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Recombinant expression of the autocatalytic complement protease MASP-1 is crucially dependent on co-expression with its inhibitor, C1 inhibitor

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ABSTRACT

MASP-1 is a protease of the lectin pathway of complement. It is homologous with MASP-2, previously thought both necessary and sufficient for lectin pathway activation. Recently MASP-1 has taken centre stage with the observation that it is crucial to the activation of MASP-2 and thus central to complement activation. Numerous additional functions have been suggested for MASP-1 and its importance is obvious. Yet, thorough analyses of proteolytic activities and physiological roles in the human scenario have been hampered by difficulties in purifying or producing full-length human MASP-1. We present the successful expression of full-length recombinant human MASP-1 entirely in the zymogen form in a mammalian expression system. We found that the catalytic activity of MASP-1 suppresses its expression through rapid auto-activation and auto-degradation. This auto-degradation was not inhibited by the addition of inhibitors to the culture medium, and it was subsequently found to occur intracellularly. Numerous mutations aimed at attenuating auto-activation or preventing auto-degradation failed to rescue expression, as did also attempts at stabilizing the protease by co-expression with MBL or ficolins or expression in hepatocyte cell lines, representing the natural site of synthesis. The active protease was finally produced through co-expression with the serine protease inhibitor C1 inhibitor. We demonstrate that the expressed protease is capable of binding MBL and auto-activating, and is catalytically active. We have generalized the concept to the expression also of MASP-2 entirely in its zymogen form and with improved yields. We suggest a general advantage of expressing aggressive, autocatalytic proteases with their cognate inhibitors.

Introduction

Complement is a central, humoral, innate immune defense mechanism, comprising three pathways of activation: the classical pathway, the lectin pathway, and the alternative pathway [1]. The lectin pathway is initiated when a complex of a recognition molecule, i.e., mannan-binding lectin (MBL)[1] or one of the ficolins, and MBL-associated serine proteases (MASPs) binds a target pattern on, e.g., a microorganism. MASP-1 is the first described protease of the lectin pathway of complement activation [2]. The protein is produced from the *MASP1* gene, which additionally gives rise to another protease, MASP-3, through alternative splicing [3], and a smaller non-enzymatic protein termed MAp44 (or MAP-1) [4,5]. The homologous *MASP2* gene gives rise to yet another protease, MASP-2, and a smaller non-enzymatic protein termed MAp19 (or sMAP) [6,7]. The MASPs are homologues of C1r and C1s, which forms a complex, C1, with C1q of the classical pathway of complement. Activated MASP-2 is able to cleave both of the complement factors C4 and C2, and until recently it was believed that MASP-2 was both necessary and sufficient for complement activation [8]. However, we and others have recently demonstrated that MASP-1 is crucial in activating MASP-2 and hence initiating the lectin pathway [9–11]. MASP-1 appears to be a very promiscuous protease [12], and numerous other substrates have been reported in the literature. MASP-1 has been found to cleave C2, thereby possibly also potentiating lectin pathway activation [13]. MASP-1 was reported in the mouse to cleave pro-fD to active fD, implicating it also in alternative pathway activation [14]. However, we have recently demonstrated that MASP-1 is not required for alternative pathway activation in man [9]. Along the lines of inflammatory responses, MASP-1 has in addition been suggested to cleave protease-activated receptor 4 (PAR4), a G-protein-coupled receptor on the surface of endothelial cells, mediating activation and induction of a pro-inflammatory response in these cells [15]. Finally, MASP-1 has been proposed to link the complement and coagulation cascades by cleaving fibrinogen and Factor XIII [16].

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[1] *Abbreviations used:* MBL, mannan-binding lectin; rMBL, recombinant MBL; MASP, MBL-associated serine protease; PRM, pattern-recognition molecule; C1INH, C1 inhibitor; PAR4, protease-activated receptor 4; wt, wild-type.

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A number of the suggested roles of MASP-1 have been corroborated by experiments in *MASP1* knock-out mice, which reportedly have no alternative pathway activity [14] and are susceptible to influenza virus infection [17], but conversely are protected from autoimmune arthritis induction [18], occlusive thrombosis formation [19], and exhibit prolonged bleeding times [20]. These latter experiments were all conducted in mice, sometimes using reconstitution with mutated recombinant mouse MASP-1 produced in insect cells, whereas the *in vitro* studies (above) have generally been carried out using truncated fragments of MASP-1, produced either in bacteria or insect cells. The activities of the MASPs are believed to play their role *in vivo* through physiological complexes with MBL and ficolins, and depend on binding of these pattern-recognition molecules (PRMs) to foreign or altered-self patterns. However, the truncated forms of MASP-1 often used are void of MBL/ficolin binding capacity. Furthermore, MASP-1 is known to be glycosylated [21], presumably of importance to its function. MASP-1 and MASP-2 are both known to be able to auto-activate and owing to its high activity MASP-1 furthermore seems prone to auto-degradation. Thus, Rossi and colleagues were unable to express significant amounts of full-length human MASP-1 in an insect cell expression system [22]. Similarly, the expression of full-length mouse MASP-1 in an insect cell system was only possible by mutating the activation site arginine to lysine [11]. Finally, the catalytic fragment of human MASP-1 could be expressed in a bacterial expression system in inclusion bodies, and was prone to auto-degradation upon refolding and purification [12]. MASP-1 can be purified in MBL/MASP complexes from human serum by affinity chromatography. However, the purification has to be carried out cold and in the presence of large amounts of synthetic protease inhibitors, and still some activation and degradation of MASP-1 is seen. Furthermore, in our hands it has been impossible to separate MASP-1 from MBL and the other MASPs and MAps present. In order to be able to study the function of native human MASP-1 under more physiological circumstances, we set out to produce non-activated recombinant human MASP-1 in a mammalian expression system.

Materials and methods

MASP-1 and MASP-2 expression constructs

Full-length human MASP-1 cDNA in pcDNA3.1(A)mycHis vector (MASP-1 wt), an active site serine to alanine mutant (MASP-1 S646A), and an activation peptide arginine to glutamine mutant, which is constitutively on the zymogen form (MASP-1 R448Q), were previously described [9]. A number of additional mutated MASP-1 expression constructs were generated (see Fig. 1) using the QuickChange II XL site-directed mutagenesis kit (Stratagene) with the primers listed in Supplementary Table 1. The supplier's instructions were followed, except for the use of TOP10 cells instead of XL-10 Gold. Full-length human MASP-2 in vector pCIneo (MASP-2 wt) was previously described [23], as was the active site serine to alanine mutant of MASP-2 (MASP-2 S633A) [9].

Recombinant expression and purified proteins

Recombinant MBL (rMBL) was produced as previously described [24]. MASP-1, MASP-2 and their mutated counterparts were produced by transient expression in FreeStyle 293F cells using Lipofectamine2000 and 293F Expression Medium (Invitrogen). For co-expression with human C1 inhibitor (C1INH), cDNA encoding this protein in the pCMV6-XL5 vector was purchased from Origene. Various ratios of C1INH construct to MASP-1 or MASP-2 constructs were employed, from 3:1 to 1:1. Co-expression with MBL [24], M-ficolin [25], and H-ficolin was performed using previously described constructs. Human L-ficolin cDNA in pENTR221 was purchased from GeneCopoeia and cloned into pCDNA3.1 using the primers in Supplementary Table 1 for amplification of cDNA and incorporation of appropriate restriction sites. MBL/MASP complexes were purified from human plasma, as described previously [26]. Purified α_2M from human serum was kindly provided by Claus Oxvig, Aarhus University. Aprotinin (Trasylol, 20,000 KIE/ml) was purchased from Bayer. Fetal calf serum (FCS) was from Gibco.

Hepatocyte cell lines

The cell lines HepG2 and Chang liver cells were purchased from LGC Promochem, Boras, Sweden (American Type Culture Collection (ATCC) cat. no. HB-8065 and CCL-13, respectively), while HuH1 and HLF were obtained from Health Science Research Resources Bank, Sennan-shi, Japan (cat. no. JCRB0199 and JCRB0405, respectively). The cells were grown in 10 cm petri dishes treated for tissue-culture (Greiner Bio-One) in DMEM with GlutaMAX, glucose and Hepes (Gibco), supplemented with penicillin, streptomycin and 10% heat-inactivated fetal calf serum (Gibco).

RT-PCR

Total RNA was purified using Trizol (Invitrogen) or a combined Trizol/Column purification procedure. Briefly, for the latter, cells were lysed in Trizol, phase-separated by mixing with 1-bromo-3-chloropropane, and the aqueous phase was loaded onto spin columns (Qiagen). The procedure for the RNeasy mini spin kit was then followed, including an on-column DNAse I digestion step (Qiagen). RNA yields were estimated using a NanoDrop 1000 spectrophotometer and cDNA synthesis was performed using iScript cDNA synthesis kit (BioRad). PCR was performed using RedTaq Readymix (Invitrogen) with the primers listed in Supplementary Table 1, followed by agarose gel analysis.

Extraction of intracellular protein

Transfected 293F cells were pelleted and solubilised by incubation end over end for 30 min at 4 °C in ice-cold Triton X-100 lysis buffer (PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4), containing 1% v/v Triton X-100 (Sigma–Aldrich) and Roche CompleteMini protease inhibitor), then centrifuged 30 min, 10,000*g* at 4 °C.

SDS–PAGE and Western blotting

Samples were run on XT-Criterion 4–12% gradient Bis-Tris polyacrylamide gels (Bio-Rad) in XT-MOPS running buffer (Bio-Rad) either reduced or non-reduced as indicated. Precision All Blue pre-stained markers (Bio-Rad) were used for the estimation of molecular sizes. The proteins in the gels were blotted onto Hybond-ECL or Hybond-PVDF membrane (GE Healthcare) in transfer buffer (25 mM Tris, 0.192 M glycine, 20% v/v ethanol, and 0.1% w/v SDS (pH 8.3)), the membrane was blocked in 0.1% Tween 20 in TBS (10 mM Tris, 140 mM NaCl, 15 mM NaN_3, pH 7.4,), and then incubated with primary Ab in primary buffer: TBS/Tw (TBS with 0.05% w/v Tween 20) with 1 mM EDTA, 1 mg of human serum albumin (HSA, CSL Behring)/ml, and 100 μg of normal human IgG (Beriglobin; CSL Behring)/ml. The blots were washed, incubated with HRP-labeled secondary Ab in secondary buffer (TBS/Tw, no azide, 1 mM EDTA, and 100 μg of human IgG/ml), and washed again before being developed with Super-Signal West Dura Extended Duration Substrate (Pierce). Images were taken using a