Activity, Specificity and Probe Design for the Smallpox Virus Protease K7L

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Background: The K7L protease is required for smallpox virus assembly.

Results: K7L requires specific substrate recognition elements, dimerization, and nucleic acid cofactors for maximum activity.

Conclusion: We have identified contributors to optimal K7L protease activity and specificity.

Significance: This first characterization of a poxvirus processing protease demonstrates the importance of dimerization and extended substrate recognition in the control of activity and specificity.

The K7L gene product of the smallpox virus is a protease implicated in the maturation of viral proteins. K7L belongs to protease Clan CE, which includes distantly related cysteine proteases from eukaryotes, pathogenic bacteria, and viruses. Here, we describe its recombinant high-level expression, biochemical mechanism, substrate preference, and regulation. Earlier studies inferred that the orthologous I7L vaccinia protease cleaves at an AG-X motif in six viral proteins. Our data for K7L suggest that the AG-X motif is necessary but not sufficient for optimal cleavage activity. Thus, K7L requires peptides extended into the P7 and P8 positions for efficient substrate cleavage. Catalytic activity of K7L is substantially enhanced by homodimerization, by the substrate protein P25K, as well as by glycerol.

RNA and DNA also enhance cleavage of the P25K protein, but not of synthetic peptides, suggesting that nucleic acids augment the interaction of K7L with its protein substrate. Library-based peptide preference analyses enabled us to design an activity-based probe that covalently and selectively labels K7L in lysates of transfected and infected cells. Our study thus provides proof-of-concept for the design of inhibitors and probes that may contribute both to a better understanding of the role of K7L in the virus life cycle and the design of novel anti-virals.

The Orthopoxviridae family of viruses infects vertebrate and invertebrate animals. Humans can be infected by several poxviruses, but the most prominent of them are variola (smallpox), monkeypox, vaccinia, and molluscum contagiosum viruses (1). The poxviruses are encoded by a large double stranded DNA genome that is translated into ~250 individual proteins within the host cytosol (2). The life cycle of the virus includes several morphogenetic stages, during one of which a spherical, immature particle transforms into a brick-shaped, infectious virion. This maturation has been associated with the proteolytic processing of several structural proteins by two viral proteases (3-8), but the enzymatic principles that govern this process remain poorly understood. Thus, the maturing
peptidases are known to pack together with their targets inside the viral core (a protein compartment surrounding the DNA), but the processes that switch their activity on are still unknown. Furthermore, the importance of these peptidases for infectivity makes them attractive targets for the development of antiviral therapies against a broad spectrum of poxviruses (9-11). Consequently, a better understanding of their substrate specificities should be beneficial for drug design.

The two variola genes that encode peptidases are H1L and K7L, while the same genes in the vaccinia virus are called G1L and I7L, respectively (12). The H1L/G1L gene products are distantly homologous to the M16 family of bacterial and eukaryotic metallopeptidases (6), which either degrade oligopeptides or truncate the signal peptides from mitochondrion/chloroplast-secreted proteins (13). The function of poxviral metalloproteinase is poorly understood, but it appears to play a complementary role in virus maturation (8,12,14).

The gene products of K7L (variola) and I7L (vaccinia) are 423-residue proteases that belong to the MEROPS Clan CE (7,15,16). The two proteases differ at only 4 residues, and are thus likely to be functionally very similar. Residues important in catalysis of other members of Clan CE are conserved in K7L/I7L, and suggest that it is a cysteine protease (confirmed experimentally by the Hruby group (17)). Based on in vivo cleavage data, I7L processes one envelope protein, P21K (A17L), and four core proteins: P4a (A10L), P4b (A3L), P17K (A12L), and P25K (L4R) (7,16,18,19), at sites with the AG-X motif (where X is generally a small residue). AG-X motifs occur in other vaccinia proteins as well, but they are not processed, suggesting that additional substrate motifs are required for recognition (12).

The closest non-viral homologs of K7L are the eukaryotic SUMO-specific proteases (15,20) and the SUMO-specific and/or ubiquitin-specific proteases from pathogenic bacteria (Supplemental Fig. 1A). The function of the SUMO-specific proteases is to process SUMO precursors and deconjugate SUMO from target proteins (21-24). In a similar way, the Clan CE proteases of pathogenic bacteria deconjugate ubiquitin-like proteins in the host cell and suppress host cell defenses. As a result, they function as virulence factors (25-28). In addition to the poxviruses, Clan CE peptidases have been identified in two other double-stranded DNA viruses: adenoviruses and African swine fever viruses (29,30). The adenoviral peptidase (adenain) processes both viral and host proteins (31-34). There is evidence that some Clan CE proteases are regulated by substrate binding. Thus, the Saccharomyces SUMO-specific protease Ulp1 is activated by the binding of its substrate protein, SMT3, at the cleft formed by the N-and C-terminal sub-domains (20). In turn, adenain is activated by binding of the 11-residue fragment of the core protein pIV (Supplemental Fig. 1B) and viral DNA (29,35,36). But it is not known if this is a general feature of viral CE proteases, including K7L.

Biochemical characterization of the K7L (or I7L) protease has not been previously performed, owing to the lack of pure, active enzyme. Here, we report the expression and biochemical characterization of the K7L variola protease. Our results lead us to propose a model for substrate recognition and regulation on the activation mechanism and additional mechanistic parameters of this essential processing protease.

### EXPERIMENTAL PROCEDURES

**Recombinant plasmids** - The full-length K7L cDNA sequence was generated by Quick Change mutagenesis (Stratagene) of a vaccinia virus I7L template. The K7L construct was cloned into the pGEX-4T vector (GE Healthcare), which encodes an N-terminally glutathione S-transferase (GST) purification tag followed by a thrombin cleavage site (Fig. 1A). The proteases I7L/K7L of variola and vaccinia viruses differ by only two non-conserved mutations 75G/R and 308T/I, all other substitutions are strain-specific and we presume that they are unlikely to affect protease activity. The substitutions are far from a putative substrate-binding site (based on threading the K7L sequence onto crystal structures of proteases from Clan C57) – see Supplemental Data for a more complete description. The gene encoding the wild-type p25K construct (C-terminally FLAG-tagged) and, the wild-type and C328A K7L mutant (each N-terminally FLAG-tagged) were cloned under the control of the T7 polymerase promoter into the mammalian expression vector pTF7-3. The cloning of p25K led to a mutation of Ser2 to Ala. The P4a and P4b genes were cloned into pcDNA3.1 vectors. The P25K gene was also
cloned into the bacterial expression vector pET151-TOPO (Invitrogen), providing it with an N-terminal His-tag and TEV protease cleavage site. The identities of the constructs were validated by DNA sequencing.

Expression of K7L protease and P25K protein in E. coli - The pET151-p25K and pGEX-K7L plasmid constructs were expressed in E. coli BL21 Codon plus-RIL cells (Stratagene). Expression was induced using 0.4-0.6 mM isopropyl-D-galactopyranoside for 17 h at 17°C. Cells were then collected by centrifugation, resuspended in 50 mM Hepes buffer, pH 7.3, containing 15% glycerol, 500 mM NaCl, 1 mM leupeptin, 1 mM PMSF, and 1 mM 2-mercaptoethanol; and lysed using a French press. The pellet was removed by centrifugation (45,000xg for 1 h). The GST-K7L construct was purified from the supernatant fraction using affinity chromatography on a glutathione-sepharose 4B column (GE Healthcare). The protein were eluted with 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM reduced glutathione, 10% glycerol, and 500 mM NaCl. The purified samples were cleaved for 16 h at 4°C using bovine thrombin (Sigma). Owing to the thrombin cleavage site, the resulting proteins contained an additional Gly-Ser at the N-terminus. Following inactivation of thrombin by 0.5 mM 4-(2-aminoethyl)-benzenesulfonylfluoride, the cleaved proteins were further purified using a HiTrap heparin HP column (GE Healthcare). Proteins were eluted using a 0.2-1.0 M gradient of NaCl concentration in 30 mM HEPES buffer, pH 7.6, containing 10% glycerol. Samples were stored in 2 mM DTT and 50% glycerol at -80°C.

His-tagged P25K was purified using a HiTrap Ni²⁺-chelating HP column (GE Healthcare). The protein was eluted using a 20–250 mM gradient of imidazole in 30 mM Tris-HCl buffer, pH 8.0, containing 500 mM NaCl; and then cleaved for 16 h at 4°C using TEV protease. Owing to the cleavage preferences of the TEV protease, the N-terminus of the resulting P25K protein included a six-residue extension (Gly-Ile-Asp-Pro-Phe-Thr). Cross-linking of K7L - K7L samples (2 µM) were cross-linked for 10-30 min at 20°C in 20 mM HEPES buffer, pH 7.6, containing 0.2 mM bis[sulfosuccinimidyl]suberate (BS3; Pierce). Reactions were stopped using 0.1 M Tris. Samples were separated on 4-12% gradient Bis-Tris SDS-PAGE and Coomassie-stained.

Sedimentation equilibrium experiments with K7L - Sedimentation equilibrium experiments were performed using a ProteomeLab XL-I analytical ultracentrifuge (Beckman-Coulter). K7L (0.075, 0.15 and 0.3 mg/ml) in 30 mM Tris-HCl buffer, pH 8.0, containing 200 mM NaCl, using 0.2 mM bis[sulfosuccinimidyl]suberate (BS3; Pierce). Reactions were stopped using 0.1 M Tris. Samples were separated on 4-12% gradient Bis-Tris SDS-PAGE and Coomassie-stained.
**K7L proteolysis of P25K** - Purified P25K (8.3 µM) was co-incubated with K7L (0.3 µM) in 30 mM Tris-HCl buffer, pH 8.0, containing 2%-45% glycerol, 100 mM NaCl, and 2 mM DTT. Where indicated, 1 mM MgCl₂, 0.1 mg/ml RNA and vaccinia DNA were added to the reactions. Reactions were stopped by adding SDS-loading buffer and then separated using 4-12% denaturing Bis-Tris SDS-PAGE gels (Invitrogen). Gels were Coomassie-stained and then digitized using a gel imager (Alpha Innotech). The specificity constants (kcat/KM) were estimated using the Ww approximation for time-dependent product accumulation (described in (38)) and the assumption of [S]<KM. The latter was validated by measuring inhibition of K7L-mediated cleavage of Ac-ISAG-7-amino-4-carbamoylmethyl coumarin (ACC) by P25K (see Results).

**Synthesis of the fluorogenic peptides** - All substrates containing the ACC fluorophore were synthesized using solid phase synthesis as described previously (39,40). Purity and structure were confirmed using analytical HPLC and mass spectrometry. The tetrapeptide substrates were also analyzed by 1H NMR (See Supplemental Methods).

**Synthesis of FAM-peptides** – FAM-labeled peptides were synthesized using an Apex 396 multiple peptide synthesizer on a Rink Amide MBHA resin (0.72 mmole/g) (NovaBiochem) using a standard Fmoc protocol and diisopropylcarbodiimide/1-hydroxybenzotriazole coupling (see Supplemental Methods).

**Synthesis of activity-based probes** - Amino-acid sidechain-protected Biotin-AEEAc-FYA-COOH, Biotin-AEEAc-EDTIFFA-COOH and Biotin-hex-EDCIFYA-COOH peptides sequences were synthesized on a solid support using 2-chlorotriyl chloride resin by coupling using HATU in DMF of the appropriate peptide sequence to vinyl methyl ester (obtained as previously described in (41)). Deprotection of the peptides was carried out in 92.5:2.5:2.5:2.5 (v/v) trifluoroacetic acid/triisopropylsilane/phenol/water. Purity and structure of the synthesized material were confirmed using analytical HPLC and mass spectrometry. Biotin-AEEAc-FYA-VME: ESI-MS=868.2; HPLC: tR=13.00 min, yield=47%, Biotin-AEEAc-EDTIFFA-VME: ESI-MS=1310.55; HPLC: tR=16.23 min, yield=43%, Biotin-hex-EDCIFYA-VME: ESI-MS=1296.55; HPLC: tR=16.39 min, yield=51%.

**Activity assays using the fluorogenic substrates** - K7L activity was measured in duplicate in 50-100 µl volumes in 96-well plates. The reaction mixtures contained K7L (0.1-0.4 µM), a fluorescent substrate (50-100 µM), glycerol (10-45%), and Tris-HCl buffer, pH 8.0, containing 50-100 mM NaCl and 2 mM DTT. The samples were incubated at 25°C for 5-60 min. The substrate cleavage rate was continuously monitored at λex=360 nm and λem=460 nm using a Spectramax Gemini EM fluorescence spectrophotometer (Molecular Devices). V, kcat and KM were calculated from the cleavage data using the equation V= kcat[E][S]/(KM + [S]).

**Activity assays using FAM-labeled peptide substrates** - Assays were performed in duplicate 96-well plates. Reaction mixtures contained K7L protease (0.2-0.6 µM), FAM-peptide substrate (15 µM), and 30 mM Tris-HCl buffer, pH 8.0, supplemented with 15-45% glycerol, 50 mM NaCl, and 2 mM DTT. Samples were co-incubated at 25°C for 5-30 min. Aliquots were withdrawn, and the enzyme inactivated by 6 M guanidine-HCl. Aliquots were chromatographed using a C4 reverse-phase column (Higgins, Proteo 300, 250x4.6 mm). Fluorescence of eluted fractions was continually monitored at λex= 490 nm and λem=530 nm using a Gilson fluorescence detector (Model 121) equipped with a narrow-band filter. Peptide yield was estimated from the peak area. The initial rate of the cleavage reactions (Vo) was then calculated based on the cleaved peptide yield, using the equation: Vo = Ap[S]/(As+Ap); where As is the area of the intact FAM-peptide, and Ap is the area of the FAM-digest product. Two experiments with independently prepared K7L samples were performed to obtain statistically significant data. Substrate depletion was minimized by collecting data below 30% product formation. Because KM for the peptide substrates significantly exceeded the substrate concentrations used in the cleavage reactions, the kcat/KM specificity constant was estimated using the equation: kcat/KM = Vo/[E] [S].

To determine the molecular mass of the cleavage products and thus the location of the scissile bonds, the digest reactions were also analyzed by MALTI-TOF MS using a Bruker AutoFlex II MALDI–TOF mass spectrometer in a cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile-0.1% TFA.
RESULTS

Recombinant variola K7L is a dimeric protease - GST-tagged K7L was expressed in E.coli as a soluble protein (1 mg/L bacterial culture), purified and cleaved with thrombin as described in Experimental Procedures (see Fig. 1). K7L migrated on SDS-PAGE with the expected size of 45 kDa (Fig. 1B). Size exclusion chromatography suggested a MW of 75-80 kDa, more consistent with homodimerization in solution (Fig. 1C). This was corroborated by cross-linking experiments, which demonstrated a dimer but no higher order polymers (Fig. 1B). Analytical ultracentrifugation (which is not affected by molecular shape) confirmed that K7L exists as a fairly strong dimer, either in the absence or the presence of 25% glycerol (Supplemental Fig. 2), with Kd values of 0.4±0.2 µM (no glycerol) and 0.2±0.1 µM, (25% glycerol). At the concentrations used in our activity assays, K7L is therefore predicted to be principally dimeric. K7L could be stored for several months in 50% glycerol at -20°C or -80°C without loss of proteolytic activity. However, the enzyme lost activity at higher temperatures, with a half-life of ~5 h at 23°C and ~30 min at 37°C. Activity measurements were therefore performed at 20-25°C.

The reported targets of K7L are P25K, P4a and P4b. To determine the activity of recombinant K7L, we incubated it with in vitro translated P25K, P4a and P4b proteins expressed from constructs encoding the highly similar vaccinia proteins (since variola DNA was not available). Variola and vaccinia P25K, P4a and P4b differ by between 1-3%, and all sequence differences are distant from the cleavage sites – see Supplemental Alignments. Since the sequences display such a high degree of identity, it is very likely that the activity of variola K7L towards vaccinia proteins would be essentially identical to the cleavage of the corresponding variola proteins. The activity assays were performed in the presence of 40% glycerol (Fig. 2). There is one putative cleavage site in P4b (Gly<sup>60</sup>Ser<sup>61</sup>) and two in P25K (Gly<sup>18</sup>Ser<sup>19</sup> and Gly<sup>32</sup>Ala<sup>33</sup>). In accord, we observed two cleavage products of the expected molecular mass for P25K (32 and 30 kDa – Fig. 2C); and one (64 kDa) for P4b (Fig. 2D). Cleavage of one of the P25K sites was especially fast, and we observed slower cleavage of the other site and P4b by K7L. By contrast, P4a was not cleaved by K7L under our experimental conditions (Fig. 2E).

Glycerol enhances K7L activity - To evaluate the activity of the K7L samples we synthesized several peptidyl substrates corresponding to the putative P25K, P4a and P4b cleavage sites (Table 1). The peptides were N-terminally acetylated, and contained ACC as the fluorophore. Activity was measured by the release of the free fluorophore using a fluorimeter operating in the kinetic mode. K7L exhibits low activity against Ac-ISAG-ACC, but its activity was dramatically enhanced in the presence of 45% glycerol (Fig. 3A). Similar to glycerol, other osmolytes such as betaine and sucrose also increased activity against Ac-ISAG-ACC (Fig. 3B). As a result, routine activity measurements were performed in the presence of 40-45% glycerol, at an optimal pH of 8.0 (Fig. 3C), unless otherwise indicated. Glycerol increased K7L activity against other fluorescent peptides distinct from Ac-ISAG-ACC and full-length protein substrates (data not shown), and thus we conclude that the osmolyte’s effect is on the enzyme, not the substrate.

Dimerization enhances K7L activity - We analyzed the effect of K7L dimerization on specific activity by observing the concentration-dependence of the cleavage of an ACC-labeled peptide substrate (Fig. 3D). The dependence had a sigmoidal shape with AC<sub>50</sub>=0.2±0.1 µM, which corresponds to the K<sub>d</sub> value determined by AUC (see above). This result clearly links the activity of K7L with dimerization.

Finally, we compared the activities of recombinant K7L produced in E. coli versus vaccinia virus-infected mammalian cells (Supplemental Fig. 3). No significant differences were observed, indicating that K7L is not differentially activated in mammalian cells (e.g., by post-translational modification).

Probing substrate specificity - The substrate specificity of vaccinia I7L has previously been defined by in vivo cleavage assays of mutant P25K (5,42,43). However, since the published data did not provide quantitative comparisons of cleavage efficiencies, we performed an in-depth analysis of K7L. The P1 site of all putative natural K7L substrates is a Gly residue. To test the tolerance of K7L quantitatively we mutated the conserved Gly at positions 18 and 32 of P25K to Ala, and compared their cleavage efficiencies with wild-type P25K. As shown in Fig. 4A, both sites of WT
P25K were fully cleaved in one and nine hours, respectively, whereas no evidence for cleavage of the double mutant was obtained after nine hours incubation. This indicates a very strong preference for Gly in the P1 position of protein substrates.

We next studied cleavage of tetrapeptide substrates, and employed a positional scanning substrate library approach to define optimal preferences at the P2, P3 and P4 positions. The P1 position was fixed as Gly, and the P2, P3 and P4 positions were each randomly altered to one of 18-20 amino acids, including norleucine and β-alanine (Fig. 4C). Since we employed similar peptide concentrations, the relative contributions of the amino acid substitutions to activity could be directly measured. The results demonstrate a high preference for Ala at P2. Selectivity at P3 was lower, but hydrophobic residues were especially well tolerated. The P4 position was more restricted than P3, and demonstrated a clear preference for hydrophobic residues (Fig. 4C). These results agree well with the sequence motifs that exist in the natural cleavage targets of K7L (Fig. 2C), and distinguish K7L from closely related proteases in Clan CE (Fig. 4D) (39).

We next utilized our peptide-based observations in a mutational analysis of the putative natural substrate P25K. We mutated P3, P2 and P1’ positions in the Gly18→Ser19 cleavage site, while introducing an Ala mutation at Gly31 to silence any contribution of the secondary cleavage (Fig. 4B). P1’ in the Gly18→Ser19 site demonstrated a clear preference for Ser and Ala, the most frequently occurring residues at all putative natural K7L cleavage sites (Fig. 2B). We also found that substitution of Ala at P2 with Gly did not affect the cleavage rate, confirming the proposal that cleavage of natural substrates may be influenced by exosites (12).

To corroborate these findings we synthesized individual tetrapeptide ACC substrates corresponding to the optimal tetrapeptide sequence and the cleavage sites of P25K, P4b, and P4a, and co-incubated them with K7L. The $k_{cat}/K_M$ parameters were then calculated from the cleavage data (Table 1), revealing a preference for Tyr and Phe at the P3 position. These results indicate that the K7L specificity in the P2, P1 and P1’ positions surrounding the scissile bond correlates well with cleavage sites of natural proteins sequences (Fig. 2C-E). However, beyond this region, correlations break down.

**Mapping the extended substrate binding site of K7L** - The discrepancies in cleavage site specificities between peptide and protein substrates beyond the catalytic cleft suggested that more distant residues than P4-P1 might affect activity. To determine the effect of distant residue positions on K7L activity we utilized two strategies. In the first strategy we extended the ACC-based peptidyl substrates to encompass the P1-P8 residues matching the P25K and P4a cleavage sites. Kinetic measurements reveal that extending both substrates to the P5 and P6 residues gave only a minor enhancement in the rate of peptide hydrolysis. In contrast, extending the substrates to the P7 and P8 positions promoted hydrolysis by up to 25-fold, while leaving the hydrolysis of the P4a-based peptide almost unchanged. Notably, negatively charged residues in P7 and P8, derived from the P25K cleavage sites, were preferred over the neutral residues found at these positions in the site P4a and P4b sequences.

In a second strategy, we synthesized peptides that covered the P9-P4’ residues of P25K, P4a and P4b, derivatized at their N-terminus with FAM. This allowed us to monitor cleavage by PR-HPLC of timed samples at low µM substrate concentrations, permitting an estimate of the catalytic efficiency ($k_{cat}/K_M$) (see Experimental Protocols). The cleavage sites were independently confirmed by MALDI-TOF mass spectrometry. In keeping with the results presented in Table 1, peptides encompassing the Gly18→Ser19 site of P25K were cleaved by K7L far more efficiently than peptides derived from other sites of P25K, P4a and P4b proteins.

Interestingly, the FAM-LDR1ITNAGTCTV peptide spanning the Gly697→Thr698 cleavage site of P4a appeared resistant to K7L proteolysis. This was in accord with data on the Ac-TNAG-ACC peptide, demonstrating that this site is highly unfavorable for cleavage by K7L (Table II). In agreement, MALDI-TOF mass spectrometry data confirmed that the FAM-LDR1ITNAGTCTV peptide was not cleaved, even by very high levels of K7L (not shown). Similar to the Ac-EDTIFFAG-ACC substrate, removing P9-P7 residues from the optimal P25K peptide sequence reduced its cleavage rate by ~40-fold. Significantly, extending substrates beyond the scissile bond in the prime direction (downstream of the scissile bond) had no major impact on
cleavage rates, and extending from P2' to P4'
provided only a modest (less than 2-fold)
enhancement in catalysis (Table II). Overall, our
cleavage data for longer peptides correlate very
well with K7L activity \textit{in vitro} against protein
substrates (P25K and P4b). Based on our data, it is
likely that the Gly$^{697}$↓Thr$^{698}$ site of P4a protein is a
sub-optimal substrate of K7L.

Evidence for allosteric exosite-mediated
activation of K7L - The substantial stimulation of
activity of K7L catalysis on substrates occupying
the P7 and P8 positions suggested two
possibilities: 1) extended concerted substrate
recognition, or 2) an exosite interaction acting as
an allosteric switch. To address these hypotheses
we tested whether P25K could enhance K7L
proteolysis of the Ac-ISAG-ACC substrate. We
co-incubated K7L (0.5 μM) with P25K (0.1-100
μM) and then measured cleavage velocity of the
fluorescent substrate. The control proteins BSA
and lysozyme had no effect on cleavage velocity,
but the P25K protein enhanced the K7L activity by
up to 2.4-fold, with a maximal stimulatory effect
at 8 μM of P25K. Higher P25K concentrations led
to the inhibition of K7L activity against Ac-ISAG-
ACC (Fig. 5A). In contrast, the \textit{FAM-}
EEDTFFAGSISE peptide showed no stimulation
of Ac-ISAG-ACC, and instead inhibited with an
IC$_{50}$ of 37 μM, similar to the concentration at
which P25K begins to inhibit cleavage of the
tetrapeptide reporter substrate (Fig. 5B). These
findings are consistent with a small allosteric
effect, but indicate that the major mechanism of
enhanced cleavage of long peptides is the presence
of extended and concerted substrate recognition.

Polynucleotides stimulate proteolysis of
P25K by K7L - P25K is a high-affinity binder of
polynucleotides (K$_{d}$=1-2 nM), including both
RNA and DNA (44). To test whether polynucleotides affect the cleavage of P25K we
co-incubated K7L with P25K in the presence and
the absence of RNA, double-stranded vaccinia
DNA, and glycerol. DNA, and especially RNA
(both at 0.1 mg/ml), substantially stimulated the
cleavage of both P25K sites, and their effect was
particularly strong at lower concentrations of
glycerol (3-15%) (Fig. 5C,D). It is possible that
polynucleotides are required to stimulate K7L
proteolysis of the P25K protein and that this
stimulation does not require the presence of high
glycerol. Importantly, RNA (and viral DNA – not
shown) both inhibited cleavage of the Ac-
EDTFFAG-ACC peptide by K7L by
approximately 4-fold (Fig. 5E), suggesting that
K7L has a propensity to bind polyanions.
Moreover, polynucleotide stimulation of catalysis
on P25K was much higher than the inhibitory
effect towards the octapeptide substrate. Because
the activation is protein-specific, we propose that
viral DNA and RNA influence the cleavage of
P25K by promoting its interaction with K7L at an
allosteric site.

Peptide-based inhibitors and activity probes -
The K7L preferred-substrate fingerprint was used
to design activity-based probes that would be
recognized by the K7L protease. For this purpose,
the FYAG and EDTFFAG peptide sequences
were functionalized with an N-terminal biotin tag
and a C-terminal vinyl methyl ester (VME)
reactive “warhead” (Fig. 6A). The selectivity and
efficiency of the Biotin-AEEAc-EDTFFA-VME
was directly confirmed in labeling experiments
using K7L. To visualize the labeled material,
samples were subjected to Western blotting with
streptavidin-HRP. While the short probe failed to
visibly label K7L, the longer Biotin-AEEAc-
EDTFFA-VME probe at 25 nM was sufficient to
label the active site of K7L, consistent with
measured inhibition rate constants (Fig. 6B).
Blocking of labeling by N-ethylmaleimide indicated
that the probe targets the active site (Fig.
6B).

To test if the biotin-EDTFFAG-VME probe
labels cellular K7L, we added 10 μM of probe to
lysates of BSC-1 cells infected with vaccinia virus.
After a short incubation, the lysates were analyzed
by Western blotting with streptavidin-HRP (Fig.
6C) and also with a K7L antibody (Supplemental
Fig 4). The results demonstrate that the probe
predominantly labeled a 48 kDa band in the virus-
infected cells. Future experiments and probe
optimization will be required to determine whether
the probes described here are able to inhibit viral
infectivity and/or replication.

CONCLUSIONS

K7L is designated by the MEROPS
classification system as a member of the C57
family of peptidase Clan CE (45), a clan that
contains processing proteases from adenovirus and
African swine fever virus, deSUMOylating
enzymes from yeast to humans, and
deubiquitinating enzymes from plant and animal
pathogenic bacteria (24-29,35). K7L orthologs are present, and presumably required, in all orthopoxviruses. By employing transient expression and conditional lethal virus analyses it has previously been demonstrated that I7L, the vaccinia ortholog of K7L, is required for core protein processing (7,16,18). A seminal study used extracts of virally-infected cells containing I7L to demonstrate that the enzyme is a cysteine protease able to cleave core protein precursors P4a, P4b and P25K (17). The authors were unable, however, to produce catalytically-active I7L synthesized *in vitro*, leading them to conclude that a cofactor is required for activity.

Our success in producing catalytically-active K7L by expression in *E. coli* leads us to propose that dimerization of K7L, and by inference I7L, is required for activity, and that the *in vitro* translation system previously employed (17) produced insufficient concentrations of protein to enable significant dimerization. Moreover, our results strongly suggest that K7L does indeed require a cofactor for maximal activity. Although we do not know its identity, we have shown that glycerol and other fold-stabilizing osmolytes have a stimulatory effect. Furthermore, we found that polyanions have differential effects on the activity of the enzyme on protein vis-à-vis peptide substrates. Together, these characteristics point to a regulation of K7L that is multivariate and more complex than for other Clan CE members, possibly as a consequence of the stringent requirement for its activity to be localized predominantly or exclusively to the condensing virion (7,16,18).

Information about other viral proteases from Clan CE is exceedingly limited, and to date only the adenoviral processing protease adenain has been characterized biochemically and structurally in significant detail (29,35,46,47). Interestingly, some properties of K7L appear to be distinct from other Clan CE members, while others are related. Thus, K7L functions most efficiently as a homodimer, which is not a reported requirement for other Clan CE enzymes. However, in common with other Clan CE members, we have provided *in vitro* evidence that co-factors enhance K7L activity, although the details vary. Thus, adenain is activated by simultaneous binding of two co-factors, the 11-residue fragment of the core protein pIV and viral DNA (29); while our data suggest that both RNA and DNA oligonucleotides can enhance K7L activity toward its cognate substrate, P25K.

Our analysis of the extended substrate binding characteristics of K7L helps to explain the specificity of the protease for its predicted substrates, revealing that the presence of negatively charged residues at the P7 and P8 positions greatly enhances cleavage of some substrates. This implies that an exosite interaction distant from the active site is required for optimal cleavage of natural substrates. Consistent with our findings, neither of the peptides that span the P4a cleavage site, Gly<sup>614</sup>↓Ser<sup>615</sup>, nor the P4a protein itself, are cleaved by recombinant K7L.

K7L has been proposed as a promising target for smallpox therapy (10,11). Indeed, a small molecule that prevents vaccinia core protein maturation has been reported (10,11), and may be an inhibitor of I7L. Our biotinylated activity-based probe analysis demonstrates that the K7L is active and targetable in lysates from infected cells. In conclusion, our results provide many novel insights into the mechanism, specificity and regulation of the K7L viral protease. Our work also provides a solid foundation as well as specific molecular tools – protease production methods, assay design, inhibitor design and synthesis - that can now be used to probe the function of K7L in a cell-based setting.
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**ABBREVIATIONS**

The abbreviations used are: ACC, 7-amino-4-carbamoylmethyl coumarin; AMC, 7-amino-4-methyl coumarin; BS3, bis[ sulfo succinimidyl] suberate; FAM, 5(6)-carboxyfluorescein; GST, glutathione S-transferase; HATU, 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate; HRP, horseradish peroxidase; I7L, *vaccinia* processing protease; K7L *variola* (smallpox) processing protease; SENP, SUMO/Sentrin-specific proteases; TFA, trifluoroacetic acid; Ulp, ubiquitin-like protein.

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The authors declare that they have no competing financial interest.
**FIGURE LEGENDS**

**Fig. 1.** Expression of K7L and analysis of its oligomeric state. A, GST fusion construct used for expression and purification of K7L. B, SDS-PAGE of (Lane 1) GST-K7L, (Lane 2) K7L after removal of GST by thrombin, and (Lane 3) K7L cross-linked with BS3. C, Gel filtration of thrombin-cleaved K7L on Superdex S200-HR1030. Numbers on the side (B) or top (C) indicate molecular weight standards.

**Fig. 2.** Presumed viral targets of K7L and their in vitro cleavage patterns. A, P4a, P4b, P25K, P17K are viral core proteins, while P21K is the envelope membrane protein. Alternative gene-based names (in parentheses) are given, as well as the putative cleavage sites and their residue numbers. B, Alignment of the cleavage sites. Acidic residues that enhanced cleavage are in bold. C, D, E, Plasmids encoding the indicated proteins were transcribed and translated in vitro. Recombinant GST-K7L was added for the indicated times, and the reaction was terminated by boiling in SDS sample buffer. SDS-PAGE gels were developed with streptavidin-HRP to reveal processed and unprocessed substrate proteins. Bands common to both P4b and P4a gels belong to nonspecifically stained proteins. Cleavage reactions employed 0.3 µM GST-K7L at 25°C in 50 mM NaCl, 2 mM DTT, 30 mM Tris, pH 8.0 buffer containing 40% glycerol and 12% v/v of the translation product.

**Fig. 3.** Stabilizing effects of osmolytes, salt, pH and enzyme concentration on K7L activity. A, Activity (velocity as a function of Ac-ISAG-ACC concentration) in the presence of 10% and 45% glycerol. B, Comparison of K7L activation by betaine, glycerol, and sucrose (velocity as a function of osmolyte concentration) in 35 µM Ac-ISAG-ACC. C, Effect of NaCl concentration on K7L activity in 35 µM Ac-ISAG-ACC. The initial velocities were measured at 25°C in 30 mM Tris pH 8.0 and 2 mM DTT, with addition of the various components. D, Effect of pH on K7L activity in the presence of 45% glycerol. E, Effect of K7L concentration on the specific activity of K7L toward 30 µM Ac-EDTIFFAG-ACC in the presence of 10% glycerol.

**Fig. 4.** Subsite profiling of K7L and comparison with related protease substrate preferences. A, P25K and its double mutant, G18A/G32A, were produced in a cell-free in vitro transcription/translation system (Promega), cleaved by recombinant K7L-GST for the indicated times, and visualized following SDS-PAGE as described in Experimental Procedures. B, Amino acid preferences at the P3-P1’ positions of the cleavage site G↓S of P25K; G32 was always mutated to Ala to facilitate quantitative analysis. The analyses were conducted in same way as shown in the panel A. Single mutations were introduced at the P3-P1’ positions of the site G↓S, as indicated on the horizontal axis. The vertical axis shows the relative cleavage rates (%). C, A library of peptides exploring the P2-P4 positions was constructed as described in Experimental Procedures, and relative activities of 0.5 µM of K7L are expressed in relation to the most efficiently cleaved substrate at each position. All reactions were conducted in 40% glycerol. The horizontal axis shows amino acids written in the standard one-letter code; the unnatural amino acids are: O, norleucine; and B, β-alanine. D, Relative preferences compared for related deSUMOylating and deubiquitinating proteases. Red indicates the highest activity; black the lowest. The preferences are clustered by their similarity to K7L.

**Fig. 5.** Influence of full-length P25K and polynucleotides on K7L activity. A, Activation by P25K of Ac-ISAG-ACC cleavage. The concentration-dependent activation fits a bell-shaped dose-response curve, with $K_A=8$ µM and $K_I=45$ µM. Bovine serum albumin (BSA) and lysozyme were used as controls of nonspecific binding. B, Inhibition by (FAM)-EEDTIFFAGSISE of Ac-ISAG-ACC cleavage, with data fitted to a first-order dose-response curve, $K_I=37$ µM. The reactions were carried out in 30 mM Tris pH 8.0 containing 40% glycerol, 50 mM NaCl, 0.2-0.5 µM of K7L and 50 µM of the Ac-ISAG-ACC.
substrate. **C**, Cleavage of P25K by K7L in the presence of various co-factors. 0.3 µM of K7L was co-incubated for the indicated times with (Left Panel) 8 µM of P25K in the presence of 1 mM Mg²⁺; (Middle Panel) human RNA (0.1 mg/ml) + Mg²⁺; or (Right Panel) viral DNA (0.1 mg/ml) + 1 mM Mg²⁺. Experiments were performed 3%, 15% or 40% glycerol, as indicated. The digests were separated by SDS-PAGE. **D**, The images in C were digitized to calculate the respective $k_{cat}/K_M$ values as described in Experimental Procedures. **E**, Effects of glycerol, human RNA and Mg²⁺ ions on the cleavage of Ac-EDTIFFAG-ACC by K7L. K7L (0.1 µM) was co-incubated at 25°C with the peptide (10 µM). The reactions contained 30 mM Tris-HCl, pH 8.0, 50 mM NaCl and 2 mM DTT, and the indicated concentrations of glycerol. Where indicated, 1 mM MgCl₂ or 0.1 mg/ml RNA were added to the reactions.

*Fig. 6. An activity-based probe labels K7L in vitro and in cell extracts.* **A**, Chemical formulae of biotinylated peptide-based probes. Inhibition constants were calculated by observing the pseudo-first-order decay of activity (kobs) in the presence of inhibitor concentration [I]. **B**, (Upper panel) K7L (0.2 µM) was labeled with the indicated concentrations of short (S) and long (L) probes for 15 min at 25°C and visualized with streptavidin-HRP by SDS-PAGE. (Lower panel) The total enzyme was visualized by Western blotting using an anti-K7L antibody on the same gel. **C**, Labeling K7L and I7L in vaccinia virus-infected (MOI=5, 24 h) BSC-1 cells. Where indicated, recombinant K7L was co-expressed from a transfected pTF7-3 vector. 10 µM of biotinylated EDTIFFAG-VME was added to homogenized samples, incubated for 15 min, and loaded onto SDS-PAGE. The biotinylated proteins were visualized with streptavidin-HRP. The black arrows indicate the positions of recombinant K7L and viral I7L on the gel. The open arrow points to the absence of the I7L band in a sample lacking the virus.
### Table I. K7L proteolysis of fluorogenic peptides.

| Sequence source | Peptide sequence | $k_{cat}/K_M$ (M⁻¹s⁻¹) | $K_M$ (µM) |
|-----------------|-----------------|------------------------|------------|
| P25K, Gly^{18}\downarrow Ser^{19} | Ac-FFAG-ACC | 300±5 | 100±15 |
| P25K, Gly^{32}\downarrow Ala^{33} | Ac-VIAG-ACC | 120±5 | NM |
| P4b, Gly^{60}\downarrow Ser^{61} | Ac-ISAG-ACC | 80±5 | 830±110 |
| P4a, Gly^{614}\downarrow Ser^{615} | Ac-FYAG-ACC | 560±90 | NM |
| P4a, Gly^{697}\downarrow Thr^{698} | Ac-TNAG-ACC | No cleavage | No cleavage |
| P25K, Gly^{18}\downarrow Ser^{19} | Ac-TIFFAG-ACC | 570±30 | 7.5±3 |
| P25K, Gly^{18}\downarrow Ser^{19} | Ac-EDTIFFAG-ACC | 14,400±300 | 26±7 |
| P4a, Gly^{614}\downarrow Ser^{615} | Ac-RYFYAG-ACC | 880±140 | NM |
| P4a, Gly^{614}\downarrow Ser^{615} | Ac-CPRYFYAG-ACC | 1010±20 | 150±30 |

Kinetic parameters were evaluated in 30 mM Tris-HCl buffer, pH 8.0, 100 nM NaCl, 2 mM DTT and 45% glycerol, with a range of peptide concentrations, and K7L from 0.2-1.0 µM. NM, not measured. Errors indicate observed standard deviation of three measurements.

### Table II. K7L proteolysis of the P9-P4’ FAM-labeled peptides.

| Protein, cleavage site | Peptide sequence | $k_{cat}/K_M$ (M⁻¹s⁻¹) 45% Glycerol | $k_{cat}/K_M$ (M⁻¹s⁻¹) 15% Glycerol |
|------------------------|-----------------|-------------------------------------|-------------------------------------|
| P25K, Gly^{18}\downarrow Ser^{19} | EEDTIFFAG\downarrow SISE | 28000±3000 | 1300±400 |
| P25K, Gly^{18}\downarrow Ser^{19} | EEDTIFFAG\downarrow SI | 17500±1500 |  |
| P25K, Gly^{18}\downarrow Ser^{19} | TIFFAG\downarrow SISE | 670 ± 220 |  |
| P25K, Gly^{32}\downarrow Ala^{33} | DDLQQVIAG\downarrow AKSK | 3500±800 | 220±100 |
| P4b, Gly^{60}\downarrow Ser^{61} | VDDDFISAG\downarrow ARNQ | 2000±500 |  |
| P4a, Gly^{614}\downarrow Ser^{615} | YCPRYFYAG\downarrow SPEG | 2100±500 |  |
| P4a, Gly^{697}\downarrow Ser^{698} | LDRIITNAG\downarrow TCTV | ND |  |
| P4a, Gly^{697}\downarrow Thr^{698} mutant | LDRIITNAG\downarrow TSTV | ND |  |

Kinetic parameters were evaluated in 30 mM Tris-HCl buffer, pH 8.0, 100 nM NaCl, 2 mM DTT and 45% glycerol, with 10 µM peptide and K7L from 0.1-1.0 µM. O (norleucine) was used in place of Met to obviate oxidation of the latter during synthesis of DDLQMVIA\downarrow AKSK sequence. Errors indicate observed standard deviation of two measurements.
Aleshin, Fig 1.

A

![Diagram of thrombin cleavage](image)

B

![Graph of protein bands](image)

C

![Graph of chromatography](image)
Aleshin, Fig 2.

A

| Protein | kDa |
|---------|-----|
| P25K (L4R) | 18 32 251 |
| P4b (A3L) | 60 (4b) |
| P4a (A10L) | (4a) 614 697 891 |
| P17K (A12L) | 56 192 |
| P21K (A17L) | 16 185 203 |

B

| Sequence | Protein | Site |
|----------|---------|------|
| EEDTIFFAG-SISE | P25K | G^{18}_{19} |
| DDLQVMAK-ASKK | P25K | G^{32}_{33} |
| VDDDDAG-ARNQ | P4b | G^{60}_{61} |
| YCPRTFAG-SPEG | P4a | G^{614}_{615} |
| LDRIITAG-TCTV | P4a | G^{697}_{698} |
| NSQTVTAG-ACDT | P17K | G^{56}_{57} |
| NMLDDFSAG-AVL | P21K | G^{6}_{17} |
| TCNKPYTATG-NKVD | P21K | G^{185}_{186} |

C

p25K

D

p4b

E

p4a
Aleshin, Fig 3.

(A) 

45% glycerol

10% glycerol

45% glycerol

10% glycerol

V_x10^9, M s^{-1}

ISAG-ACC, mM

(B) 

[Betaine, Glycerol, Sucrose]

V_x10^9, M s^{-1}

Osmolyte, %

(C) 

V_x10^9, M s^{-1}

pH

(D) 

V / [K7L] \times 10^2, s^{-1}

log[K7L], M
Aleshin, Fig 4.
Aleshin, Fig 5.
Aleshin, Fig 6.

A

\[ \text{Biotin-AEEAc-FYAG-VME} \quad k_{\text{obs}}/I = 14.5 \pm 1.1 \]

\[ \text{Biotin-AEEAc-EDTFFAG-VME} \quad k_{\text{obs}}/I = 4400 \pm 1100 \]

B

\[
\begin{array}{cccccc}
25 & 50 & 100 & 200 & 200 \\
- & S & L & S & S & L & S^* L^* \\
\end{array}
\]

\[
\begin{array}{cccccc}
kDa & 66 & 45 & 31 & 200 \\
- & - & - & - & - & - \\
\end{array}
\]

Streptavidin-HRP

Anti-K7L

C

VacV Probe pM1-K7L

\[
\begin{array}{cccccc}
1 & 2 & 3 & 4 & 1 & 2 & 3 & 4 \\
188 & 62 & 49 & 38 & 188 & 62 & 49 & 38 \\
98 & 62 & 49 & 38 & 98 & 62 & 49 & 38 \\
62 & 49 & 38 & 28 & 62 & 49 & 38 & 28 \\
49 & 38 & 28 & 17 & 49 & 38 & 28 & 17 \\
38 & 28 & 17 & 6 & 38 & 28 & 17 & 6 \\
28 & 17 & 6 & 1 & 28 & 17 & 6 & 1 \\
17 & 6 & 1 & 6 & 17 & 6 & 1 & 6 \\
6 & 1 & 6 & 1 & 6 & 1 & 6 & 1 \\
1 min exposure & 5 min exposure
\end{array}
\]
Activity, specificity and probe design for the smallpox virus protease K7L
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