic infection, the KSHV-infected B-cell line, BCBL-1, was employed. This line harbors the viral genome in latent form, and treatment of these cells with TPA induces lytic replication (54). Equivalent amounts of poly(A)-enriched RNA from uninduced or induced BCBL-1 cells in the presence or absence of PFA were separated on a formaldehyde-agarose gel, transferred to a membrane, and hybridized to a probe made from the 1.5-kb fragment containing the Pr/AP ORF of KSHV (Fig. 4A). A band migrating at approximately 950 bases, the expected size of the AP transcript, hybridizes weakly to the probe in lane 1, which contains RNA from uninduced cells; production of this species is strongly inhibited by PFA, an inhibitor of herpesviral DNA replication, as noted by its absence in lane 2, which contains RNA isolated from uninduced, PFA-treated cells. The band in lane 1 is presumably due to the small amount of spontaneous lytic reactivation in the BCBL-1 cells (54). This RNA species is not present when a single-stranded antisense probe specific to the Pr domain (i.e., that does not contain the AP transcript) is hybridized to RNA from either induced or uninduced BCBL-1 cells (Fig. 4C, lanes 1 and 2) but hybridizes strongly to the 1.5-kb single-stranded antisense probe for Pr/AP (Fig. 4C, lanes 3 and 4). Expression of the AP transcript is strongly upregulated upon lytic induction of KSHV (Fig. 4A, lane 3); after induction there is also a very weak band migrating around 1.8 kb, a size consistent with what would be expected from the transcript encoding the Pr/AP ORF. This species hybridizes to a single-stranded probe specific for the Pr domain (Fig. 4C, lane 2), indicating that this RNA species is the Pr transcript. There are also slower-migrating species (roughly 3 and 4 kb) that have the same temporal induction pattern as the Pr transcript; the origin of these RNAs is unclear, but they may emanate from read-through into flanking viral sequences. Indeed, there is a canonical poly(A) signal approximately 2 kb downstream from the predicted Pr/AP poly(A) site. The strong band observed at approximately 3 kb is presumably read-through of the AP transcript, since it does not hybridize to the Pr-specific single-stranded probe. The roughly 4-kb, weaker signal may represent the read-through of the Pr/AP transcript. Both the 950-bp and 1.8-kb bands are inhibited in the presence of PFA upon TPA induction, as are the slower-migrating bands, indicating that they are late transcripts made primarily after viral DNA replication.

DISCUSSION

In this report, we present the sequence of the Pr/AP coding region of KSHV and demonstrate that the translated Pr domain is catalytically active. The similarity of the genomic coding organization of the KSHV Pr/AP region to those of other herpesviruses strongly suggests that the functional role of the KSHV Pr in lytic replication mirrors that described for its cognates in the related viruses. We also identify the abundant AP transcript and the low-abundance Pr transcript as late genes, strongly upregulated by TPA and inhibited in the presence of PFA, a viral replication inhibitor.

Comparison of the sequence of KSHV Pr with those of other herpesviral Pr's allows instructive inferences to be made about

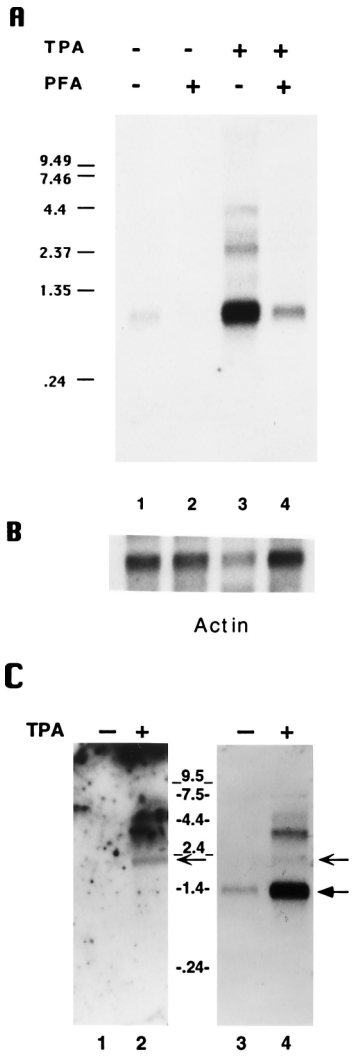

FIG. 4. Northern blots of BCBL-1 RNA hybridized to probes from Pr or Pr/AP. (A) Total RNA (300 μg) from BCBL-1 cells that were uninduced (lane 1), uninduced in the presence of PFA (lane 2), induced with TPA (lane 3), or induced with TPA in the presence of PFA, was poly(A) enriched, separated on a 1% agarose–formaldehyde gel, and transferred to a nylon membrane. The membrane was hybridized to a probe made from sequences in Pr/AP (see Materials and Methods) and exposed to Kodak XAR5 film. (B) Same blot as shown in panel A, but hybridized to an actin probe. (C) Poly(A)-enriched RNA from uninduced BCBL-1 cells (lanes 1 and 3) and TPA-induced BCBL-1 cells (lanes 2 and 4) was hybridized to single-stranded antisense probes to the Pr domain alone (lanes 1 and 2, probe from the 0.8 kb *Sac*I fragment described earlier) and exposed to film for 2 days or was hybridized to the Pr/AP probe and exposed for 5 h (lanes 3 and 4). The bottom arrow indicates a transcript corresponding to the predicted size of the AP transcript, and the top arrows indicate RNA species corresponding to the predicted size of the Pr/AP transcript. Values adjacent to the gels are molecular sizes (in kilobases).

the KSHV enzyme. With HCMV, affinity labelling experiments using diisopropyl fluorophosphate identified Ser132 as the putative active-site nucleophile of its Pr (18). Replacing Ser132 with an alanine abolished proteolytic activity, supporting this identification. His63 was shown to be the second member of the catalytic triad by mutagenesis studies. The three-dimensional structure of HCMV Pr confirmed the identities of Ser132 and His63 as catalytic residues and suggested His157 as the third member of the triad (10, 53, 60, 65). KSHV Pr has a serine at position 114 in CD3, a histidine at position 46 in CD2, and a histidine at position 134 in CD1; these are presumably the corresponding catalytic residues (Fig. 2). The assignment of Ser114 as the active-site nucleophile is supported by our kinetic analysis of the S114A-substituted KSHV Pr.

The high sequence identity, 45%, between the KSHV and HCMV Pr's suggests that the two enzymes have similar three-dimensional structures. The unique fold of proteases of the herpesvirus family creates a Ser-His-His catalytic triad in contrast to the Ser-His-Asp active site of other serine proteases such as trypsin, subtilisin, and yeast carboxypeptidase (49). The latter, digestive enzymes are significantly more active, exhibiting peptide hydrolysis rates 3 to 5 orders of magnitude greater than those of the viral Pr's. A trypsin variant in which the active-site aspartic acid has been replaced with an asparagine, trypsin D102N, exhibits an activity on synthetic peptide substrates that is similar to that of the KSHV Pr (Table 1). Trypsin D102H, not yet expressed or characterized, would perhaps present a more accurate comparison but is not necessary to draw conclusions regarding the loss of Asp within the catalytic triad. The instructive value of D102H versus D102N is likely to be subtle, although it is an issue which may deserve further experimental consideration.

A less active protease can be understood in the context of the more selective processing activity associated with the herpesvirus life cycle. Indeed, the slightly higher k_{cat}/K_m value for KSHV Pr directed toward the HCMV substrate may be indicative of this. Perhaps it is favorable for the KSHV M-site to be processed slightly more slowly than other sites. It is also possible that the difference in M-site composition between the two

ATG GCA CAG GGC CTG TAC GTC GGA GGG TTT GTA GAT GTT GTG TCC TGC CCC AAG CTG GAG
CAA GAG CTC TAT CTC GAT CCG GAT CAG GTG ACG GAT TAT CTC CCA GTC ACA GAA CCC CTT
CCA ATA ACA ATC GAA CAC CTA CCA GAG ACA GAA GTG GGC TGG ACA CTG GGT CTA TTT CAA
GTG TCC CAC GGT ATT TTC TGC ACC GGA GCC ATC ACG TCG CCA GCC TTC CTA GAG CTG GCA
TCC AGG CTG GCG GAC ACC TCC CAC GTG GCC AGA GCC CCC GTG AAA AAT CTC CCT AAG GAA
CCA CTG TTG GAG ATA CTC CAC ACG TGG CTC CCG GGG CTG TCT TTA TCG TCC ATA CAT CCC
CGC GAG TTA TCC CAG ACT CCC AGC GGT CCC GTG TTT CAA CAC GTA TCA CTA TGC GCC CTG
GGG CGC CGA CGC GGC ACA GTG GCC GTG TAC GGA CAC GAC GCC GAG TGG GTG GTT TCC AGA
TTC TCA TCA GTA TCT AAG TCG GAG CGC GCC CAC ATC CTC CAG CAC GTA AGT AGC TGC AGG
CTG GAG GAC CTT TCC ACA CCA AAT TTC GTC AGT CCC CTG GAG ACC TTA ATG GCA AAA GCT
ATA GAT GCC AGC TTC ATA CGG GAC CGC CTC GAC CTA TTG AAA ACT GAC AGA GGT GTG GCC
AGC ATA TTG AGC CCG GTG TAT TTA AAG GCC AGC CAA TTC CCG GCC GGC ATC CAA GCC GTC
ACA CCA CCC AGA CCA GCC ATG AAC AGC TCT GGT CAA GAG GAT ATC ATA TCC ATC CCC AAA
TCC GCC TTC CTG AGC ATG CTA CAA AGC AGC ATC GAT GGA ATG AAG ACC ACA GCG GCA AAA
ATG TCA CAT ACA CTT TCA GGG CCA GGC CTA ATG GGG TGT GGG GGC CAG ATG TTC CCC ACC
GAC CAT CAC CTA CCT TCG TAT GTT TCA AAC CCA GCG CCA CCA TAC GGC TAC GCT TAC AAG
AAC CCA TAC GAT CCA TGG TAT TAC TCG CCA CAG CTG CCT GGA TAT AGG ACG GGG AAG CGC
AAG CGC GGC GCA GAG GAC GAC GAA GGA CAC CTC TTT CCA GGA GAG GAG CCG GCG TAT CAC
AAG GAT ATC TTG TCC ATG TCA AAG AAC ATA GCG GAA ATA CAG TCT GAA CTC AAA GAG ATG
AAA CTG AAC GGT TGG CAC GCA GGG CCA CCG CCG TCC TCC TCT GCA GCA GCA GCC GCA GTA
GAT CCA CAC TAC AGG CCC CAC GCC AAT TCA GCG GCC CCG TGT CAA TTC CCG ACA ATG AAG
GAG CAC GGA GGA ACC TAC GTA CAC CCA CCC ATT TAC GTG CAG GCG CCA CAC GGT CAG TTC
CAG CAA GCG GCG CCC ATC CTT TTT GCT CAG CCA CAT GTG AGC CAC CCG CCA GTC TCT ACA
GGA CTC GCG GTA GTT GGC GCA CCA CCC GCT GAA CCC ACC CCC GCC TCC AGC ACG CAG AGC
ATC CAA CAA CAG GCA CCG GAG ACC ACG CAT ACA CCA TGC GCG GCG GTG GAG AAA GAC GCT
CCT ACG CCG AAC CCT ACA TCG AAC CGC CTT GAA GCC AGC AGT CGC TCT AGT CCA AAA TCT
AAA ATT CGC AAG ATG TTC TGC GAG GAG CTC CAG CTT TTG GTT CCC TTT AGT GAG GGT TAA

FIG. 5. DNA sequence of KSHV Pr/AP.

synthetic substrates (P5-P6′ for KSHV and P6-P5′ for HCMV) is responsible for this difference, a fact that has been observed with HSV Pr, for which peptide substrates containing positions P9-P8′ were required for optimal hydrolytic activity (19). In addition, the different fluorophore moieties could contribute to altered subsite binding properties; indeed, trypsin and trypsin D102N appear to discriminate between the two substrates as well. The absence of the aspartic acid in the active site of the viral Pr, while reducing the enzyme's velocity, also results in less sensitivity to standard serine protease inhibitors such as phenylmethylsulfonyl fluoride and tosyl lysyl chloromethyl ketone (8, 14, 39, 62, 66). This was similarly observed for trypsin D102N (15). Hence, subsequent inhibitor design for this new class of serine proteases must take into account the highly reduced nucleophilicity of the active-site serine and may, in fact, be aided by parallel investigations of this issue with trypsin D102N or D102H.

The prospect for specific inhibitor design is now enhanced by the availability of KSHV Pr. The bacterially expressed, His_6-tagged Pr molecule is capable of catalyzing peptide bond hydrolysis of related viral processing sites. Preliminary analysis of the KSHV Pr reaction products by mass spectrometric methods (data not shown) indicates that three sites within the 11-amino-acid KSHV M-site peptide are cleaved by KSHV Pr (Ser6-Ser7, Ala5-Ser6, and Arg8-Ser9) and that two sites within the 11-amino-acid HCMV M-site peptide (Ala6-Ser7 and Ser7-Ser8) are hydrolyzed by the enzyme. The expected Ala*Ser bond is cleaved in both cases. The basis for the relaxed proteolytic specificity of KSHV Pr toward these substrates is currently under investigation, as is a more complete kinetic characterization of the enzyme. This will allow direct comparison of the kinetic behavior of members of the herpesvirus family of maturational Pr's with that of the now-identified KSHV enzyme. Results of this ongoing study will aid in the understanding of the molecular recognition and chemical-mechanistic properties of KSHV Pr.

Although the various Pr members of the herpesvirus family are highly similar in their primary structure, there are several differences, especially with regard to their individual substrate specificities. This can be seen in the kinetic analysis of various synthetic substrates (64), as well as differences in the amino acid sequences of the release sites of the viral Pr's. In particular, amino acid differences between the P3, P2, P2′, P3′, and P4′ sites of KSHV Pr and the R-sites of other members of the family can be seen (Fig. 2). These differences may permit the design of specific inhibitors of the KSHV protease. Furthermore, the prospect of achieving antiviral specificity with limited host cell toxicity may be enhanced by the fact that herpesvirus Pr's have no known cellular homologs.

Finally, the three-dimensional structure of the HCMV Pr reveals a dimeric assembly stoichiometry, consistent with both sedimentation studies (12) and the concentration dependence profile of its enzymatic activity (16). Not unexpectedly, this assembly requires an extensive interface between its constituent promoters. A major component of this interface is an α-helix composed of residues 217 to 230 as well as residues from CD7. This region is conserved in its entirety in KSHV Pr and strongly suggests a linkage between dimerization and enzymatic activity for this newly identified enzyme as well. This likely feature of the KSHV protease provides the opportunity to develop KSHV-specific or more broadly active antiherpesviral agents, through disruption of Pr or Pr/AP homodimer formation. This strategy, using small-molecule or dominant-negative inhibitors of dimerization, has proven to be effective in inhibiting the HIV Pr (4, 35, 42). Such inhibitors can be used to dissect the role of the Pr in the KSHV viral life cycle and to examine the impact of antiviral therapy on the natural history of KSHV infection.

APPENDIX

During the preparation of this manuscript, the genomic sequence of KSHV was published (56). Comparison of our KSHV Pr DNA sequence and that published showed a difference at codon 217, resulting in a Thr in the sequence presented in this manuscript and an Arg in the above-mentioned publication. This difference could be due to strain differences, cloning artifacts, or sequencing errors. The sequence we obtained is provided here (Fig. 5).

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REFERENCES

1. **Albrecht, J.-C., J. Nicholas, D. Biller, K. R. Cameron, B. Biesinger, C. Newman, S. Wittmann, M. A. Craxton, H. Coleman, B. Fleckenstein, and R. W. Honess.** 1992. Primary structure of the herpesvirus saimiri genome. J. Virol. **66:**5047–5058.
2. **Ambroziak, J. A., D. J. Blackbourn, B. G. Herndier, R. G. Glogau, J. H. Gullett, A. R. McDonald, E. T. Lennette, and J. A. Levy.** 1995. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. Science **268:**582–583.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Wiley & Sons, New York, N.Y.
4. **Babé, L. M., J. Rosé, and C. S. Craik.** 1992. Synthetic "interface" peptides alter dimeric assembly of the HIV 1 and 2 proteases. Protein Sci. **1:**1244–1253.
5. **Beral, V.** 1991. Epidemiology of Kaposi's sarcoma. Cancer Surv. **10:**5–22. (Erratum, **12:**225, 1992.)
6. **Beral, V., T. A. Peterman, R. L. Berkelman, and H. W. Jaffe.** 1990. Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? Lancet **335:**123–128.
7. **Boshoff, C., T. F. Schulz, M. M. Kennedy, A. K. Graham, C. Fisher, A. Thomas, J. O. McGee, R. A. Weiss, and J. J. O'Leary.** 1995. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat. Med. **1:**1274–1278.
8. **Burck, P. J., D. H. Berg, T. P. Luk, L. M. Sassmannshausen, M. Wakulchik, D. P. Smith, H. M. Hsiung, G. W. Becker, W. Gibson, and E. C. Villarreal.** 1994. Human cytomegalovirus maturational proteinase: expression in *Escherichia coli*, purification, and enzymatic characterization by using peptide substrate mimics of natural cleavage sites. J. Virol. **68:**2937–2946.
9. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865–1869.
10. **Chen, P., H. Tsuge, R. J. Almassy, C. L. Gribskov, S. Katoh, D. L. Vanderpool, S. A. Margosiak, C. Pinko, D. A. Matthews, and C. C. Kan.** 1996. Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad. Cell **86:**835–843.
11. **Chuck, S., R. M. Grant, E. Katongole-Mbidde, M. Conant, and D. Ganem.** 1996. Frequent presence of a novel herpesvirus genome in lesions of human immunodeficiency virus-negative Kaposi's sarcoma. J. Infect. Dis. **173:**248–251.
12. **Cole, J. L.** 1996. Characterization of human cytomegalovirus protease dimerization by analytical centrifugation. Biochemistry **35:**15601–15610.
13. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Grahm, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivoprakash, et al.** 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:**569–571.
14. **Cox, G. A., M. Wakulchik, L. M. Sassmannshausen, W. Gibson, and E. C. Villarreal.** 1995. Human cytomegalovirus proteinase: candidate glutamic acid identified as third member of putative active-site triad. J. Virol. **69:**4524–4528.
15. **Craik, C. S., S. Roczniak, C. Largman, and W. J. Rutter.** 1987. The catalytic role of the active site aspartic acid in serine proteases. Science **237:**909–913.
16. **Darke, P. L., J. L. Cole, L. Waxman, D. L. Hall, M. K. Sardana, and L. C.**

Kuo. 1996. Active human cytomegalovirus protease is a dimer. J. Biol. Chem. **271:**7445–7449.
17. **Desai, P., S. C. Watkins, and S. Person.** 1994. The size and symmetry of B capsids of herpes simplex virus type 1 are determined by the gene products of the UL26 open reading frame. J. Virol. **68:**5365–5374.
18. **Dilanni, C. L., J. T. Stevens, M. Bolgar, D. R. O'Boyle, S. P. Weinheimer, and R. J. Colonno.** 1994. Identification of the serine residue at the active site of the herpes simplex virus type 1 protease. J. Biol. Chem. **269:**12672–12676.
19. **Dilanni, C. L., C. Mapelli, D. A. Drier, J. Tsao, S. Natarajan, D. Riexinger, S. M. Festin, M. Bolgar, G. Yamanaka, S. P. Weinheimer, C. A. Meyers, R. J. Colonno, and M. G. Cordingley.** 1993. In vitro activity of the herpes simplex virus type 1 protease with peptide substrates. J. Biol. Chem. **268:**25449–25454.
20. **Ensoli, B., G. Barillari, and R. C. Gallo.** 1991. Pathogenesis of AIDS-associated Kaposi's sarcoma. Hematol. Oncol. Clin. N. Am. **5:**281–295.
21. **Evnin, L. B., J. R. Vasquez, and C. S. Craik.** 1990. Substrate specificity of trypsin investigated by using a genetic selection. Proc. Natl. Acad. Sci. USA **87:**6659–6663.
22. **Ganem, D.** 1994. Viruses, cytokines and Kaposi's sarcoma. Curr. Biol. **5:**4693.
23. **Gao, M., L. Matusick-Kumar, W. Hurlburt, S. F. DiTusa, W. W. Newcomb, J. C. Brown, P. J. McCann III, I. Deckman, and R. J. Colonno.** 1994. The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. J. Virol. **68:**3702–3712.
24. **Gao, S. J., L. Kingsley, M. Li, W. Zheng, C. Parravicini, J. Ziegler, R. Newton, C. R. Rinaldo, A. Saah, J. Phair, R. Detels, Y. Chang, and P. S. Moore.** 1996. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. Nat. Med. **2:**925–928.
25. **Gibson, W.** 1981. Structural and nonstructural proteins of strain Colburn cytomegalovirus. Virology **111:**516–537.
26. **Gibson, W., A. I. Marcy, J. C. Comolli, and J. Lee.** 1990. Identification of precursor to cytomegalovirus capsid assembly protein and evidence that processing results in loss of its carboxy-terminal end. J. Virol. **64:**1241–1249.
27. **Gibson, W., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10:**1044–1052.
28. **Gibson, W., A. R. Welch, and M. R. T. Hall.** 1994. Assemblin, a herpes virus serine maturational proteinase and new molecular target for antivirals. Perspect. Drug Discov. Desn. **2:**413–426.
29. **Hall, D. L., and P. L. Darke.** 1995. Activation of the herpes simplex virus type 1 protease. J. Biol. Chem. **270:**22697–22700.
30. **Holskin, B. P., M. Bukhtiyarova, B. M. Dunn, P. Baur, J. de Chastonay, and M. W. Pennington.** 1995. A continuous fluorescence-based assay of human cytomegalovirus protease using a peptide substrate. Anal. Biochem. **227:**148–155.
31. **Holwerda, B. C., A. J. Wittwer, K. L. Duffin, C. Smith, M. V. Toth, L. S. Carr, R. C. Wiegand, and M. L. Bryant.** 1994. Activity of two-chain recombinant human cytomegalovirus protease. J. Biol. Chem. **269:**25911–25915.
32. **Hong, Z., M. Beaudet-Miller, J. Durkin, R. Zhang, and A. D. Kwong.** 1996. Identification of a minimal hydrophobic domain in the herpes simplex virus type 1 scaffolding protein which is required for interaction with the major capsid protein. J. Virol. **70:**533–540.
33. **Huang, Y. Q., J. J. Li, M. H. Kaplan, B. Poiesz, E. Katabira, W. C. Zhang, D. Feiner, and A. E. Friedman-Kien.** 1995. Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. Lancet **345:**759–761.
34. **Irmiere, A., and W. Gibson.** 1985. Isolation of human cytomegalovirus intranuclear capsids, characterization of their protein constituents, and demonstration that the B-capsid assembly protein is also abundant in noninfectious enveloped particles. J. Virol. **56:**277–283.
35. **Junker, U., S. Escaich, I. Plavec, J. Baker, F. McPhee, J. R. Rosé, C. S. Craik, and E. Böhnlein.** 1996. Intracellular expression of human immunodeficiency virus type 1 (HIV-1) protease variants inhibits replication of wild-type and protease inhibitor-resistant HIV-1 strains in human T-cell lines. J. Virol. **70:**7765–7772.
36. **Kaiser, E., R. L. Colescott, C. D. Bossinger, and P. I. Cook.** 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal. Biochem. **34:**595–598.
37. **Kedes, D. H., E. Operskalski, M. Busch, R. Kohn, J. Flood, and D. Ganem.** 1996. The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nat. Med. **2:**918–924. (Comments.)
38. **King, D. S., C. G. Fields, and G. B. Fields.** 1990. A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. Int. J. Pept. Protein Res. **36:**255–266.
39. **Liu, F., and B. Roizman.** 1992. Differentiation of multiple domains in the herpes simplex virus 1 protease encoded by the UL26 gene. Proc. Natl. Acad. Sci. USA **89:**2076–2080.
40. **Liu, F. Y., and B. Roizman.** 1991. The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. J. Virol. **65:**5149–5156.
41. **Loutsch, J. M., N. J. Galvin, M. L. Bryant, and B. C. Holwerda.** 1994. Cloning and sequence analysis of murine cytomegalovirus protease and capsid assembly protein genes. Biochem. Biophys. Res. Commun. **203:**472–478.
42. **McPhee, F., A. C. Good, I. D. Kuntz, and C. S. Craik.** 1996. Engineering HIV-1 protease heterodimers as macromolecular inhibitors of viral maturation. Proc. Natl. Acad. Sci. USA **93:**11477–11481.
43. **Moore, P., L. Kingsley, S. Holmberg, T. Spira, P. Gupta, D. Hoover, J. Parry, L. Conley, H. Jaffe, and Y. Chang.** 1995. Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. AIDS **10:**175–180.
44. **Moore, P. S., and Y. Chang.** 1995. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and without HIV infection. N. Engl. J. Med. **332:**1181–1185.
45. **Moore, P. S., S. J. Gao, G. Dominguez, E. Cesarman, O. Lungu, D. M. Knowles, R. Garber, P. E. Pellett, D. J. McGeoch, and Y. Chang.** 1996. Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. J. Virol. **70:**549–558.
46. **Nagase, H., C. G. Fields, and G. B. Fields.** 1994. Design and characterization of a fluorogenic substrate selectively hydrolyzed by stromelysin 1 (matrix metalloproteinase-3). J. Biol. Chem. **269:**20952–20957.
47. **Nicholson, P., C. Addison, A. M. Cross, and J. Kennard.** 1994. Localization of the herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires the abundant scaffolding protein VP22A. J. Gen. Virol. **75:**1091–1099.
48. **O'Callaghan, D. J., and C. C. Randall.** 1976. Molecular anatomy of herpesviruses: recent studies. Prog. Med. Virol. **22:**152–210.
49. **Perona, J. J., and C. S. Craik.** 1995. Structural basis of substrate specificity in the serine proteases. Protein Sci. **4:**337–360.
50. **Preston, V. G., M. F. al-Kobaisi, I. M. McDougall, and F. J. Rixon.** 1994. The herpes simplex virus gene UL26 proteinase in the presence of the UL26.5 gene product promotes the formation of scaffold-like structures. J. Gen. Virol. **75:**2355–2366.
51. **Preston, V. G., J. A. Coates, and F. J. Rixon.** 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. J. Virol. **45:**1056–1064.
52. **Preston, V. G., F. J. Rixon, I. M. McDougall, M. McGregor, and M. F. al-Kobaisi.** 1992. Processing of the herpes simplex virus assembly protein ICP35 near its carboxy terminal end requires the product of the whole of the UL26 reading frame. Virology **186:**87–98.
53. **Qiu, X., J. S. Culp, A. G. DiLella, B. Hellmig, S. S. Hoog, C. A. Janson, W. W. Smith, and S. S. Abdel-Meguid.** 1996. Unique fold and active site in cytomegalovirus protease. Nature **383:**275–279.
54. **Renne, R., W. Zhong, B. Herndier, M. McGrath, N. Abbey, D. Kedes, and D. Ganem.** 1996. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat. Med. **2:**342–346.
55. **Rixon, F. J., A. M. Cross, C. Addison, and V. G. Preston.** 1988. The products of herpes simplex virus type 1 gene UL26 which are involved in DNA packaging are strongly associated with empty but not with full capsids. J. Gen. Virol. **69:**2879–2891.
56. **Russo, J. J., R. A. Bohenzky, M.-C. Chien, J. Chen, M. Yan, D. Maddalena, J. P. P. Parry, D. Peruzzi, I. S. Edelman, Y. Chang, and P. S. Moore.** 1996. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc. Natl. Acad. Sci. USA **93:**14862–14867.
57. **Schalling, M., M. Ekman, E. E. Kaaya, A. Linde, and P. Biberfeld.** 1995. A role for a new herpes virus (KSHV) in different forms of Kaposi's sarcoma. Nat. Med. **1:**705–706.
58. **Schechter, I., and A. Berger.** 1968. On the active site of proteases. 3. Mapping the active site of papain; specific peptide inhibitors of papain. Biochem. Biophys. Res. Commun. **32:**898–902.
59. **Sherman, G., and S. L. Bachenheimer.** 1988. Characterization of intranuclear capsids made by ts morphogenic mutants of HSV-1. Virology **163:**471–480.
60. **Shieh, H. S., R. G. Kurumbail, A. M. Stevens, R. A. Stegeman, E. J. Sturman, J. Y. Pak, A. J. Wittwer, M. O. Palmier, R. C. Wiegand, B. C. Holwerda, and W. C. Stallings.** 1996. Three-dimensional structure of human cytomegalovirus protease. Nature **383:**279–282.
61. **Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M. F. d'Agay, J. P. Clauvel, M. Raphael, L. Degos, et al.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood **86:**1276–1280.
62. **Stevens, J. T., C. Mapelli, J. Tsao, M. Hail, D. N. O'Boyle, S. P. Weinheimer, and C. L. Diianni.** 1994. In vitro proteolytic activity and active-site identification of the human cytomegalovirus protease. Eur. J. Biochem. **226:**361–367.
63. **Thomsen, D. R., L. L. Roof, and F. L. Homa.** 1994. Assembly of herpes simplex virus (HSV) intermediate capsids in insect cells infected with recombinant baculoviruses expressing HSV capsid proteins. J. Virol. **68:**2442–2457.
64. **Tigue, N. J., P. J. Matharu, N. A. Roberts, J. S. Mills, J. Kay, and R. Jupp.** 1996. Cloning, expression and characterization of the proteinase from human herpesvirus 6. J. Virol. **70:**4136–4141.
65. **Tong, L., C. Qian, M. J. Massariol, P. R. Bonneau, M. G. Cordingley, and L. Lagace.** 1996. A new serine-protease fold revealed by the crystal structure of human cytomegalovirus protease. Nature **383:**272–275.

66. **Welch, A. R., L. M. McNally, M. R. T. Hall, and W. Gibson.** 1993. Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine. J. Virol. **67:**7360–7372.
67. **Welch, A. R., A. S. Woods, L. M. McNally, R. J. Cotter, and W. Gibson.** 1991. A herpesvirus maturational proteinase, assemblin: identification of its gene, putative active site domain, and cleavage site. Proc. Natl. Acad. Sci. USA **88:**10792–10796.
68. **Whitby, D., M. R. Howard, M. Tenant-Flowers, N. S. Brink, A. Copas, C. Boshoff, T. Hatzioannou, F. E. Suggett, D. M. Aldam, A. S. Denton, et al.** 1995. Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet **346:**799–802.
69. **Wood, L. J., M. K. Baxter, S. M. Plafker, and W. Gibson.** 1997. Human cytomegalovirus capsid assembly protein precursor (pUL80.5) interacts with itself and with the major capsid protein (pUL86) through two different domains. J. Virol. **71:**179–190.
70. **Zhong, W., H. Wang, B. Herndier, and D. Ganem.** 1996. Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus B) genes in Kaposi sarcoma. Proc. Natl. Acad. Sci. USA **93:**6641–6646.