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Dissociation and re-association studies on the interaction domains of mannan-binding lectin (MBL)-associated serine proteases, MASP-1 and MASP-2, provide evidence for heterodimer formation

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ABSTRACT

Activation of the lectin pathway of complement begins with the activation of mannan-binding lectin (MBL)-associated serine proteases, MASP-1 and MASP-2, which are bound to the recognition molecules, MBL and ficolins. MASPs are Ca^{2+}-dependent dimers. Dimerization and Ca^{2+}-dependent association with the recognition molecules occurs via the first 3 domains, the CUB1-EGF-CUB2 region. The CUB1-EGF-CUB2 (D1-3) regions of MASP-1 and MASP-2, and also their tagged versions, were expressed in *E. coli*, refolded and purified. The first three domains of MASP-1 are identical with the respective regions of MASP-3 and MAp44, which are also associated with MBL and ficolins. The functionality of the fragments was checked by inhibition of C3 deposition from human serum. Time-course of the dissociation and re-association was examined by size exclusion chromatography. Both refolded proteins are tight Ca^{2+}-dependent dimers, as expected. In buffer containing EDTA MASP-1_D1-3 dissociated to monomers, however it took about 1 h to reach an equilibrium. Upon re-calcification dimers were re-formed, but this process was even slower; only after overnight incubation was the dimerization completed. MASP-2_D1-3 showed a somewhat different behavior: dissociation by EDTA was even slower, less complete, and higher MW aggregates also appeared. Heterodimer formation was detected by native PAGE. As modeled by the D1-3 fragments, MASP-1 and MASP-2 can readily form heterodimers after dissociation and re-association, however, in the presence of Ca^{2+} exchange of subunits is slow between the homodimers. MASP-1:MASP-3 heterodimer formation was modeled by the tagged and untagged D1-3 fragments, and data indicate that subunits of these proteins are readily exchanged even in the presence of Ca^{2+}. The existence of heterodimers influences the current view on the composition of lectin pathway complexes and their activation.

1. Introduction

Complement is a vital effector arm of the immune system. It can be activated via 3 routes: the classical, the alternative and the lectin pathways. The 3 routes merge into a common terminal pathway, and as a result, the complement system can lead to elimination of invading microorganisms or altered self structures by direct lysis or opsonization (Ricklin et al., 2010). The classical and the lectin pathways share many common features. The serine protease components are bound to the oligomeric recognition molecules, which are composed of collagen-like triple helices and head domains. The head domains recognize the activating surface , typically antibody–antigen complexes for the classical pathway, and carbohydrate arrays on microorganisms for the lectin pathway (Wallis et al., 2010; Gál et al., 2009).

The exact structure and composition of the lectin pathway activating complexes are still enigmatic. The recognition molecules, mannan-binding lectin (MBL[4]), H-, L-, and M-ficolins, collectin K-1 (CL-K1), are made of a different number of the basic trimeric unit. For example, MBL the most frequent oligomeric forms in the blood are the trimers and the tetramers of the basic unit, but dimers and

Abbreviations: MBL, mannan-binding lectin; MASP, MBL-associated serine protease; MAp, MBL-associated protein; CUB, C1r/C1s-Uegf-BMP domain; EGF, epidermal growth factor domain; CCP, complement control protein domain; SP, serine protease domain; TRIFMA, time-resolved immunofluorimetric assay; CBD, chitin-binding domain; IC_{50}, 50% inhibitory concentration; SEC, size exclusion chromatography; TBS, Tris-buffered saline; GuHCl, guanidine-hydrochloride; SPR, surface plasmon resonance; PMSF, phenylmethanesulfonyl fluoride; AUC, sedimentation velocity analytical ultra centrifugation.

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higher oligomers also exist. It was proposed (Chen and Wallis, 2001) that especially to the higher oligomeric forms not just one, but two homodimers of the associated proteases and proteins can bind.

The MBL-associated serine proteases (MASP-1, MASP-2, MASP-3), and MBL-associated proteins (MAp19, and MAp44) are the products of two genes. MASP-1, MASP-3 and MAp44 (also known as MAP-1) are alternative splice products of the *MASP1* gene, whereas MASP-2 and MAp19 (also known as sMAP) are those of the *MASP2* gene (Dahl et al., 2001; Degn et al., 2009; Skjoedt et al., 2010; Takahashi et al., 1999; Stover et al., 1999). MASPs are composed of 5 regulatory domains (CUB1-EGF-CUB2-CCP1-CCP2) and a serine protease (SP) domain. The regulatory regions of MASP-1 and MASP-3 are identical, as they are derived from the same gene, only their SP domains are different (Dahl et al., 2001). MAp44 also shares its first four domains with MASP-1 and MASP-3 and contains only an additional short peptide (Degn et al., 2009; Skjoedt et al., 2010). Crystal structure of the CUB1-EGF-CUB2 fragment of MASP-1/3 clearly shows a head to tail arrangement (Teillet et al., 2008) and Ca^{2+} ions bound to all three domains. It is the CUB1-EGF region, which is responsible for the Ca^{2+}-dependent dimerization, but all three domains (CUB1-EGF-CUB2) are involved in the Ca^{2+}-dependent association with the recognition molecules (Wallis and Dodd, 2000; Thielens et al., 2001).

MASP dimers and MBL-MASP complexes exhibit an unusual behavior upon addition of EDTA. It was shown that MBL-MASP complexes dissociate only in the presence of EDTA and 1 M NaCl, while at physiological salt concentration EDTA alone is not sufficient to disrupt the interaction between MBL and MASPs, hence the MBL-MASP complexes were regarded Ca^{2+}-independent (Tan et al., 1996; Thiel et al., 2000). On the other hand, surface plasmon resonance (SPR) measurements and binding assays showed no interaction in EDTA-containing buffer between MBL and MASPs or their fragments (Wallis and Dodd, 2000; Thielens et al., 2001).

Sedimentation studies with the CUB1-EGF fragment of MASP-1 and MASP-2 showed dimers both in the presence of Ca^{2+}, or in the presence of EDTA (Thielens et al., 2001). The conclusion was that although dimer formation is Ca^{2+}-dependent for both MASP-1 and MASP-2, the Ca^{2+} ions are not accessible to EDTA. They could not detect, however, any interaction between MASP-1 and MASP-2, and concluded that both MASP-1 and MASP-2 form separate homodimers.

It has been shown recently that heterocomplexes, i.e. MBL-MASP complexes containing two kinds of MASP, are possible both in cell culture and in serum (Degn et al., 2013). When MASP-1 and -2 were co-expressed heterodimers were detected by an ELISA-type immunological (TRIFMA) assay, however the extent of heterodimer formation was not quantified (Degn et al., 2013). Degn et al. (2013) also tried to detect heterodimers from serum, but oddly they performed the immunological assay in the presence of EDTA and 1 M NaCl. They concluded that although complexes containing MBL and both MASP-1 and -2 do exist in circulation, heterodimers are not present, rather the heterocomplexes contain two different homodimers. Studies are in progress by others (Rosbjerg et al., 2013) in an attempt to detect heterodimers between MASP-1, MASP-3, and MAp44. Nevertheless heterodimers between these three proteins should not pose any structural problem since their interaction domains are identical. On the other hand, heterodimers between MASP-1 and MASP-2 could theoretically be prohibitive if the interaction surfaces are incompatible. The related complement protease C1s of the classical pathway can form homodimers, but it preferably interacts with C1r forming the C1s-C1r-C1r-C1s heterotetramer, so a similar scenario is not unprecedented. An important difference between the classical and the lectin pathway, however, is that tetramer formation is not possible for the lectin pathway proteases, since neither MASP-1 nor MASP-2 is able to dimerize through their catalytic region unlike C1r does (Gál et al., 2009).

In an attempt to clarify the dimerization properties of MASPs we expressed the N-terminal 3 domains of both MASP-1 and MASP-2 in *E. coli*, then refolded and purified the proteins to produce sufficient amounts of pure material. Here, we provide semi-quantitative data on their dissociation properties in the presence of EDTA, and also on their re-association rates upon re-calcification using size exclusion chromatography (SEC). We also provide direct evidence using native PAGE that heterodimer formation between MASP-1 and MASP-2 is structurally allowed, moreover they form dimers just as favorably with each other as with themselves. On the other hand, exchange studies in the presence of Ca^{2+} indicate that there is only minimal exchange of monomeric subunits between pre-formed MASP-2 and MASP-1 dimers even after prolonged incubation. Modeling the possible exchange reaction between MASP-1 and MASP-3 resulted in a different outcome: here subunit exchange was significant in the time-frame of a few days.

2. Materials and methods

2.1. Recombinant MASP-1 and MASP-2 fragments: expression and purification

The chitin-binding domain (CBD)-tagged human MAPS-1 CUB1-EGF-CUB2 (briefly MASP-1_D1-3_CBD) and the CBD-tagged human MAPS-2 CUB1-EGF-CUB2 (briefly MASP-2_D1-3_CBD) encoding DNAs were ordered as a synthetic genes from Entelechon GmbH (Regensburg, Germany). The coding sequences were codon optimized for expression in *E. coli* with the Leto software by the manufacturer. The gene sequences and translation are provided as a supplement (supplementary Table S1). The NdeI-XhoI fragments of the synthetic genes were subcloned into the pET17b (Novagen) vector that was cleaved with the same enzymes. The resulting plasmids are named pET17b-MASP-1_D1-3_CBD, and pET17b-MASP-2_D1-3_CBD. The synthetic genes were constructed so that cleavage with EcoRV and HpaI followed by re-ligation removes the linker region and the CBD encoding region and creates a stop codon at the end of the gene. The resulting plasmids pET17b-MASP-1_D1-3, and pET17b-MASP-2_D1-3 will produce the tag-free native sequences of the CUB1-EGF-CUB2 domains plus a Gly at the C-terminus (supplementary Table S1).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.12.003.

Proteins were expressed in *E. coli* BL21 (DE3) pLysS (Novagen), and inclusion bodies were prepared using standard procedures as described (Ambrus et al., 2003). Inclusion bodies were dissolved in solubilization buffer (7 M GuHCl, 50 mM Tris, 50 mM DTT, pH 8.0) in a minimal volume sufficient to dissolve all protein. The concentrations of the expressed proteins were estimated by SDS-PAGE analysis. The dissolved proteins were further diluted with solubilization buffer to final concentrations of 5 mg/mL. Proteins were refolded by diluting 20 mL of the 5 mg/mL solution into 1 L of 0.75 M Arg, 200 mM $CaCl_2$, 3 mM glutathione, 2 mM oxidized glutathione, pH 8.0 refolding buffer. After (at least) a week at 4 °C each refolding mixture was dialyzed against 10 L of water, then 10 L of 10 mM Tris–HCl, 10 mM $CaCl_2$ at 4 °C. Following filtration on 0.22 μm membranes samples were applied to a Source 30Q (GE Healthcare) 16 mm × 100 mm column and eluted with a linear gradient of 0–300 mM NaCl, in 20 mM Tris–HCl, pH 8.0 buffer containing 10 mM $CaCl_2$. Fractions containing the desired protein were concentrated and applied to a 16 mm × 900 mm Superdex 200 (GE Healthcare) column equilibrated with TBS-Ca buffer (150 mM NaCl, 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0) and eluted with the same buffer. Fractions containing the desired fragment of MASP-1 or MASP-2 were combined and concentrated on 10 kDa