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Elucidation of the substrate specificity of the MASP-2 protease of the lectin complement pathway and identification of the enzyme as a major physiological target of the serpin, C1-inhibitor☆

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Abstract

Complement is a central component of host defence, but unregulated activation can contribute to disease. The system can be initiated by three pathways: classical, alternative and lectin. The classical and lectin pathways are initiated by the C1 and mannose-binding lectin (MBL) or ficolin complexes, respectively, with C1s the executioner protease of the C1 complex and MASP-2 its counterpart in the lectin complexes. These proteases in turn cleave the C4 and C2 components of the system. Here we have elucidated the cleavage specificity of MASP-2 using a randomised substrate phage display library. Apart from the crucial P_1 position, the MASP-2 S_2 and S_3 subsites (in that order) play the greatest role in determining specificity, with Gly residues preferred at P_2 and Leu or hydrophobic residues at P_3. Cleavage of peptide substrates representing the known physiological cleavage sequences in C2, C4 or the serpin C1-inhibitor (a likely regulator of MASP-2) revealed that MASP-2 is up to 1000 times more catalytically active than C1s. C1-inhibitor inhibited MASP-2 50-fold faster than C1s and much faster than any other protease tested to date, implying that MASP-2 is a major physiological target of C1-inhibitor.

Keywords: Mannose-binding lectin-associated serine protease-2; Complement; Active site specificity; C1s; C1-inhibitor; Serpin; Protease

1. Introduction

The complement system can be activated in three ways: the classical, lectin and alternative pathways (Goldsby et al., 2003). The former two pathways are both activated following binding of a recognition molecule to pathogenic surfaces, these being C1q for the classical pathway and mannose-binding lectin (MBL) or ficolin for the lectin pathway (Fujita et al., 2004). Following binding of the recognition molecule, associated serine proteases are activated, which in turn cleave the complement C4 and C2 proteins. The C3 convertase (C4bC2a) complex then activates C3 and the rest of the complement system in turn, resulting in the formation of the membrane attack complex and release of small pro-inflammatory protein fragments, such as the potent anaphylotoxin, C5a. The initiating proteases of both the classical and lectin pathways are regulated by the serpin, C1-inhibitor

Abbreviations: NHMec, 7-amino-4-methyl coumarin group; Abz, 2-aminobenzoyl; Lys(Dnp), lysine dinitrophenyl; DMF, dimethyl formamide; PoPS, prediction of protease specificity program; MBL, mannose-binding lectin; MASP, mannose-binding lectin-associated serine protease; C1-Inh, C1-inhibitor; FQS, fluorescence-quenched substrate

☆ The nomenclature for residues in substrates is based on that outlined by Schechter and Berger (1967) for the substrates of proteases. The residues are numbered from the cleaved bond (P_1–P_1') as follows: P_n–···–P_4–P_3–P_2–P_1–P_1'–P_2'–P_3'–P_4'–···–P_n'; the corresponding subsites of the enzyme are denoted as S_n–···–S_4–S_3–S_2–S_1–S_1'–S_2'–S_3'–S_4'–···–S_n'.

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(C1-Inh) (Bos et al., 2002; Caliezi et al., 2000; Rossi et al., 2001; Presanis et al., 2004; Ambrus et al., 2003).

The lectin pathway appears to be predominantly activated via complexes containing the MBL-associated protease-2 (MASP-2) (Vorup-Jensen et al., 2000), which autoactivates following lectin binding to target molecules on pathogen surfaces (Gál et al., 2005), while the classical pathway is initiated by the C1 complex, where sequential activation of the C1r and C1s proteases takes place following C1q binding. The initiating proteases of the classical and lectin pathways share similar domain structure (Sim and Tsiftsoglou, 2004), with the N-terminal CUB-EGF-CUB domains playing a role in binding of the C1q and lectin recognition proteins, while the C-terminal CCP1-CCP2-serine protease domains recognize and cleave substrates. While the CCP domains clearly play a role in binding to the C4 protein substrate, it appears that the active site of the serine protease plays a dominant role in interactions with the C2 substrate (Rossi et al., 2001, 2005; Ambrus et al., 2003).

In contrast to C1r and C1s, the three MASPs are less characterized in terms of their substrate specificity and function. MASP-2 functions as the lectin pathway counterpart of C1s, playing the major role in cleaving C4 and C2 (Vorup-Jensen et al., 2000), while the functions of the MASP-1 and MASP-3 proteases are essentially unknown. The three-dimensional structure of MASP-2 (Harmat et al., 2004) suggests that the architecture of the enzyme's active site is different from C1s (Gaboriaud et al., 2000), although the specificity for protein substrates is similar. Having recently characterized the full substrate specificity of C1s (Kerr et al., 2005), we show here using substrate phage display technology that MASP-2 has broader specificity than C1s, but its active site specificity allows optimal cleavage at the required positions in itself and C2. Our data indicates that the demonstrated exosite interactions involved in cleavage of the C4 substrate (Ambrus et al., 2003; Rossi et al., 2001, 2005) are vital to provide efficient and correct cleavage of this protein. In addition, MASP-2 has a higher catalytic efficiency than C1s and a very high rate of interaction with C1-Inh, suggesting that it is a major physiological target of this serpin.

2. Experimental procedures

2.1. Materials

Fluorescence quenched substrates (FQS) [C2 P_4–P_4' substrate (2Abz-SLGRKIQI-Lys(Dnp)-NH_2), C4 P_4–P_4' substrate (2Abz-GLQRALEI-Lys(Dnp)-NH_2) and C1-Inh P_4–P_4' substrate (2Abz-SVARTLLV-Lys(Dnp)-NH_2)] were synthesised at greater than 80% purity by Auspep (Melbourne, Victoria, Australia). High performance nickel-Sepharose beads were purchased from Amersham Biosciences (Sydney, Australia). Commercial PCR clean-up kits were purchased from Millipore (Billerica, MA, USA). The Novagen T7SelectUP and DOWN PCR primers were from the T7Select Cloning Kit (Novagen). BIG-DYE version 3.1 was purchased from the Monash University Micromon DNA sequencing facility. Recombinant human MASP-2 CCP1-2-SP domain was expressed and purified as described previously (Rossi et al., 2001). C1-Inh was purified from human plasma as described previously (Pilatte et al., 1989). Human C1s and C1r were purchased from Calbiochem (La Jolla, CA, USA) and human factor XIa (FXIa) was from Haematologic Technologies Inc. (Essex Junction, VT, USA). Human plasma kallikrein, N-t-Boc-Leu-Gly-Arg-7-amido-4-methylcoumarin (LGR) and Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (FR) were from BaChem (Bubendorf, Switzerland). All other chemicals were of analytical grade or the best quality commercially available.

2.2. Phage display

The T7Select1-1b Phage Display system (Novagen) was used to generate a randomised protease substrate library as described previously (Kerr et al., 2005; Kaiserman et al., 2006) following the approach of Cwirla et al. (1990). Approximately 10^9 *pfu* of amplified phage in phage extraction buffer were bound to nickel-Sepharose beads at 4 °C. Unbound phage were removed by washing the beads with phage wash buffer (850 mM NaCl, 0.1% (v/v) Tween-20 in PBS), followed by 1 mM $MgSO_4$ in PBS. Phage selection commenced by the addition of 100 nM MASP-2 protease to the treatment tube for rounds 1–5 of selection. An equal volume of 1 mM $MgSO_4$ in PBS was added to the control tube instead of protease. Both the treated and control tubes were incubated overnight at 37 °C. Cleaved phage were recovered from the supernatant, titrated and amplified to form the sub-library for the next round of selection. Phage that remained bound to the beads were eluted with 0.5 M imidazole and titrated to assess cleavage efficiency. After 5 rounds of protease selection, the amount of MASP-2 added to the treatment tube was decreased to 25 nM for rounds 6–10 of selection. Randomly selected individual phage plaques from rounds 5, 7 and 10 were chosen for sequencing as described previously (Kerr et al., 2005).

2.3. Use of PoPS to investigate models of cleavage

The PoPS program (Boyd et al., 2005) was used to computationally analyse how the specificity data from phage display data relates to the known cleavage of the C2 and C4 substrates and C1-Inh, as well as self-cleavage of MASP-2. The PoPS program requires a specificity model, which was created from the phage sequence data. The position-specific scoring matrix (PSSM) of the PoPS model was obtained by calculating the frequency of the residues at each of the nine positions in the randomised phage sequences. The fixed arginine was assumed to represent the P_1 position in each sequence, with the PSSM representing the S_5–S_4' subsites. These frequencies were then adjusted according to the expected residue frequency using the codon frequencies of the T7 phage display system (Matthews et al., 1994). The PSSM was then normalized to the −5.0 to +5.0 range, as required by the PoPS program. PoPS allows weightings to be assigned to the subsites, to indicate relative importance. For this model, the weights were all set to 1.0, because the relative importance was implicit in the residue frequencies calculated in the PSSM. No dependency rules were added to the model. The model was then used in the PoPS program to predict cleavage sites in each of