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The action of MBL-associated serine protease 1 (MASP1) on factor XIII and fibrinogen

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ABSTRACT

The complement system is an important recognition and effector mechanism of the innate immune system that upon activation leads to the elimination of foreign bodies. It can be activated through three pathways of which the lectin pathway is one. The lectin pathway relies on the binding of mannan-binding lectin (MBL) or the ficolins and the subsequent activation of the MBL-associated serine proteases (MASPs), namely, MASP1, 2 and 3 which all form complexes with both MBL and the ficolins. Major substrates have only been identified for MASP2 i.e. C4 and C2. For MASP1 only a few protein substrates which are cleaved at a low rate have been identified while none are known for MASP3. Since chromogenic substrate screenings have shown that MASP1 has thrombin-like activity, we wanted to investigate the catalytic potential of MASP1 towards two major proteins involved in the clotting process, fibrinogen and factor XIII, and compare the activity directly with that of thrombin. We found that rMASP1 and thrombin cleave factor XIII A-chain and the fibrinogen β-chain at identical sites, but differ in cleavage of the fibrinogen α-chain. The thrombin turnover rate of factor XIII is approximately 650 times faster than that of rMASP1 at 37°C, pH 7.4. rMASP1 cleavage of fibrinogen leads to the release of the proinflammatory peptide fibrinopeptide B. Thus rMASP1 has similar, but not identical specificity to thrombin and its catalytic activity for factor XIII and fibrinogen cleavage is much lower than that of thrombin. Nevertheless, rMASP1 can drive the formation of cross-linked fibrinogen. Since MASP1 is activated on contact of MBL or the ficolins with microorganisms, fibrinogen and factor XIII may be involved in the elimination of invading pathogens.

1. Introduction

The immune system is a complex but highly efficient network of both cellular and humoral mechanisms that prevents the vast majority of microorganisms becoming pathogenic. One of these immune system mechanisms is the complement system, which is a protein cascade comprising more than 30 soluble and cell-bound recognition and effector proteins. The complement system has multiple anti-microbial activities of which the direct lysis and opsonization of pathogens as well as its proinflammatory role are of the greatest importance. Additionally complement is involved in maintaining homeostasis in the body, since it is involved in the removal of circulating immune complexes and dying host cells [1]. The complement system can become activated through three independent pathways: the classical, the alternative and the lectin pathway each of which results in the formation of a C3 convertase complex (an unstable protease which activates C3) on the surface of the complement-activating target. Activation through the classical pathway occurs when the C1 complex (composed of C1q, C1r and C1s) binds to charged or hydrophobic clusters on antibodies, microorganisms or other targets [2,3]. The binding of Clq induces a conformational change to the entire C1 complex enabling the serine protease proenzyme C1r to autoactivate and subsequently activate another serine protease C1s [4]. The activated Cls can then cleave C4 and C2 forming the C3 convertase complex C4b2a. The alternative pathway depends on the continuous hydrolysis of C3 in the fluid-phase and the lack of complement inhibitors on the surface of pathogens.

The lectin pathway was discovered when purified preparations of mannan-binding lectin (MBL) were found to promote complement-mediated lysis of erythrocytes [5]. It was later discovered that this ability of MBL to activate complement was due to the association of MBL with the MBL-associated serine proteases (MASPs), which are homologues of the classical pathway proteases C1r and C1s. MBL is associated with three different MASPs i.e. MASP1, 2 and 3 as well as a non-enzymic protein called MAp19 [6–9]. The ficolin protein family is composed of three members, namely L-ficolin, H-ficolin and M-ficolin, which are similar in structure to MBL and also capable of activating complement through association with the MASPs [10–12].

The MASPs and MAp19 are alternative splicing products of 2 genes, the MASP1/3 and MASP2/MAp19 genes. MASP2 has been identified as the protein responsible for the cleavage of C4 and C2 [7] and it has

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recently also been reported to activate prothrombin [13]. For MASP1 and MASP3 no major substrates have been discovered but MASP1 has been observed to have a low catalytic activity towards a limited number of proteins. Since MASP1 was originally believed to be involved in the activation of the complement system the catalytic activity of MASP1 towards the complement components C2, C4 and C3 has been investigated. It was reported that MASP1 cleaves both C2 and C3 [14,15]. The C3 cleavage is not believed to be physiologically relevant since MASP1 was found only to cleave the hydrolyzed form of C3 (C3(H_2O)) rather than the biologically active C3 [16,17]. In the case of C2 MASP1 was found to have approximately a 10-fold lower proteolytic activity than MASP2 [17], but since MASP1 has no activity towards C4 this was regarded as being of little physiological relevance since C4 and C2 have to be cleaved essentially simultaneously (as can occur with MASP2 and C1s) to generate the C3 convertase C4b2a. Recently it has been suggested that MASP1 and MASP2 may cooperate for the generation of the C4b2a convertase complex since a serum depleted of all the MASPs was found to have a higher C3 activation potential when both MASP1 and MASP2 were added back, than with either of them alone [18].

Studies with tripeptide substrates have revealed MASP1 to have thrombin-like specificity [19] and MASP1 has been shown to be capable of cleaving and polymerizing fibrinogen [16]. This process is dependent on simultaneous activation of two individual proteins, fibrinogen and factor XIII. Fibrinogen is a heterohexameric protein composed of two α-, β- and γ-chains with an elongated structure [20]. During coagulation it becomes activated by thrombin which cleaves the α- and β-chains releasing fibrinopeptides A and B [21]. This activation enables the fibrin strands to polymerize with other fibrin molecules and to form non-covalent interactions with nearby surfaces [21]. Simultaneously thrombin also activates factor XIII which is a tetraheteromeric protein complex composed of 2 A-chains and 2 B-chains. Thrombin cleaves the A-chains enabling the A-chain and B-chain dimers to dissociate. This leaves the functional transglutaminase (the cleaved A-chain) free to cross-link fibrin molecules and other suitable substrates by forming bonds between lysine and glutamine sidechains on polypeptides [22,23].

We report here the characterization of MASP1 in terms of thrombin-like activity by investigating its ability to cleave and activate factor XIII and fibrinogen.

2. Materials and methods

2.1. Recombinant human MASP1 construct (rMASP1)

The MASP1 construct used in this study was a truncated MASP1 construct (catalytic fragment of MASP1) composed of the CCP_1CCP_2SP-domains, that during preparation becomes fully activated by autolytic cleavage of the Arg_{448}-Ile_{449} bond, and was prepared as described by Ambrus et al. [17]. Upon storage under experimental conditions the rMASP1 undergoes autolytic degradation through the cleavage of the SP-domain at the Arg_{504}-Asp_{505} bond [17]. This cleavage results in an approximate 50% loss of activity compared to the intact protease when compared using tripeptide substrates (unpublished observation, Krarup A.). Autolytic degradation at 37°C resulted in an 80% cleavage of the SP-domain accompanied by a 35% loss of activity after 8h of incubation of rMASP1 at 0.1μg/ml in 20mM HEPES, 140mM NaCl, 5mM $CaCl_2$, 0.05% (v/v) Tween20, pH 7.4 (activation buffer) as measured by rate of VPR-AMC turnover as described in [19] (unpublished observation, Krarup A.).

2.2. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli system [24] with stacking gels of 3% (w/v) acrylamide and separation gels containing 10% (w/v) acrylamide or using NUPAGE® precast 4–12% (w/v) gradient gels using MOPS as running buffer (Invitrogen, Paisley, UK). The samples were prepared by mixing them in a 1:1 (v/v) ratio with 0.2M TRIS–HCl, 8M Urea, 2% (w/v) SDS, 0.005% (w/v) bromophenol blue, and 20mM dithiothreitol, pH 8.0 (SDS-PAGE sample buffer) [25]. The samples were incubated for 5min at 95°C and loaded on the gel. Protein Marker 2–212kDa (New England Biolabs Ltd., Herts, UK) was run alongside the samples to enable approximate protein size estimation. The gels were stained with Coomassie Brilliant Blue. Densitometry was carried out using a Syngene Chemigenius Bioimaging System (Synoptics Ltd., Cambridge, UK) and the Genesnap and Genetools software (Synoptics Ltd.).

2.3. rMASP1 activation of factor XIII

Human factor XIII (66μg/ml) (Haematologic Technologies Inc, HTI, Essex Junction, VT) was incubated with either 10μg/ml rMASP1 or 1U/ml human thrombin (Sigma-Aldrich, Poole, UK) in 15μl activation buffer at 37°C and the reaction was stopped by addition of 1volume of SDS-PAGE sample buffer and analyzed on SDS-PAGE.

2.4. Fibrinogen cleavage by rMASP1

Human fibrinogen (Enzyme Research Laboratories Ltd, Swansea, UK) (300μg/ml) was incubated with a 2-fold dilution series of thrombin or rMASP1 starting at 10U/ml or 32μg/ml, respectively. The reactions were carried out in a total volume of 10μl activation buffer with or without 2mM iodoacetamide (IAM). Most commercial fibrinogen preparations contain factor XIII, and IAM is added as an irreversible inhibitor of factor XIIIa. The samples were incubated for 24h at 37°C, the reaction stopped by addition of SDS-PAGE sample buffer, as above, and analyzed reduced on SDS-PAGE.

2.5. Fibrinopeptide A and B detection

Fibrinogen (1mg/ml) was incubated for 16h with 1U/ml thrombin or 16μg/ml rMASP1 or alone in 250μl activation buffer. A preparation of thrombin and fibrinogen at the same concentrations was also incubated for only 5min under identical conditions. As an additional control commercially available human fibrinopeptides A and B (Bachem, Bubendorf, Switzerland) were solubilized in activation buffer at a relative ratio of 4:1, respectively and incubated with 1U/ml thrombin for 16h at 37°C. To some samples 1mM IAM was added. After incubation the samples were filtered using Ultrafree®-MC centrifugal filter devices with a 10kDa cutoff (Millipore, Billerica, MA). Fibrinopeptides A and B (Mw 1.5kDa) pass through the filter. The samples which passed through the filter (200μl) were run on a Dionex P680 high-performance liquid chromatography (HPLC) system (Dionex Ltd, Leeds, UK) using a Phenomenex Jupiter 5μ (300A pore-size) C18 reverse-phase column (150mm × 2mm) (Phenomenex Ltd, Macclesfield, UK). The bound peptides were eluted with a 2–50% (w/v) linear gradient of solvent B mixing with starting solvent A. Solvent A was 0.1% (w/v) trifluroacetic acid in water and solvent B was 0.09% (w/v) trifluroacetic acid, 80% (v/v) acetonitrile in water. Flow rate was 200μl/min. Peptides eluted were detected by OD_{215} and fractions were collected by hand. For matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF) mass spectrometry the samples were dried by using a Speedvac® (Thermo Fisher Scientific, Inc., Waltman, MA) and the dried peptides were resuspended in a suitable volume (1–5μl) of 0.1% (w/v) trifluroacetic acid in water. The samples were mixed 1:1 (v/v) with 10mg/ml α-cyano-4-hydroxycinnamic acid (LaserBio Labs, Sophia-Antipolis Cedex, France) and the peptide masses were estimated using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences Ltd).

2.6. N-terminal sequencing

N-terminal sequencing was performed by running protein samples on a 10% Novex BIS–TRIS NuPAGE precast gel using 2-(*N*-morpholino)ethanesulfonic acid buffer in a Novex X Cell II Mini-Cell gel apparatus. The protein bands were electroblotted onto a Novex 0.2μm polyvinylidene difluoride membrane (Invitrogen) using a Novex Blot module. The membrane was stained with Coomassie Brilliant Blue and target bands were excised. These were washed with 10% methanol and sequenced on a 494A Procise protein sequencer (Applied Biosystems, Warrington, UK) for 10cycles using standard sequencing cycles [26].

3. Results

The study showing that MASP1 was capable of polymerizing fibrinogen was carried out by Hajela et al. [16] and was done using a MASP1 and MASP3 mixture isolated from human serum. The cleavage was established to be due to MASP1 and not MASP3 because it was inhibitable by C1-inhibitor, which does not inhibit MASP3 [17,27]. We investigated further the ability of MASP1 to cleave and activate fibrinogen and factor XIII.

Factor XIII was incubated with either thrombin or rMASP1 and samples were taken at different time points. These samples were analyzed on SDS-PAGE to monitor the activation of factor XIII by the appearance of the activated A-chain (A'-chain). In Fig. 1 the activation of factor XIII by thrombin (A) or rMASP1 (B) is shown. As can be observed in Fig. 1A at 0min two bands are visible: the B-chain (top) and the A-chain. With time, the A-chain is converted into the A'-chain and at 16min of incubation only about 50% of the A-chain remains. When factor XIII is incubated with rMASP1 (Fig. 1B) the same trend is observed. As time progresses a larger proportion of the A-chain is converted into the A'-chain. In Fig. 2 part of the N-terminal sequence of the factor XIII A-chain is shown and the activation peptide, which is