

Structural insights into the substrate specificity of human granzyme H – the functional roles of a novel RKR motif

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1 Introduction

Human granzyme H (GzmH), a chymotrypsin-like serine protease, is constitutively expressed in NK cells, suggesting its critical role in NK cells-mediated immune responses. It has been shown that GzmH is capable of inducing tumor cell death via caspase-dependent apoptosis with DNA fragmentation through cleavage of ICAD to release CAD. Increasing reports have established the pivotal viral clearance role of GzmH. GzmH can directly hydrolyze important viral proteins, such as the adenovirus DNA-binding protein for viral DNA replication and the adenovirus 100K assembly protein for virus assembly. Degradation of 100K also unleashes its inhibitory effect on pro-apoptotic GzmB, suggesting the cooperative action of Gzms to thwart viruses. Recently, GzmH has been shown to cleave HBx, an important factor required for the HBV replication, to facilitate the eradication of HBV. GzmH also degrades a multifunctional phosphoprotein La to inhibit hepatitis C virus (HCV)-internal ribosome entry site-mediated translational activity. Nevertheless, substrate specificity determinants of GzmH remain largely unknown. For lacking of mouse homolog, a GzmH knockout mouse model for anti-viral and anti-tumor investigation in vivo is not available. Chemical inhibition of human GzmH is an important alternative for studying the physiological roles of this enzyme. However, currently available inhibitors for GzmH are not specific, which hinder further research for GzmH functions.

2 Experiment

Crystals were generated in 2- μ L hanging drops containing equal amounts of protein (8-10 mg/mL) and mother liquor equilibrated over 200 μ L of reservoir solution at 16°C. D102N-GzmH was crystallized in a buffer containing 0.2 M Li₂SO₄, 0.1 M Bicine (pH 8.5), 25% (w/v) PEG3350 after one week incubation. For the generation of D102N-GzmH-decapeptide and DNH-inhibitor complexes, crystals of D102N-GzmH were soaked in the above mother liquor supplemented with either 10 mM decapeptide for 2 days or 2 mM inhibitor for 18 h. For data collection, crystals were dehydrated in a solution containing 0.2 M Li₂SO₄, 0.1 M Bicine (pH8.5), 30% (w/v) PEG3350 for 2 h and then fast frozen with liquid nitrogen. All data sets were collected at 100 K. Data for D102N-GzmH, D102N-GzmH-decapeptide,

D102N-GzmH-inhibitor complexes were respectively collected at the beamline BL6A ($\lambda=1.0\text{\AA}$), BL17A ($\lambda=0.98\text{\AA}$), NE3A ($\lambda=1.0\text{\AA}$) of Photon Factory (Japan). All diffraction data were indexed, integrated and scaled with the program HKL2000.

3 Results and Discussion

To provide structural insights into the substrate recognition mechanisms for GzmH, we solved the crystal structures of GzmH alone and in complex with a decapeptide substrate and an inhibitor to 2.2 \AA , 2.4 \AA and 2.7 \AA , respectively. The Thr189, Gly216 and Gly226 specificity triad in the S1 pocket of GzmH defines its preference for bulky, aromatic residues (Tyr and Phe) at the P1 position. Notably, we discovered that an unusual RKR motif (Arg39-Lys40-Arg41), conserved only in GzmH, helps define the S3' and S4' pockets, indicating the preference for acidic residues at the P3' and P4' sites. Disruption of the RKR motif or the acidic P3' and P4' residues in the substrate abolished the proteolysis of GzmH. We designed a tetrapeptide chloromethylketone (CMK) inhibitor, Ac-PTSY-CMK, which can specifically and efficiently block the enzymatic and cytotoxic activity of GzmH, providing a useful tool for further studies on the function of GzmH.

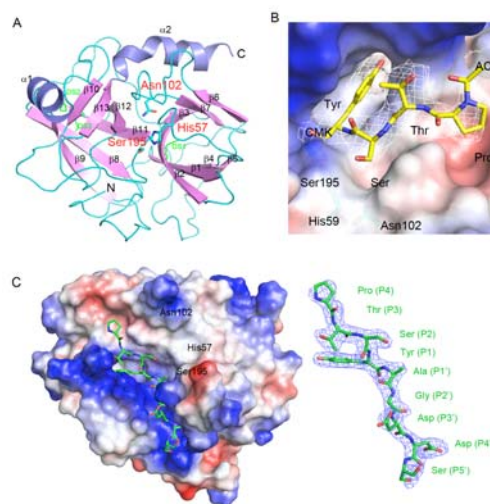


Fig. 1: Crystal structure of GzmH alone and complexes with a synthetic inhibitor and a decapeptide substrate.

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