Mechanism of gasdermin D recognition by inflammatory caspases and their inhibition by a gasdermin D-derived peptide inhibitor

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The inflammasomes are signaling platforms that promote the activation of inflammatory caspases such as caspases-1, -4, -5, and -11. Recent studies identified gasdermin D (GSDMD) as an effector for pyroptosis downstream of the inflammasome signaling pathways. Cleavage of GSDMD by inflammatory caspases allows its N-terminal domain to associate with membrane lipids and form pores that induce pyroptotic cell death. Despite the important role of GSDMD in pyroptosis, the molecular mechanisms of GSDMD recognition and cleavage by inflammatory caspases that trigger pyroptosis are poorly understood. Here, we demonstrate that the catalytic domains of inflammatory caspases can directly bind to both the full-length GSDMD and its cleavage site peptide, FLTD. A GSDMD-derived inhibitor, *N*-acetyl-Phe-Leu-Thr-Asp-chloromethylketone (Ac-FLTD-CMK), inhibits GSDMD cleavage by caspases-1, -4, -5, and -11 in vitro, suppresses pyroptosis downstream of both canonical and noncanonical inflammasomes, as well as reduces IL-1 β release following activation of the NLRP3 inflammasome in macrophages. By contrast, the inhibitor does not target caspase-3 or apoptotic cell death, suggesting that Ac-FLTD-CMK is a specific inhibitor for inflammatory caspases. Crystal structure of caspase-1 in complex with Ac-FLTD-CMK reveals extensive enzyme-inhibitor interactions involving both hydrogen bonds and hydrophobic contacts. Comparison with other caspase-1 structures demonstrates drastic conformational changes at the four active-site loops that assemble the catalytic groove. The present study not only contributes to our understanding of GSDMD recognition by inflammatory caspases but also reports a specific inhibitor for these caspases that can serve as a tool for investigating inflammasome signaling.

inflammasome | inflammatory caspases | gasdermin D | pyroptosis | covalent inhibitor

The inflammasomes are signaling platforms that play crucial roles in inflammatory responses to infections and danger signals (1–3). The canonical inflammasome complex is composed of a sensor protein, an adaptor protein called ASC (apoptosis-associated speck-like protein containing a CARD), and inflammatory caspases such as caspase-1. Activation of the canonical inflammasomes promotes the maturation of caspase-1, which cleaves both proinflammatory cytokines (4) and gasdermin D (GSDMD) (5–7). GSDMD is suggested to adopt an autoinhibited conformation (through intramolecular domain interactions) (8–10), which is released by caspase cleavage to allow its N-terminal p30 fragment to interact with membrane lipids and to assemble pores that induce pyroptosis, a lytic form of cell death that releases intracellular contents (8, 11–15). The GSDMD pores contribute to the release of mature cytokines such as IL-1 β and IL-18, which in turn recruit more immune cells to promote inflammation (16). The noncanonical inflammasomes play important roles in immune

response to intracellular bacterial infections (17–19). The caspase activation and recruitment domains (CARDs) of caspases-4, -5, and -11 can directly bind to lipopolysaccharides (LPSs) and mediate the activation of the noncanonical inflammasomes (20). This leads to cleavage of GSDMD and induction of pyroptosis. Similar to GSDMD, the N-terminal domains of other gasdermin family members such as GSDMA/GSDMA3, GSDMB, GSDMC, and GSDME/DFNA5 can induce pyroptosis in mammalian cells and kill bacteria (8, 21). As a result, pyroptosis was redefined as gasdermin-mediated programmed necrotic cell death (22).

Caspase is a family of cysteine proteases that are expressed as zymogens activated by protease cleavage (23, 24). The mature enzymes predominantly cleave at P1-site aspartate residues within their substrates and play crucial roles in cell death, tissue remodeling, and differentiation (25–27). Caspases can be divided

Significance

The inflammasomes are signaling platforms that promote the activation of inflammatory caspases such as caspases-1, -4, -5, and -11, which cleave gasdermin D (GSDMD) to induce pyroptotic cell death. The mechanisms of GSDMD recognition by inflammatory caspases remain poorly understood. Here, we demonstrate that the catalytic domains of inflammatory caspases can directly bind to GSDMD or its cleavage site peptide, FLTD. A GSDMD-derived inhibitor, *N*-acetyl-Phe-Leu-Thr-Asp-chloromethylketone (Ac-FLTD-CMK), inhibits GSDMD cleavage in vitro and suppresses pyroptosis downstream of both canonical and noncanonical inflammasomes. By contrast, the inhibitor does not target caspase-3 or apoptosis, suggesting that it is specific for inflammatory caspases. Structure of caspase-1 in complex with Ac-FLTD-CMK reveals extensive enzyme-inhibitor interactions that shed light on GSDMD recognition by inflammatory caspases.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID code 6BZ9).

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Fig. 3. Ac-FLTD-CMK specifically inhibits inflammatory caspases. (A) Chemical structure of the GSDMD-derived peptide inhibitor Ac-FLTD-CMK. (B–D) Enzymatic activities of human caspase-1 (B), caspase-4 (C), caspase-5 (D), and caspase-3 (E) in the presence of Ac-FLTD-CMK. Data shown are representative of mean $\pm$ SD of at least three independent experiments.

Results

Inflammatory Caspases Directly Bind GSDMD. To investigate the interaction between caspases and GSDMD, FLAG-tagged catalytic domain of murine caspase-11 (FLAG-mCasp11 CAT) and Myc-tagged full-length murine GSDMD (Myc-mGSDMD) were coexpressed in HEK-293T cells (Fig. 1). The full-length GSDMD was coimmunoprecipitated by both wild-type (WT) and catalytically inactive (C254A) caspase-11, with GSDMD partially processed by the former (Fig. 1B). By contrast, no obvious binding was detected between the caspase-11 CARD and GSDMD (*SI Appendix, Fig. S1A*), suggesting that the catalytic domain of caspase-11 is sufficient for binding to GSDMD. To test the direct association between inflammatory caspases and GSDMD, recombinant proteins for murine caspase-11 catalytic domain (C254A) or human caspase-1 catalytic domain (C285A) was incubated with murine or human full-length GSDMD, followed by analysis with size-exclusive chromatography. The catalytically inactive mutant caspases were used to prevent cleavage of and dissociation from GSDMD. When purified full-length GSDMD protein and caspases were mixed together, the appearance of a larger protein complex containing both GSDMD and caspases-1 and -11 was observed (Fig. 1C and D and *SI Appendix, Fig. S1B and C*), suggesting direct association of inflammatory caspases with GSDMD.

GSDMD Cleavage Site Peptide and Its Derivative Bind Inflammatory Caspases. Since GSDMD directly associates with caspase-1 and caspase-11, we hypothesized that peptides containing the GSDMD cleavage site residues FLTD (*SI Appendix, Fig. S2A*) may participate in caspase binding. A thermal shift assay was used to examine

the binding between caspase-1 and the FLTD peptide. A 3.7 °C shift of the melting temperature for the human caspase-1 C285A mutant was observed in the presence of the peptide, indicating that the cleavage site peptide is sufficient for caspase-1 binding (Fig. 2A). To quantify this binding, we performed fluorescence polarization assays between caspases and an FLTD peptide containing N-terminal fluorescent label carboxyfluorescein (FAM), followed by an aminohexanoic acid (Ahx) spacer. The binding affinities of FAM-Ahx-FLTD to human caspase-1 (C285A) and to human caspase-4 (C258A) are 21.7 μ M and 29.6 μ M, respectively, compared with no significant binding to human caspase-3 (C163A) within the range of concentrations tested (Fig. 2 and *SI Appendix, Fig. S2B and C*). This direct binding is further confirmed by Biacore analysis of the peptide association with human caspase-1 (C285A) (*SI Appendix, Fig. S2D*). Collectively, our data suggest direct and specific binding between the FLTD peptide and inflammatory caspases, in agreement with previous findings that GSDMD is cleaved by inflammatory, rather than apoptotic, caspases (5).

A GSDMD-Derived Inhibitor Specifically Targets Inflammatory, but Not Apoptotic, Caspases. Based on the specific binding of the FLTD peptide to inflammatory caspases and on previous studies of caspase inhibitors using halomethyl ketone groups to covalently modify the catalytic site Cys residue (31), the GSDMD-derived peptide inhibitor Ac-FLTD-CMK (Fig. 3A) was designed to target inflammatory caspases. To investigate the effects of Ac-FLTD-CMK on caspase activities, we performed in vitro caspase activity assays using synthetic caspases-1, -4, and -5 substrate *N*-acetyl-Trp-Glu-His-Asp-7-amido-4-methylcoumarin (Ac-WEHD-AMC, Enzo Life Sciences) and caspase-3 substrate Ac-DEVD-AMC. Ac-FLTD-CMK potentially inhibited the enzymatic activities of caspases-1, -4, and -5, but not caspase-3 (Fig. 3B–E and *SI Appendix, Fig. S3A*). In addition to the synthetic caspase substrates, cleavage of the physiological substrate GSDMD was investigated in the presence of Ac-FLTD-CMK and z-VAD-FMK, a pan-caspase inhibitor. As expected, caspases-1, -4, and -5 cleaved human

Fig. 4. Ac-FLTD-CMK inhibits in vitro GSDMD cleavage by inflammatory caspases. Ac-FLTD-CMK inhibits MBP-hGSDMD cleavage by human caspase-1 (hCasp1) (A), hCasp4 (B), hCasp5 (C), and MBP-mGSDMD cleavage by murine (m)Casp11 (D) at 10 μ M concentration.

Fig. 6. Crystal structure of human caspase-1 in complex with Ac-FLTD-CMK. (A) Overview of the crystal structure of human caspase-1 catalytic domain in complex with the Ac-FLTD-CMK inhibitor with the $2F_o - F_c$ electron density map (magenta) contoured at 1σ . The p20 and p10 subunits of caspase-1 are colored cyan and marine, respectively, and the inhibitor is colored orange. (B) The electrostatic charge surface of caspase-1 at its catalytic pocket is shown on a scale of -5 kT/e (red) to 5 kT/e (blue), with the four catalytic site loops L1 to L4 marked. The bound Ac-FLTD-CMK is shown as a ball-and-stick model. (C) Detailed interaction between the inhibitor and caspase-1 is shown, with hydrogen bonds indicated as gray dotted lines. The inhibitor is shown in orange sticks. (D) Comparison of the four active-site loops from the caspase-1–Ac-FLTD complex (cyan), caspase-1 ligand free structure (green; PDB ID code 1SC1), and caspase-1 zymogen structure (gray; PDB ID code 3E4C).

human caspase-1 in complex with Ac-FLTD-CMK at $1.8\text{-}\text{\AA}$ resolution (PDB ID code 6BZ9). The crystallographic asymmetric unit contains a p20/p10 heterodimer bound to an Ac-FLTD-CMK inhibitor at the catalytic groove (Fig. 6A), cradled by L1 to L4 loops (Fig. 6B), similar to a previous structure of caspase-1 in complex with z-VAD-FMK (*SI Appendix, Fig. S5A*) (34). Both hydrophilic and hydrophobic interactions contribute to the extensive interaction between Ac-FLTD-CMK and caspase-1 (Fig. 6C), which buries $550\text{ \AA}^2$ of the total $820\text{ \AA}^2$ of solvent-accessible surface area for the inhibitor. Specifically, at the P1 site, the Asp residue from Ac-FLTD-CMK forms main-chain–main-chain hydrogen bonds with G238 and S339, a main-chain–side-chain hydrogen bond with H237, and side-chain hydrogen bonds with R179, Q283, and R341 from caspase-1. Such interaction is strengthened by a covalent bond formed by the CMK moiety and C285 from caspase-1. The P2-site Thr residue forms a main-chain–side-chain hydrogen bond with R341 and hydrophobic contacts with V338 and W340. The P3-site Leu residue forms two main-chain–main-chain hydrogen bonds with R341, resembling those in an antiparallel β sheet. The side chain of the P4-site Phe residue forms extensive hydrophobic and van der Waals interactions with the side chains of W340, H342, M345, and R383. The N-terminal acetyl moiety in Ac-FLTD-CMK is close to H342 and P343, which may allow for additional hydrogen bonds and hydrophobic contacts with P5 residue in the full-length GSDMD protein. Superposition of our structure with a previously reported caspase-1 zymogen structure (PDB ID code 3E4C) (35)

and a caspase-1 structure in the absence of bound ligand (PDB ID code 1SC1) (36) demonstrates drastic conformational change at the four active-site loops, L1 to L4 (Fig. 6D), in agreement with previous analyses, revealing key roles of these loops in mediating the recognition of caspase substrates (25, 26).

Structure-based sequence alignment of inflammatory and apoptotic caspases reveals that the L1 and L2 loop residues engaging the inhibitor are identical among caspases-1, -3, -4, -5, and -11, whereas the L3 and L4 loop residues are less conserved, with the L3 loop harboring the most extensive interaction (Fig. 6C and *SI Appendix, Fig. S5B*). In fact, three of seven inhibitor-binding residues in the caspase-1 L3 loop (H342, P343, and M345) are not conserved among the different caspases, neither is the L4 loop residue R383. Since the M345 and R383 residues are involved in hydrophobic and van der Waals contact with the P4-site residue of the inhibitor (Fig. 6C), it is possible that the negatively charged Asp residues in caspase-3 at these two equivalent positions may disrupt such interactions. As a result, divergent L3 and L4 loop residues may dictate the specific recognition of GSDMD by inflammatory, rather than apoptotic, caspases.

Discussion

Despite the identification of GSDMD as an effector of pyroptosis, it remains unclear how inflammatory caspases specifically recognize GSDMD, which leads to its cleavage and formation of membrane pores by its N-domain. In the current study, we examined the interactions between inflammatory caspases and