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Chlamydia Protease-like Activity Factor (CPAF): Characterization of Proteolysis Activity in Vitro and Development of a Nanomolar Affinity CPAF Zymogen-Derived Inhibitor

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Supporting Information

ABSTRACT: During infection of epithelial cells, the obligate intracellular pathogen *Chlamydia trachomatis* secretes the serine protease *Chlamydia* protease-like activity factor (CPAF) into the host cytosol to regulate a range of host cellular processes through targeted proteolysis. Here we report the development of an in vitro assay for the enzyme and the discovery of a cell-permeable CPAF zymogen-based peptide inhibitor with nanomolar inhibitory affinity. Treating *C. trachomatis*-infected HeLa cells with this inhibitor prevented CPAF cleavage of the intermediate filament vimentin and led to the loss of vimentin cage surrounding the intracellular vacuole. Because *Chlamydia* is a genetically intractable organism, this inhibitor may serve as a tool for understanding the role of CPAF in pathogenesis.

The obligate intracellular pathogen *Chlamydia trachomatis* is the causative agent of ocular and genital diseases of significant clinical and economic importance such as corneal scarring, pelvic inflammatory disease, ectopic pregnancy, and infertility.[1] *C. trachomatis* is the leading cause of infectious blindness (trachoma) worldwide and the most common sexually transmitted pathogen in the developed world. *C. trachomatis* replicates within a parasitophorous vacuole, termed an inclusion, and secretes enzymes and proteins that efficiently modulate a wide range of host cellular processes such as lipid and membrane transport,[2−4] the actin cytoskeleton,[5] microtubule-based motors,[6] lysosomal recognition of the inclusion,[7] ERK/MEK signaling pathways,[8] and the onset of programmed cell death.[9] Unfortunately, *C. trachomatis* is refractory to genetic manipulation;[10] thus, the molecular mechanisms underlying chlamydial cooption of host cellular functions are poorly understood. In light of this limitation, enzymology and chemical biology approaches may provide insight into the function of specific enzymes important in *C. trachomatis* pathogenesis.

The serine protease CPAF (*Chlamydia* protease-like activity factor) is secreted by *C. trachomatis* into the cytosol of host cells, where it cleaves several host proteins, including transcription factors (USF-1 and RFX5) required for MHC transcription[11] and pro-apoptotic proteins (Bad, Puma, and Bid).[12] In addition, CPAF mediates expansion of the inclusion by cleaving host intermediate filaments such as keratin 8 and vimentin.[12−15] As such, mounting evidence points to CPAF as an important target for therapeutic intervention.

Beyond sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) or Western blotting activity assays with impure enzyme preparations, detailed investigations of CPAF have been somewhat limited because of the lack of a robust in vitro assay suitable for obtaining activity and inhibition kinetic parameters. In response to this, we developed a facile assay for CPAF proteolysis that would support kinetic studies and serve as a platform for inhibitor discovery.

Few proteolysis sites have been mapped at specific residues because CPAF is known to proteolyze several different proteins and most of these substrates are degraded by multiple proteolytic events. However, Valdivia and co-workers determined the primary site of cleavage of human vimentin to be between Ser72 and Ser73 by N-terminal sequencing of immunoprecipitated vimentin cleaved by CPAF.[15] We envisioned that peptides derived from the vimentin primary sequence surrounding Ser72 might serve as a substrate for development of an in vitro assay for CPAF hydrolytic activity. Using solid-phase methods, we prepared a peptide substrate derived from the sequence of human vimentin (see the Supporting Information). Peptide Abz-VRLRSSVPGV-NH_2 (**1**) contained an N-terminal 2-anthranilic acid (Abz) moiety for fluorescence detection following high-performance liquid chromatography (HPLC) separation of the products from CPAF-mediated proteolysis. Figure 1 depicts a typical chromatogram of the reaction. Mass spectrometry confirmed that CPAF proteolyzed this substrate into two peptide fragments at the anticipated scissile bond. Apparent kinetic parameters obtained for CPAF proteolysis of substrate **1** were 13.2 s^{-1}, 0.88 mM, and 1.5 × 10^4 M^{-1} s^{-1} for k_{cat}^{app}, K_M^{app}, and k_{cat}^{app}/K_M^{app}, respectively.

CPAF is highly resistant to conventional broad-spectrum serine protease inhibitors such as fluorophosphonates and fluorosulfonates and has one only known inhibitor, the natural product lactacystin.[16] Lactacystin acylates Ser499 within the Ser449-His105-Glu558 catalytic triad.[16,17] To further confirm the utility of substrate **1** for inhibitor discovery, we examined the effect of lactacystin on CPAF activity in vitro. Lactacystin

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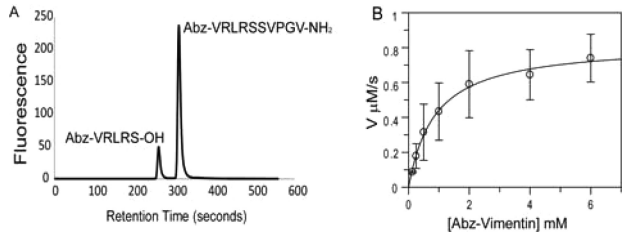

Figure 1. HPLC CPAF assay using Abz-VRLRSSVPGV-NH_2. (A) Representative HPLC trace showing **1** and the Abz-containing hydrolysis product Abz-VRLRS-OH (**2**) as viewed by fluorescence detection at 420 nm (λ_{em}). (B) Determination of apparent kinetic parameters for CPAF for **1**. Assays were performed in a total volume of 100 μL containing assay buffer, CPAF (62.5 nM), and varying concentrations of Abz-VRLRSSVPGV-NH_2 (0–6 mM).

exhibited an CPAF IC_{50} of 10.2 ± 2.3 μM, a value comparable to concentrations required for full ex vivo inhibition.[11]

Indeed, the prospects of utilizing lactacystin as a probe of CPAF's function in chlamydial cooption of mammalian host cell function are improbable because this agent is an irreversible, potent inhibitor of the human proteasome. To design a CPAF-specific inhibitor, we referred to the recent structural data for the zymogen and active forms of CPAF.[17] CPAF is produced as a catalytically inactive 609-amino acid zymogen that undergoes concentration-dependent autoproteolysis and self-activation by excision of an internal 40-amino acid inhibitory sequence (residues 243–283), releasing a 29 kDa C-terminal fragment and a 32 kDa N-terminal fragment that reassociate to form a stable heterodimer. Removal of the 40-mer internal sequence allows the serine protease catalytic triad to assemble from residue contributions from both subunits, as was revealed from the recent crystal structure of recombinant CPAF (rCPAF) determined by Chai and co-workers.[17] A mutated fragment of this 40-mer sequence fused to glutathione *S*-transferase exhibited modest inhibitory activity against CPAF.[17] Building upon these precedents, we hypothesized that binding of a 25-amino acid portion within the $^{243-283}$CPAF sequence that was predominantly α-helical (residues 258–283) would likely bind within the mature CPAF heterodimer's active site (Figure 2). The enzyme-bound α-helix may therefore compete for substrate binding to CPAF and emulate the inactive zymogen conformation by repositioning active site residues at the interface of the heterodimer. Because base strength amplification is the proposed mechanism of Ser499 nucleophilic activation,[17] altering the relative positions of the three catalytic residues that were donated by two separate polypeptide chains may abrogate CPAF activity.

We subsequently prepared the peptide inhibitor **3** (H-SLFYSPMVPHFWAELRNHYATSGLK-NH_2) that corresponded to $^{258-283}$CPAF by solid-phase peptide synthesis (see the Supporting Information) and showed that this sequence was capable of inhibiting CPAF proteolysis of **1** in vitro using rCPAF. Peptide **3** exhibited an IC_{50} of 1.6 ± 0.6 μM, which was 6-fold more potent than lactacystin (Figure 2A).

Because *C. trachomatis* is an obligate intracellular pathogen, we designed a cell-permeable variant of inhibitor **3** by introducing a nona-arginine C-terminal addition to this sequence, generating [H-SLFYSPMVPHFWAELRNHYATSGLKRRRRRRRRR-NH_2 (**4**)]. When examined in vitro, peptide **4** yielded an IC_{50} of 50 ± 7 nM, a value ~200-

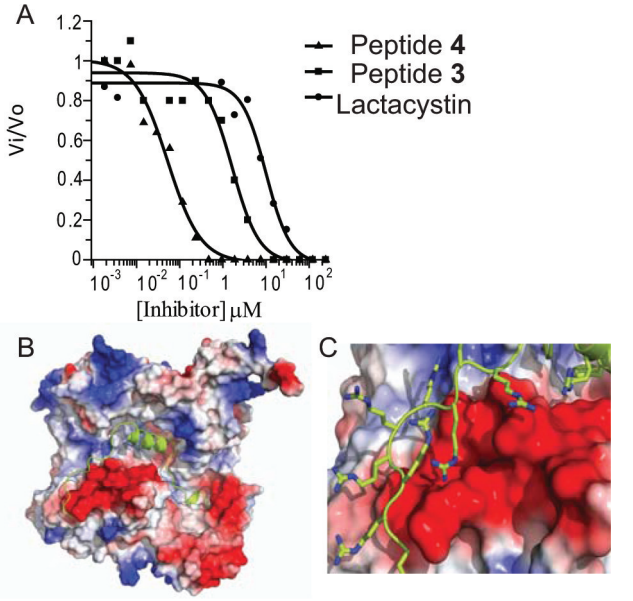

Figure 2. CPAF inhibition assay comparison of lactacystin with inhibitors **3** and **4**. (A) HPLC-based assays were performed in a final volume of 100 μL containing assay buffer, CPAF (62.5 nM), substrate **1** (0.5 mM), and varying concentrations of inhibitor (0–240 μM). IC_{50} values for lactacystin, peptide **3**, and peptide **4** were 10.2 ± 2.3 μM, 1.6 ± 0.6 μM, and 50 ± 7 nM, respectively. (B) Three-dimensional structure of CPAF with peptide **4** modeled into the active site of heterodimeric CPAF. (C) Expansion view of the inhibitor **4** bound CPAF model showing predicted polyarginine binding to a region of negative electrostatic potential.

fold greater than that of lactacystin and ~30-fold greater than that of inhibitor **3** (Figure 2A), making **4** the most potent CPAF inhibitor presently known. By analysis of the model of inhibitor **4** bound to mature CPAF (Figure 2B), we attribute the increase in inhibitory activity to enhanced binding due to favorable electrostatic interactions between the nona-arginine C-terminus and a large region of electronegative potential proximal to the active site where the helical 25-mer is predicted to bind. We are currently performing structural studies of the peptide **4**–rCPAF complex to confirm this hypothesis.

Valdivia and co-workers demonstrated that treating *C. trachomatis* LGV-L2 434-infected HeLa cells with lactacystin resulted in fiber oligomerization of vimentin due to inhibition of CPAF-mediated proteolysis.[15] To establish if CPAF was inhibited by peptide **4** during infection, we assessed vimentin cleavage in *C. trachomatis* LGV-L2 434-infected HeLa cells after they had been treated with a range of concentrations (2–10 μM).[15] Under similar conditions, infected HeLa cells were treated with a sequence-scrambled control peptide that possessed no CPAF inhibitory activity in vitro [H-NFALSHFRLPLSTYKEMPYVSHWAGRRRRRRRRR-NH_2 (**5**)]. Peptide **4**, but not the scrambled peptide **5**, markedly inhibited CPAF-mediated degradation of vimentin in a dose-dependent manner (Figure 3B). This result strongly suggested that peptide **4** not only penetrated the cell membrane but also selectively targeted CPAF activity ex vivo. The permeability of these peptides is most likely modest with respect to the percentage of peptide being delivered; however, the ability to inhibit still renders them useful.

Chlamydia remodels and recruits cytoskeletal components of the host cell such as F-actin and vimentin to form a dynamic

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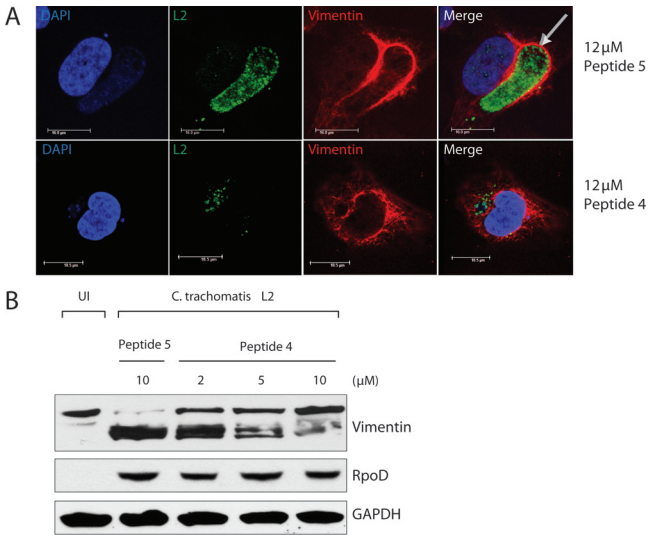

Figure 3. HeLa cells were infected with *C. trachomatis* L2, treated with control peptide **5** and peptide **4**, and harvested at 30 hpi. (A) Immunofluorescence microscopy via staining with an anti-vimentin antibody (red) and an anti-*Chlamydia* L2 antibody (green). Note the loss of the vimentin cage (arrow) surrounding the inclusion in cells treated with peptide **4**. (B) Cells were lysed in the presence of protease inhibitors, resolved by SDS–PAGE, and subjected to immunblot analysis. Vimentin, RpoD (bacterial loading control), and GAPDH (host loading control) were visualized with substrate-specific antibodies.

scaffold or "cage" that provides structural stability to the inclusion.[15] As the inclusion expands, secreted CPAF progressively modifies the intermediate filament scaffold, presumably to increase the inclusion's flexibility and accommodate the increased bacterial load. In infected cells, CPAF processing of vimentin filaments occurs several hours after the hour postinfection (hpi) at which CPAF can be detected in the cytosol.[15] Treatment of *C. trachomatis*-infected HeLa cells with peptide **4**, but not the control peptide **5**, resulted in a loss of vimentin processing (Figure 3B) and increased disorder in the position of the vimentin cage surrounding the inclusion (Figure 3A). These data suggest that peptide **4** selectively inhibits CPAF activity, which prevents vimentin cleavage and proper deposition of vimentin surrounding the intracellular vacuole (Figure 3A). Because intermediate filaments like vimentin are stable structures that provide mechanical support to maintain vacuole integrity in infected cells,[15] it is likely that peptide **4** has altered the integrity of the vacuole, which may have a broader impact on bacterial survival within the host.

The development of an in vitro activity assay for CPAF coupled with the discovery of a cell-permeable nanomolar affinity inhibitor furthers our understanding of the role of CPAF in chlamydial pathogenesis. Because genetic manipulation of *Chlamydia* is not possible at this time, we hope that these enzymology and chemical biology tools will allow investigations into the kinetic and molecular mechanism of this unique enzyme, as well as analyses of the substrate specificity, small molecule inhibitor discovery, mechanism of action, and role in pathogenesis in vivo.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary experimental methods and enlarged figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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