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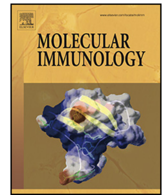

A recombinant two-module form of human properdin is an inhibitor of the complement alternative pathway

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ABSTRACT

Properdin upregulates the alternative complement pathway by binding and stabilising the C3 convertase complex (C3bBb). Properdin is a soluble glycoprotein and its flexible rod-like 53 kDa monomers form cyclic polymers (dimers, trimers, tetramers and pentamers). The properdin monomer consists of seven thrombospondin type I repeats (TSR 0–6), which are similar and homologous to domains found in circumsporozoite and thrombospondin-related anonymous proteins of *Plasmodium* species, ETP100 of *Eimeria tenella*, various complement components C6-C9, and thrombospondin I and II. Using deletion constructs, TSR4 and TSR5 of human properdin were implicated in C3b binding and stabilising C3 convertase. However, individually expressed TSR4 or TSR5 failed to bind properdin ligands. Here, we have expressed and characterized biologically active TSR4 and TSR5 together (TSR4 + 5) in tandem in *Escherichia coli*, fused to maltose-binding protein. MBP-TSR4 + 5 bind solid-phase C3b, sulfatides and glycosaminoglycans. In addition, functionally active recombinant TSR4 + 5 modules inhibit the alternative pathway of complement.

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1. Introduction

The alternative pathway of the complement system is activated by surface features of microorganisms and can be triggered by antibody-antigen complexes of IgG or IgA (Carroll and Sim, 2011). A current explanation for activation of the alternative pathway is that C3 undergoes hydrolysis, followed by a conformational change, to a form called C3(H_2O). This then binds factor B (FB). FB in the complex is cleaved by a serine protease Factor D (FD) forming Ba and Bb fragments. Bb remains bound in a C3(H_2O) Bb complex, which itself is a protease, called a C3 convertase, which cleaves C3 at a single site (Carroll and Sim, 2011). This leads to the generation of C3a and C3b, the latter binding to surfaces of target cells through a reactive thioester moiety. The surface bound C3b binds FB, which is again cleaved by FD to form a surface-bound C3 convertase (C3bBb), which is unstable with a half-life of about 90 s. Properdin binds and stabilises the C3bBb complex, increasing its half-life by 5–10 fold (Pillemer et al., 1954; Le et al., 2007). Further C3b is generated in an amplification loop. A C3b molecule binding directly to the convertase forms the complex $C3b_2Bb$, which acts as a C5 convertase, also stabilised by properdin, leading to assembly of the C5b-9 membrane attack complex (MAC) and cell lysis. Properdin is, therefore, a positive regulator of the alternative pathway.

In addition to liver, spleen and bone marrow (Ponten et al., 2008), properdin is synthesised by neutrophils (Camous et al., 2011), monocytes (Whaley, 1980), dendritic cells (Reis et al., 2007), endothelial cells (Bongrazio et al., 2003), T cells and a bone marrow progenitor cell line reviewed in (Kouser et al., 2013). Properdin mRNA expression was undetectable in HEP G2 cells derived from human hepatocytes (Schwaeble et al., 1993). Properdin can be released from intracellular stores of neutrophils upon stimulation by *N*-formyl-methionine-leucine-phenylalanine (fMLP) or tumor

Abbreviations: GAG, glycosaminoglycan; CSA, chondroitin sulphate A; TSR, thrombospondin type I repeat; MAC, membrane attack complex; PBS, phosphate buffered saline; *E. coli*, *Escherichia coli*; fMLP, N-formyl-methionine-leucine-phenylalanine; TNF-α, tumor necrosis factor alpha; MBP, maltose binding protein; FD, complement factor D; FB, complement factor B; EDTA, Ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; β-ME, 2-Mercaptoethanol.

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necrosis factor-alpha (TNF-α) (Camous et al., 2011; Wirthmueller et al., 1997). Properdin recognises apoptotic T cells via sulfated glycosaminoglycans (GAGs) and promotes phagocytosis and removal of apoptotic T cells by macrophages (Kemper and Hourcade, 2008; Xu et al., 2008; Ferreira et al., 2010). Heparin is thought to interact with properdin as well as with factor H. However, binding kinetics and dissociation constants (*K*d) are quite dissimilar (Yu et al., 2005). Properdin has also been shown to bind to sulfated glycoconjugates, including dextran sulfate and fucoidan (Holt et al., 1990).

Properdin is a highly positively charged (pI > 9.5) glycoprotein (Fearon and Austen, 1975) and present in plasma at a concentration of about 25 μg/ml. It is an oligomeric protein composed of identical 53 kDa subunits, each 26 nm long and 2.5 nm diameter (Camous et al., 2011) that bind to each other in a head-to-tail orientation (DiScipio, 1981; Nolan et al., 1992) and form cyclic dimers, trimers, tetramers and pentamers (Alcorlo et al., 2013; Smith et al., 1984). The monomer is a flexible rod-like structure, 442 amino acids long and composed of seven non-identical tandem repeats of about 60 amino acids called thrombospondin type I (TSR) repeats (Goundis and Reid, 1988; Higgins et al., 1995). TSRs are also found in cell adhesion molecules, called thrombospondins, in human blood platelets. Alignments of properdin and thrombospondin sequences show a 46% similarity of the first 26 amino acids to a section of malaria circumsporozoite (CS) protein (Goundis and Reid, 1988).

Initially, each monomer of properdin was thought to have five and a half TSR modules, each having 58–67 residues with additional N-terminal and C-terminal domains (Sun et al., 2004). However, further sequence alignments have indicated that there is a sixth TSR module, which is larger than the rest due to a sequence insertion. Thus, properdin is considered to contain seven TSRs, as the N-terminal module (TSR-0, a truncated region) has the same six conserved Cys residues as in TSR1–TSR6 (Sun et al., 2004). The human properdin gene comprises 10 exons spread over ~6 kb of genomic DNA (Nolan et al., 1992). The first exon contains the promoter region and encodes part of the 5′ untranslated region, while the second exon encodes the translation start site as well as 24 amino acids of signal peptide. The N-terminal region (TSR-0) is encoded by exon 3. Exons 4–8 encode TSRs 1–5. Exon 9 encodes the first 38 amino acids of TSR6, while the C-terminal parts of TSR6 is encoded by exon 10 (Nolan et al., 1992; Higgins et al., 1995).

Properdin purified from human serum was reported to bind to *Neisseria gonorrhoeae*, allowing the C3 convertase (C3bBbP) complex to form, which leads to opsonization of bacteria (Agarwal et al., 2010). However, it has been suggested that purified properdin undergoing freeze thawing forms higher order oligomers of properdin, which may bind surfaces that native properdin would not (Farries et al., 1987). Agarwal et al. found that properdin does not bind directly to *Neisseria meningitidis* or *N. gonorrhoeae* but enhances the deposition of C3 on the bacterial surface by stabilizing the alternative pathway C3 convertase (Agarwal et al., 2010). Another report, however, has shown that native properdin (dimer, trimer, and tetramer) binds to *Chlamydia pneumoniae* and enhances C3b deposition and alternative pathway activation (Cortes et al., 2011). It has recently been reported that a higher polymeric recombinant form of properdin expressed in Chinese hamster ovary cells, can augment the alternative pathway activity and can be used therapeutically to drastically reduce morbidity and mortality in experimental mouse models of *N. meningitidis* and *Streptococcus pneumoniae* infection (Ali et al., 2014).

A domain deletion study has demonstrated that properdin lacking TSR4 forms dimers and binds to C3b, but not to C3bBb. Properdin without TSR5 does not bind to C3b, and thus for both deletions, the C3bBb complex is not stabilized (Higgins et al., 1995). Properdin lacking TSR3 is still able to form oligomers (dimers, trimers, and tetramers) as well as monomers. This deletion does not prevent properdin from binding C3b and the C3bBb complex. TSR4 and TSR5, therefore, appear to play an important role in the binding of properdin to C3b, sulfatides (sulfated galactosylceramides), and in the stabilization of C3 convertase of the alternative pathway. TSR5 is involved in the binding of sulfated glycoconjugates but the binding region for C3bBb is distinct (Perdikoulis et al., 2001). Deletion of TSR6 and the short C-terminal charged region prevents the formation of oligomers (Higgins et al., 1995).

Here, we have expressed in *Escherichia coli* TSR4 + 5 modules together and examined their interaction with C3b, sulfatides, chondroitin sulphate A and heparin. We show that the two-module recombinant protein is biologically active and that the TSR4 and TSR5 modules are involved in the binding of C3b and therefore in stabilising the C3 convertase by properdin. We also show that the double module acts as an inhibitor of the complement alternative pathway.

2. Materials and methods

All reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

2.1. Cloning TSR4 + 5 in pMAL-c

TSR4 + 5 DNA sequences were sub-cloned into pMAL-c plasmid (New England Biolabs, MA, USA) downstream to *E. coli* maltose-binding protein (MBP) under the P_{tac} promoter (di Guan et al., 1988; Maina et al., 1988). The TSR4 + 5 were PCR-amplified at 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s, 35 cycles using TSR4 forward primer (FP) 5′ GCCCGGGAATTCGTGGCTGGGGGCTGGG 3′ and TSR5 reverse primer (RP) 5′ GCCAAGCTTCTAAGGGGCAGTGCTGGATG 3′ using cDNA clone of human properdin isolated from PMA-stimulated U937 cells (Higgins et al., 1995; Nolan et al., 1991). The PCR product containing TSR4 + 5 was purified using Peqlab cycle pure kit by applying it to a PerfectBind DNA column (Peqlab) to remove any dNTPs. The PCR product was cleaved using engineered sites for EcoRI and HindIII, as was the pMAL-c vector. The EcoRI—HindIII double-digested pMAL-c vector and the PCR fragment were ligated using 10 × T4 ligase buffer and T4 ligase (New England Biolabs) in a 20 μl reaction volume using D.H_20 (molecular biology grade; Fisher scientific, catalogue no. BP2819-100). The ligation mix was then used to transform TOP10 *E. coli* competent cells (Life Technologies, Paisley, UK, catalogue no. 10368022). The ampicillin-resistant *E. coli* colonies were screened for the recombinant construct via double-digestion with EcoRI and HindIII that generated a fragment of 367 base pairs detected via electrophoresis on a 0.7% agarose gel.

2.2. Expression in E. coli and purification of the recombinant modules as MBP fusion proteins

The recombinant proteins fused with MBP were expressed as described in (Perdikoulis et al., 2001) using *E. coli* BL21 strain (Life Technologies, catalogue no. C601003). The bacterial cells were grown in Luria-Bertani medium (1 l) containing 100 μg/ml ampicillin with shaking at 37 °C until an A_{600} of 0.6 was reached. Cells were then induced with 0.4 mM isopropyl beta-D-thiogalactoside (IPTG) (Sigma, Poole, UK), and left for 3 h shaking (200 rpm) at 37 °C. The cells were then centrifuged at 4500 rpm at 4 °C for 10 min. The cell pellet was suspended in 50 ml lysis buffer [20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 0.25% v/v Tween 20, 5% v/v glycerol, 100 μg/ml lysozyme (Sigma, Poole, UK), and 0.1 mM Phenylmethanesulfonyl fluoride (Sigma, Poole, UK)] and incubated for 1 h at 4 °C on a rotary shaker. The cell lysate was then sonicated (using a Soniprep 150; MSE, London, UK) at 60 Hz for 30 s with an interval of 2 min (12 cycles). The sonicate was centrifuged at 13000 rpm for 15 min at 4 °C. The supernatant (50 ml) was collected

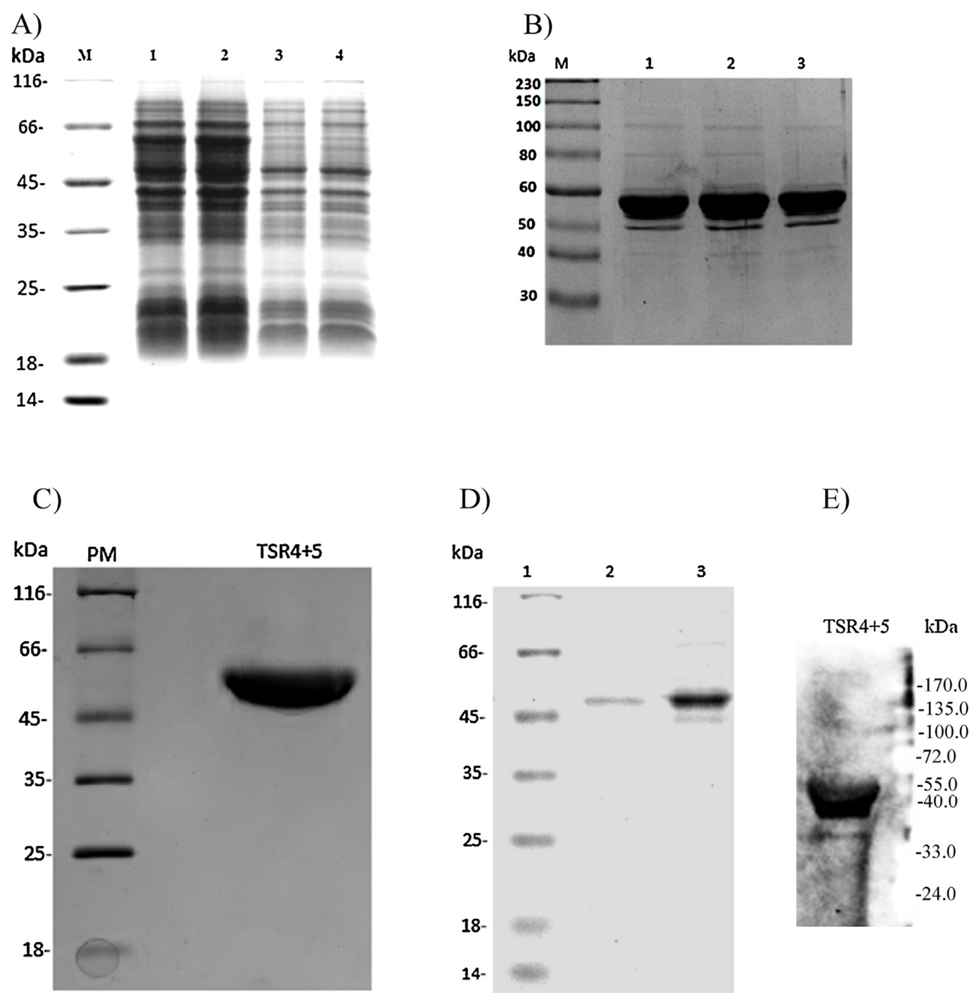

Fig. 1. Expression and purification of MBP-TSR4 + 5 (a) 12% acrylamide SDS-PAGE, under reducing conditions, Lane M: standard protein marker, lane 1 and 2 *E. coli* lysate containing MBP-TSR4 + 5 expression vector induced with 0.4 mM IPTG; lane 3 and 4 same *E. coli* culture before induction with IPTG. A difference between induced and un-induced sample can be observed. The induced MBP-TSR4 + 5 band is evident at ~55 kDa. The bands were visible after staining with comassie blue and then destained to remove exessive stain, as described under methods and Materials. (b) 12% SDS-PAGE, showing amylose-affinity purified MBP-TSR4 + 5. Protein marker, lane M; lanes 1–3 show successive elution fractions of MBP fused TSR4 + 5 protein purification after amylose resin purification step eluted with 10 mM maltose. (c) SDS-PAGE showing purified MBP-TSR4 + 5 single band at ~55 kDa after elution from a Q Sepharose ion exchange column; (d) individual MBP-TSR4 and MBP-TSR5 modules as reported (Perdikoulis et al., 2001). Lane 2 shows elution of purified MBP-TSR4 and lane 3 shows MBP-TSR5. Both bands appear at about 48 kDa (e) Western Blot of MBP-TSR4 + 5 using anti- human properdin (polyclonal) antibody showing a band at ~55 kDa.

and diluted 5-fold with buffer I (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.25% v/v Tween 20), and passed through an amylose resin column (New England Biolabs, catalogue no E8021L), previously equilibrated in buffer I. The affinity column was washed with buffer I without Tween 20 and with 1 M NaCl, followed by buffer II (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). The MBP-TSR fusion protein was eluted with 100 ml of buffer II containing 10 mM maltose (Sigma, Poole, UK) (elution buffer). Minor contaminants were further removed by applying the fusion protein to a Q-Sepharose column (Sigma, catalogue Q1126). The affinity purified fusion protein in elution buffer was applied to the ion-exchange (5 ml bed) column and washed with 3 column volumes of low salt buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, pH 7.5. After extensive washing with low salt buffer, the fusion protein eluted at 0.2 M NaCl using an NaCl gradient (50 mM–1 M).

2.3. Purification of native properdin

Properdin was purified from human plasma (TCS Biosciences, catalogue no. PR100) using a method described earlier (Sun et al., 2004). Sodium EDTA, pH 7.4 was added to 1 l of plasma to a final concentration of 5 mM. Plasma was filtered by Whatman filter paper to remove cell debris, particles and lipids. Plasma (in batches of 200 ml) was then passed through an IgG-Sepharose column (10 ml bed volume) to deplete serum of C1q, as described by Tan et al. (2010). The column was made from human non-immune IgG (~26 mg IgG per ml of Sepharose) coupled to CNBr-activated Sepharose (GE Healthcare, UK). An anti-human properdin antibody column (8 ml) was set up by coupling mouse monoclonal antibody 2 mg/ml of Sepharose; (Perdikoulis et al., 2001) raised against human properdin to CNBr-activated Sepharose. The antibody column was washed with 3 column volumes of HEPES buffer (10 mM HEPES, 140 mM NaCl, 0.5 mM EDTA, pH 7.4). Plasma devoid of C1q was then applied to the monoclonal anti-properdin column and washed through with the same HEPES buffer. Bound properdin was eluted with 3 M $MgCl_2$. The peak eluted fractions were dialysed against HEPES buffer overnight at 4 °C. Minor contaminants were further removed by applying the pooled fractions to a HiTrap Q FFSepharose (GE Healthcare) ion exchange column. The column (5 ml) was washed with 3 column volumes of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA. Properdin did not bind to the

Q Sepharose column and appeared in the flow-through free from contaminants. SDS-PAGE analysis was carried out to confirm the presence of properdin and confirm its molecular size (53 kDa).

2.4. SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli (1970) to analyse and detect plasma properdin, recombinant MBP-TSR4 + 5, or individual recombinant MBP-TSR4 or MBP-TSR5. Properdin, MBP-TSR4 + 5, MBP-TSR4, or MBP-TSR5 were diluted in 1:1 v/v ratio in 2 × Laemmli sample buffer (Bio-Rad, Hertfordshire, catalogue no. 161-0737) containing β-ME (Bio-Rad, Hertfordshire, UK catalogue no. 161-0710). The proteins were then denatured for 10 min at 95 °C before loading onto SDS-PAGE. Standard protein marker (Peqlab, Leicestershire UK, catalogue no. 271010) was also loaded to assess the size of the native and recombinant proteins. The SDS-PAGE was stained using staining solution (1 g of brilliant blue, 50% v/v methanol, 10% v/v acetic acid, 40 ml d.H_2O), and left overnight on a rotary shaker. The gel was then de-stained using a de-staining solution (40% v/v methanol, 10% v/v acetic acid) for 1 h on rotary shaker or until the protein bands were visible.

2.5. Western blotting

20 μl of 1 mg/ml MBP-TSR4 + 5 was subjected to SDS-PAGE. The gel was run until the dye front reached the end of the gel. Then protein bands were transferred on to a nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM Glycine, 20% v/v methanol, pH ~8.3) at 320 mA for 2 h. The membrane was incubated in blocking solution made up of 5% semi-skimmed milk powder (Tesco, UK) in PBS, pH 7.4 (Sigma, Poole, UK, catalogue no. P4417), in order to reduce non-specific binding, overnight at 4 °C. The membrane was then washed in PBS + Tween 20 (0.05%), pH 7.4 (PBST) three times for 10 min each. Anti- human properdin (0.92 mg IgG/ml) (polyclonal) antibodies (Goundis and Reid, 1988; di Guan et al., 1988) were diluted 1:2500 (Higgins et al., 1995; Perdikoulis et al., 2001) in PBST and incubated with the membrane for 1 h at room temperature. The membrane was washed again with PBST as above. The secondary probe, Protein A-HRP (Thermo Scientific, Massachusetts, USA, catalogue no. 32400) was added to the blot at 1:5000 dilution in PBST and incubated for 1 h at room temperature. The bands were detected using Enhanced chemiluminescence reagent (ECL) (Bio-Rad, catalogue no. 17050605).

2.6. C3b binding assay

Maxisorp 96-well plates (Fisher scientific, catalogue no. 10450343) were coated with 5 μg/ml, 100 μl/well, C3b purified as described in ref. (Micklem et al., 1984) in 0.05 M sodium carbonate/bicarbonate, pH 9.6 (Sigma catalogue no. C3041), and left overnight at 4 °C. After washing 3 times with PBST, the wells were blocked with 2% BSA in PBS for 2 h at 37 °C. Serial two-fold dilutions (100 μl/well) of human properdin (starting at 25 μg/ml) and MBP-TSR4 + 5 (6 μg/ml) were added to wells and incubated for 2 h at 37 °C. Single module recombinant proteins (25 μg/ml of MBP-TSR4 or MBP-TSR5) were used as controls, and MBP (20 μg/ml) was also used as a negative control (same controls were used for all binding assays; see below). The wells were washed three times with PBST and polyclonal rabbit anti-human properdin (0.92 mg IgG/ml) (Higgins et al., 1995; Perdikoulis et al., 2001) (1:2500 in PBST) was added to the wells coated with properdin, whereas mouse anti-MBP monoclonal antibody (Sigma, Poole, UK, catalogue no. M6295) (1:5000 in PBST) was added to the wells containing TSRs. The plate was incubated for 1 h at 37 °C, washed again 3 times with PBST, and then incubated with Protein A—HRP (1:5000) conjugate in PBST for probing anti-human properdin primary antibody, and anti-mouse IgG HRP (1:5000) (Promega, Southampton, UK, catalogue no W4021) for probing anti-MBP monoclonal antibody. Colour was developed by adding o-phenylenediamine dihydrochloride (OPD) (Sigma, Poole, UK, catalogue no. P8287) and read at 415 nm using iMark Microplate Absorbance Reader (Bio Rad, Hertfordshire, UK, catalogue no. 168-1130).

2.7. Sulfatide binding assay

Sulfatides (from bovine brain; Sigma, Poole, UK, catalogue no. S1006) were dissolved in methanol/chloroform in a ratio 95:5 (v/v) to a concentration of 5 mg/ml. 10 μg in 100 μl of methanol/chloroform was placed in each well of a maxisorp 96-well plate, air dried at room temperature for 2 h, and blocked overnight at 4 °C with 2% BSA in PBS. Wells were washed three times with PBS and then 100 μl of serial two-fold dilutions of properdin (starting concentration 25 μg/ml) or MBP-TSR4 + 5 in PBS (starting concentration 6 μg/ml) were placed in wells and left at 37 °C for 2 h. Wells were washed again three times with PBS and bound proteins were detected with either rabbit anti-human properdin polyclonal antibody (1:2500 in PBS) for properdin, or anti-MBP monoclonal antibody (1:5000 dilution in PBS) for MBP-TSR4 + 5. Secondary conjugates and substrate were used as described above.

2.8. Heparin binding assay

Maxisorp 96-well plates were coated with 10 μg/ml of heparin purified as described in ref. (Clark et al., 2006) in carbonate/bicarbonate buffer, pH 9.6 (Sigma, catalogue no. C3041) and left at 4 °C overnight. The plate was washed and the wells were blocked as described for the C3b assay above. Properdin and MBP-TSR4 + 5 binding was tested as above, except that colour was developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) (Biolegends, London, UK, catalogue no. 421101) and read at 450 nm.

2.9. Chondroitin sulfate A (CSA) binding assay

Microtitre wells of a maxisorp 96-well plate were coated with 10 μg/ml of CSA (Sigma, Poole, UK, catalogue no. C8529) using carbonate/bicarbonate buffer, pH 9.6 at 4 °C, overnight. Assay of properdin and MBP-TSR4 + 5 binding was done as described for heparin above.

2.10. Properdin vs TSR4 + 5 competitive binding assay

Maxisorp 96-well plates were coated with C3b or sulfatide or CSA or heparin as above (see binding assays), blocked with 2% BSA in PBS, and washed three times with PBST (or PBS for sulfatide assays). Serially diluted MBP-TSR4 + 5 (starting at 6 μg/ml), 100 μl/well, and a constant amount of properdin (25 μg/ml) 100 μl/well (in PBS) was added to each well, and incubated at 37 °C for 1 h and then at 4 °C for 1 h. Wells were washed again three times with PBST or PBS, incubated with primary antibody monoclonal (mouse) anti-human properdin (1.19 mg/ml) (1:2500 dilution), probed with secondary antibody anti-mouse IgG HRP in PBS (1:5000 dilution), and colour developed using TMB. Positive control was binding of constant properdin (25 μg/ml) carried out as above. MBP (20 μg/ml) was also used together with constant properdin (25 μg/ml) as control. Monoclonal anti-properdin antibody does not detect MBP-TSR4 or MBP-TSR5, however polyclonal anti-properdin is able to recognise MBP-TSR4 + 5.

2.11. Assay of erythrocyte lysis by the alternative pathway

Red blood cells from rabbit blood (TCS Biosciences Ltd., Buckingham, UK, catalogue no. RB053) in Alsever's solution were washed

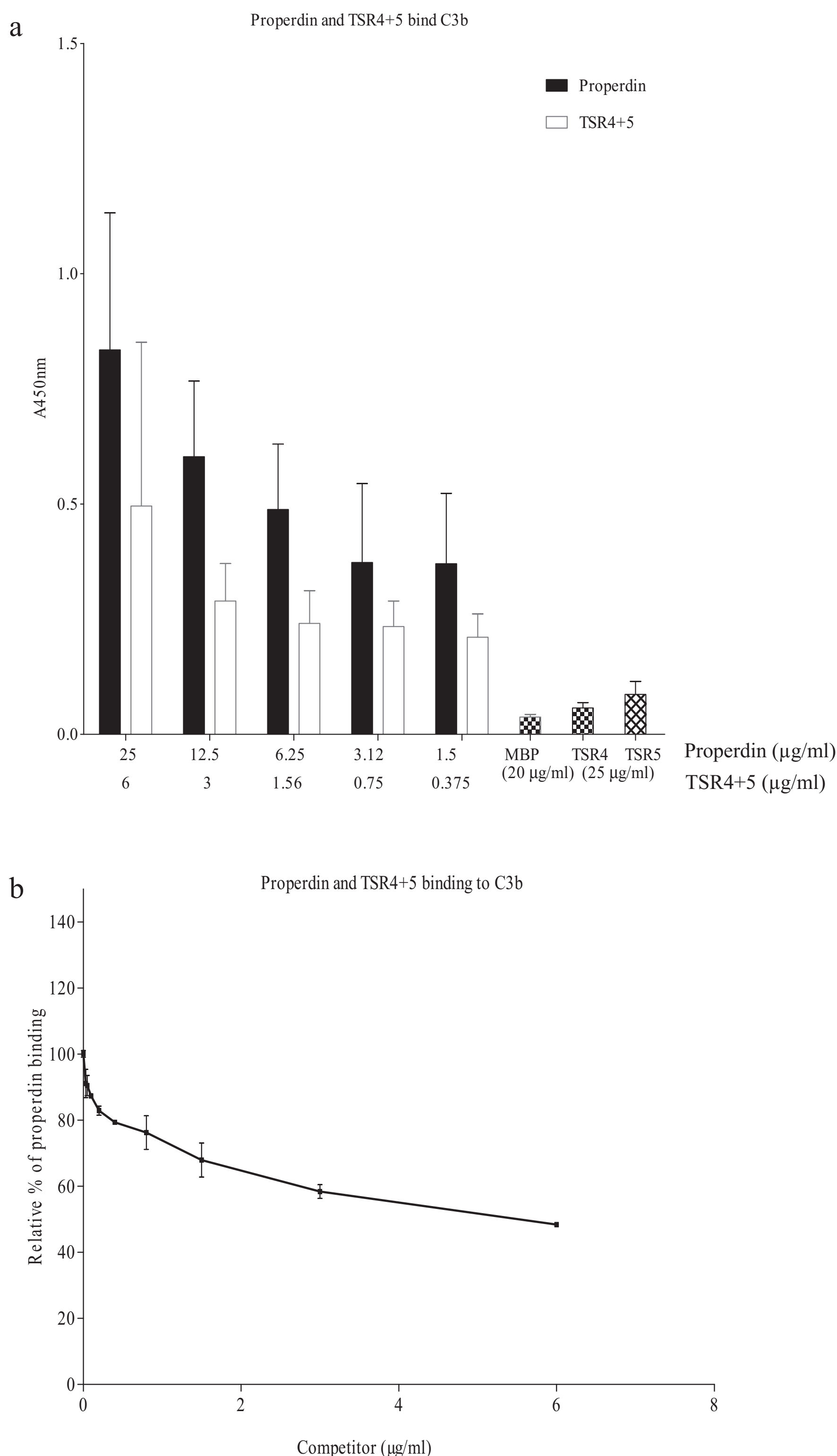

Fig. 2. (a). ELISA to examine the interaction of MBP-TSR4 + 5 with C3b. The ELISA shows binding of properdin and MBP-TSR4 + 5 to C3b. Properdin and MBP-TSR4 + 5 showed a dose dependent binding to C3b coated to maxisorp 96-well plates. MBP-TSR4 + 5 modules bound C3b whereas the individual TSRs (25 μg/ml) did not show significant binding to C3b, and neither did MBP (20 μg/ml). Binding was detected with polyclonal anti-human properdin to detect properdin, and monoclonal anti-MBP to detect MBP-TSR4 + 5. The binding of properdin and TSR4 + 5 cannot be quantitatively compared as different detection systems are used. (b) Competitive inhibition assay to examine binding of properdin to C3b in the presence of MBP-TSR4 + 5. The OD value for Properdin binding in the presence of 20 μg/ml MBP was taken as 100% binding (average of 6 replicates) (data not shown). MBP-TSR4 + 5 (starting at 6 μg/ml) was serially diluted and 25 μg/ml of properdin was added to all wells to allow for interaction with C3b. MBP-TSR4 + 5 inhibited properdin binding as the amount of MBP-TSR4 + 5 increased in concentration. All experiments were done in triplicate and error bars represent standard deviation. Molar concentration of 53 kda properdin at 25 μg/ml is 0.47 μm; for 12.5 μg/ml is 0.23 μM; for 6.25 μg/ml is 0.11 μM; for 3.12 μg/ml is 0.058 μM; for 1.56 μg/ml is 0.029 μM. Molar concentration of TSR4 and TSR5 at ~48 kda for 25 μg/ml is 0.52 μm. The molar concentration of TSR4+5 at 6 μg/ml is 0.10 μM; for 3 μg/ml is 0.05 μM; for 1.56 μg/ml is 0.028 μM; for 0.75 μg/ml is 0.013 μM; and for 0.37 μg/ml is 0.0067 μM. The molar ratio used for both properdin and TSRs is at a comparable level based on their weight and concentrations.

with PBS + 5 mM EDTA and the cell concentration was adjusted using DGVB-Mg-EGTA (2.5 mM sodium barbital, 71 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA, 2.5% w/v glucose, 0.1% gelatin, pH 7.4) to 1×10^9 cells/ml. Different concentrations of MBP-TSR4 + 5 were incubated with normal human serum (1/40 dilution and 1/120 dilution in DGVB-Mg-EGTA) 50 μl of TSR4 + 5 solution and 50 μl of serum after dilution, giving a final starting concentration of TSR4 + 5 25 μg/ml and 1/40 or 1/120 dilution of serum (100 μl total volume), in maxisorp 96-well plate for 1 h at 37 °C. Then, 100 μl of erythrocytes (1×10^9) were added to the serum dilutions (200 μl total final volume) and incubated again for 1 h at 37 °C. Cells were centrifuged (3000 rpm, 5 min) and the supernatant was read at 541 nm to determine the percentage of cell lysis.

2.12. C3b deposition assay

Maxisorp 96-well plates were coated with zymosan (1 μg/well) in carbonate bicarbonate buffer and left overnight at 4 °C. The wells were blocked with 2% BSA in PBS for 1 h at 37 °C, and then washed three times with PBST. Different concentrations of TSR4 + 5 (starting from final concentration of 20 μg/ml) were mixed with a dilution of normal human serum (final dilution 1/100) using DGVB-Mg-EGTA buffer (100 μl total volume), and left in the coated wells at 37 °C for 30 min. MBP or TSR4 or TSR5 or no added proteins were used as controls. DGVB-5 mM EDTA was also used as control (with 1/100 dilution of serum), and these OD values subtracted from the test samples. C3b was detected by using rabbit anti-human C3 polyclonal (MRC Immunochemistry Unit, Oxford) (1:5000) in PBST (incubated at room temperature for 1 h), probed with secondary antibody protein A HRP (Sigma, Poole, UK, catalogue no. 101023) (1:5000) in PBST (incubated at room temperature for 1 h). Colour was developed using TMB and read at 450 nm.

3. Results

3.1. Expression and purification of MBP-TSR4 + 5 in tandem

The cDNA sequences spanning TSR4 and TSR5 were cloned in tandem into pMAL-c vector, which has a Mal*E* gene coding for maltose binding protein (MBP) under the P_{tac} promoter. The recombinant construct was transformed into competent *E. coli* BL21 cells. The MBP-TSR4 + 5 fusion proteins were examined for expression by inducing with 0.4 mM IPTG. Analysis of SDS-PAGE showed an induced band at ~55 kDa (Fig. 1a). A clear difference can be seen between induced and uninduced samples. The size of the fusion protein at ~55 kDa is consistent with a combined mol. wt. of MBP (42 kDa) and TSR4 + 5 (~13 kDa). MBP-TSR4 + 5 following affinity purification on amylose resin had minor contaminants (Fig. 1b), which were successfully removed by Q Sepharose ion exchange chromatography. The yield of purified protein was about 25 mg/L similar to the single TSR modules purified in the same way (di Guan et al., 1988). A single band can be seen on SDS-PAGE representing peak pooled fraction following recovery from Q Sepharose ion exchange chromatography (Fig. 1c). For comparative functional studies, individual MBP-TSR4 as well as MBP-TSR5 were also expressed and purified as described earlier (Perdikoulis et al., 2001). As expected, both modules when expressed separately migrated at ~48 kDa (Fig. 1d). MBP-TSR4 + 5 was detected using anti-human properdin (polyclonal) antibody and probed with Protein A HRP as shown in western blot analysis (Fig. 1e).

3.2. Properdin and MBP-TSR4 + 5 bind to C3b

Microtitre wells were coated with C3b to examine the interaction of properdin and MBP-TSR4 + 5 with C3b. Properdin and MBP-TSR4 + 5 showed binding to C3b in a dose-dependent manner. The extent of binding cannot be directly compared as different detection systems are used (Fig. 2a). Individual MBP-TSR4 and 5 (25 μg/ml) did not show binding to C3b, as has previously been reported (Perdikoulis et al., 2001). In addition, MBP-TSR4 + 5 also competed with properdin in binding to C3b (Fig. 2b).

3.3. Properdin and MBP-TSR4 + 5 bind sulfatides, heparin and CSA

Properdin and MBP-TSR4 + 5 bound solid-phase sulfatides, heparin and CSA in a dose-dependent manner. As for Fig. 2, note that extent of binding cannot be compared quantitatively here, as two different detection systems are used. Individual MBP-TSR 4 and 5 again did not show binding to these GAGs, or sulfatide, suggesting that MBP-TSR4 + 5 modules, when together, both contribute to properdin binding to GAGs or sulfatides. Binding of MBP-TSR4 + 5 to sulfatides was dose-dependent (Fig. 3a) and MBP-TSR4 + 5 was also able to inhibit the binding of native properdin to sulfatides. About 50% inhibition was achieved for properdin binding to sulfatides (Fig. 3b). Similarly, MBP-TSR4 + 5 bound immobilised CSA in a dose-responsive manner (Fig. 4a), and was able to inhibit properdin-CSA interaction (Fig. 4b). At the highest concentration tested (~6 μg/ml), only about 20% inhibition was achieved.

Heparin is a glycosaminoglycan, which can interfere with many steps of the complement system. Yu et al. (2005) have shown that most soluble complement proteins such as C1–C9, C1INH, factor I, factor H, factor B and properdin bind to heparin but with different binding kinetics and dissociation constants (Kd), ranging from 2–320 nM (Yu et al., 2005). Properdin and MBP-TSR4 + 5 bound to heparin in a dose-dependent manner (Fig. 5a). Again, MBP-TSR4 + 5 inhibited properdin-heparin interaction. About 20% inhibition was observed at 6 μg/ml concentration (Fig. 5b). These results may suggest that polymeric structure of properdin is more relevant for high affinity interaction with GAGs.

Since MBP-TSR4 + 5 bound solid-phase C3b well and competed out native properdin effectively, we examined if MBP-TSR4 + 5 was able to act as an inhibitor of the complement alternative pathway.

3.4. Alternative pathway assay

MBP-TSR4 + 5 could potentially bind to C3b generated in the alternative pathway assay using rabbit erythrocytes, and thus not allow properdin in the serum to stabilise the C3bBb complex. When a 1:40 serum dilution was used, the highest concentration of MBP-TSR4 + 5 used (25 μg/ml) inhibited haemolysis by >60% (Fig. 6). However, at 1:120 dilution, <1 μg of MBP-TSR4 + 5 was able to bring down haemolysis to <20%, suggesting a reasonably strong interaction between C3b and MBP-TSR4 + 5. This result shows that MBP-TSR4 + 5 is an effective inhibitor of the action of properdin in complement activation. These results are consistent with the view that TSR4 + TSR5 encompass the C3b binding site of properdin (Higgins et al., 1995; Perdikoulis et al., 2001; Nolan et al., 1991).

3.5. Inhibition of C3b deposition by TSR4 + 5

C3b deposition in zymosan-coated microtitre plate wells, in the buffer DGVB-Mg_EGTA was tested, using 1/100 diluted human serum. MBP-TSR4 + 5 inhibits the C3b deposition in a dose dependent fashion. Inhibition was observed even at ng levels of MBP-TSR4 + 5, and close to 100% inhibition was seen at 5–20 μg/ml of TSR4 + 5 (Fig. 7). This supports the view of interaction of TSR4 + 5 with C3b and its ability to inhibit the binding of properdin to C3b.

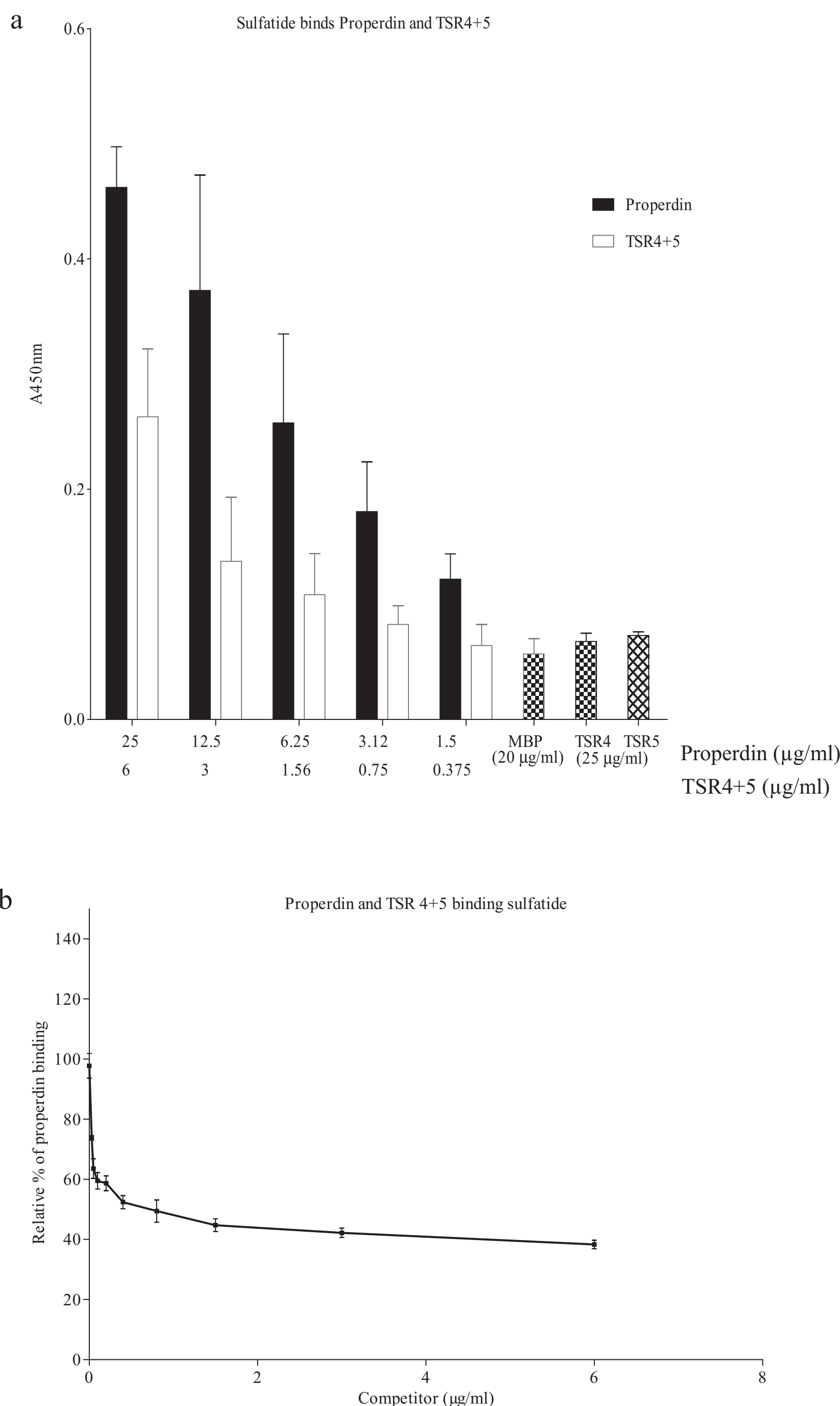

Fig. 3. (a) ELISA to examine Properdin and MBP-TSR4 + 5 binding to sulfatides. Individual MBP-TSR4 and MBP-TSR5 (25 μg/ml) did not show any significant binding to sulfatides, whereas properdin and MBP-TSR4 + 5 bound sulfatides dose-dependently. MBP (20 μg/ml) alone did not show binding to sulfatides. Binding was detected with polyclonal anti-human properdin to detect properdin, and monoclonal anti-MBP to detect MBP-TSR4 + 5. The binding of properdin and TSR4 + 5 cannot be quantitatively compared as different detection systems are used (b) Competitive inhibition ELISA between properdin and MBP-TSR4 + 5 for binding to sulfatides. The OD value for Properdin binding in the presence of 20 μg/ml MBP was taken as 100% binding (average of 6 replicates) (data not shown). Sulfatide was coated in microtitre wells, and various concentrations of MBP-TSR4 + 5 (starting at 6 μg/ml) were serially diluted and constant properdin (25 μg/ml) was added to each well, to test competition binding to sulfatides. 100% properdin binding was calculated in the presence of 20 μg/ml of MBP. MBP-TSR4 + 5 inhibited the binding of properdin to sulfatides. The percentage of inhibition of properdin is increased with higher concentration of MBP-TSR4 + 5, giving about 60% decrease of signal. Binding of properdin in the presence of TSR4 + 5 was detected by anti-human properdin (monoclonal), which does not detect TSR4 + 5. MBP co-incubated with properdin did not show any inhibition, as no decreased signal was observed. All experiments were done in triplicate and error bars represent standard deviation. See Fig. 2 legend for the molar ratios of properdin and TSRs.

4. Discussion

Properdin, as an upregulator of the complement alternative pathway, has been shown to bind C3b and stabilise the C3bBb complex. In addition, properdin also interacts with GAGs (Kemper et al., 2008). Attempts have previously been made to localise ligand binding sites within the thrombospondin (TSR) modules of

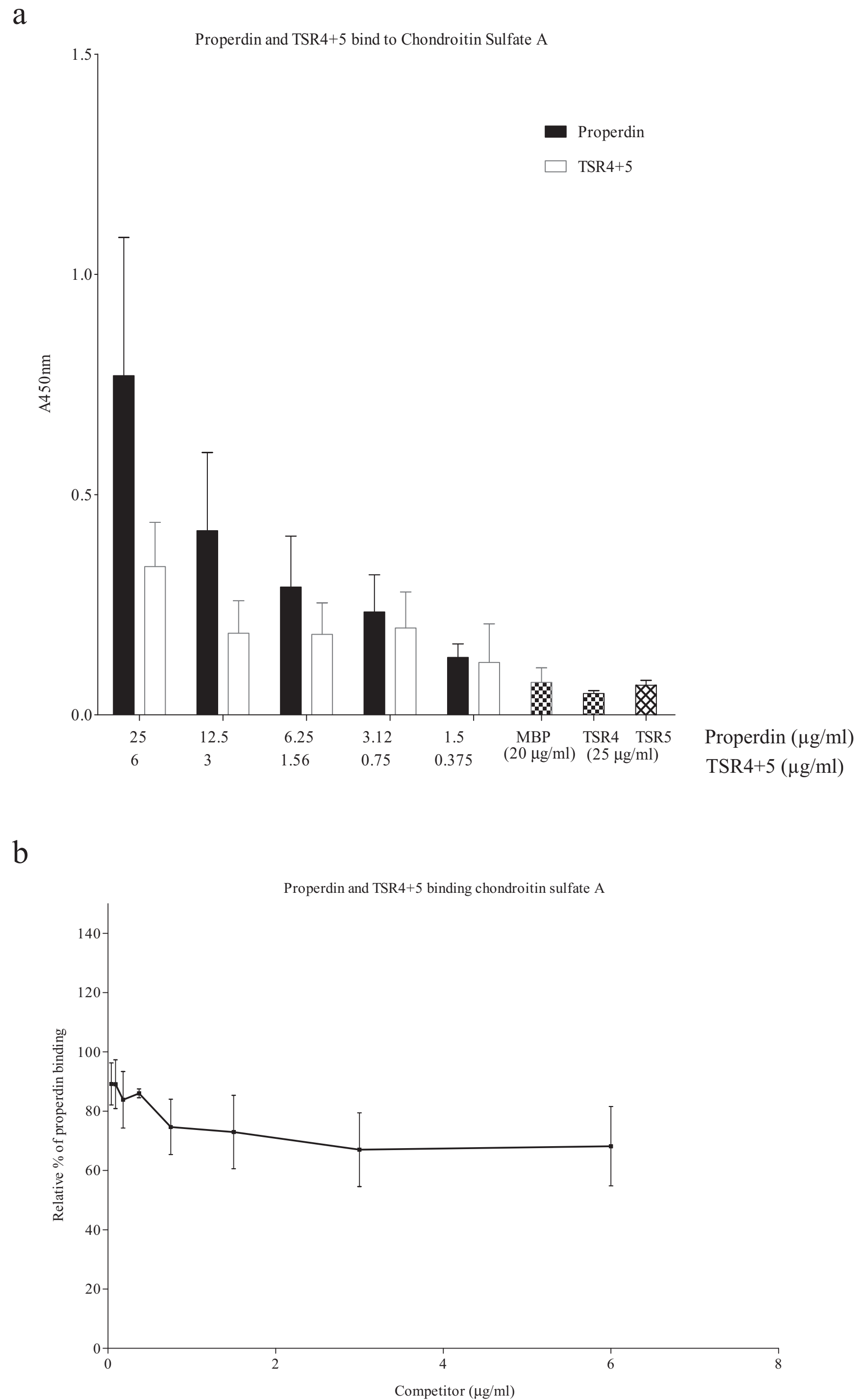

Fig. 4. (a). ELISA to examine the interaction of MBP-TSR4 + 5 with chondroitin sulfate A. Chondroitin sulfate A (CSA) was coated in microtitre well plates, and different concentrations of MBP-TSR4 + 5 (starting at 6 μg/ml) and properdin (starting at 25 μg/ml) were placed in each well. CSA bound properdin and MBP-TSR4 + 5 dose-dependently. Individual MBP-TSR4 and MBP-TSR5 (25 μg/ml) modules or MBP (20 μg/ml) did not show binding to CSA Binding was detected with polyclonal anti-human properdin to detect properdin, and monoclonal anti-MBP to detect MBP-TSR4 + 5. The binding of properdin and TSR4 + 5 cannot be quantitatively compared as different detection systems are used. (b) Competitive inhibition assay to examine binding of properdin to CSA in the presence of MBP-TSR4 + 5. The OD value for Properdin binding in the presence of 20 μg/ml MBP was taken as 100% binding (average of 6 replicates) (data not shown). Plate wells were coated with 10 μg/ml of CSA. Various concentrations of MBP-TSR4 + 5 (starting at 6 μg/ml) were serially diluted and constant properdin (25 μg/ml) was added to each well, to test competition binding to CSA. In this assay MBP-TSR4 + 5 inhibited the binding of properdin up to ∼20%. All experiments were done in triplicate and error bars represent standard deviation. See Fig. 2 legend for the molar ratios of properdin and TSRs.

properdin. Limited treatment of human properdin with trypsin that cleaves a peptide bond within TSR5 has been shown to prevent binding to C3b, without losing its ability to interact with sulfatides, suggesting the importance of TSR5 for C3b interaction (Higgins et al., 1995). In an interesting domain deletion approach using inverted PCR strategy, properdin variants lacking individual TSRs have been expressed in Chinese Hamster Ovary (CHO) cells and characterised for their oligomeric state via gel filtration, ability to bind C3b (and sulfatides), and stabilise the C3bBb complex (via alternative pathway haemolytic assay) (Higgins et al., 1995).

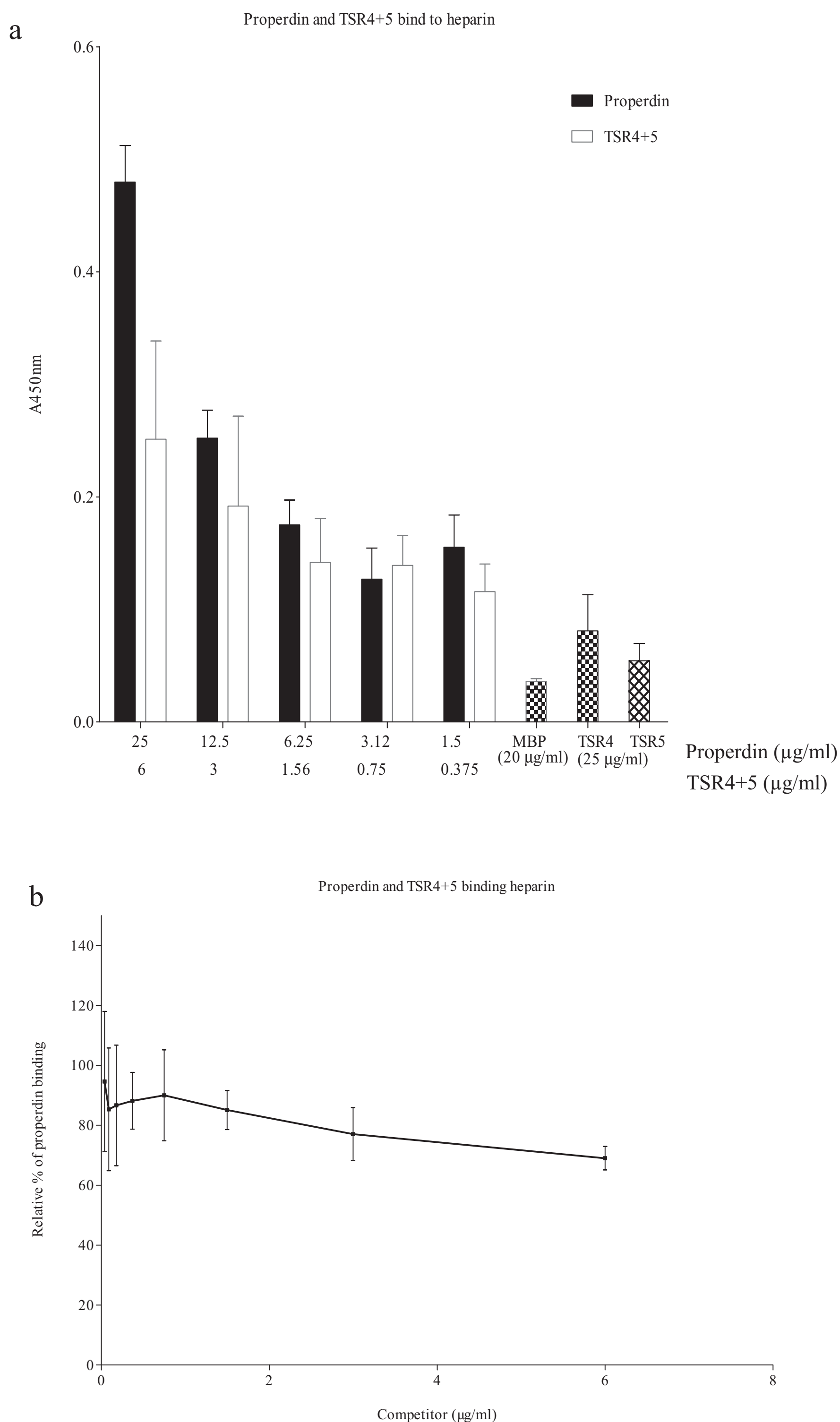

Fig. 5. (a). ELISA to examine the interaction of MBP-TSR4 + 5 with heparin. To analyse the interaction of properdin and MBP-TSR4 + 5, microtitre wells were coated with heparin. Serial dilutions of Properdin (starting concentration from 25 µg/ml) and MBP-TSR4 + 5 (starting concentration 6 µg/ml) were added to appropriate wells. A dose dependence response can be seen for properdin and MBP-TSR4 + 5 binding to heparin. MBP (20 µg/ml) or individual MBP-TSRs (25 µg/ml) used as controls did not show binding to heparin. Binding was detected with polyclonal anti-human properdin to detect properdin, and monoclonal anti-MBP to detect MBP-TSR4 + 5. The binding of properdin and TSR4 + 5 cannot be quantitatively compared as different detection systems are used. (b). Competitive inhibition assay to examine binding of properdin to heparin in the presence of MBP-TSR4 + 5. The OD value for Properdin binding in the presence of 20 µg/ml MBP was taken as 100% binding (average of 6 replicates) (data not shown). To assess whether MBP-TSR4 + 5 competes with the binding of properdin to heparin, both properdin and MBP-TSR4 + 5 were incubated with heparin coated wells. Properdin binding to heparin is decreased at higher doses of MBP-TSR4 + 5. At 6 µg of MBP-TSR4 + 5, there is about 30% inhibition of properdin binding to heparin. All experiments were done in triplicates and error bars represent standard deviation. See Fig. 2 legend for the molar ratios of properdin and TSRs.

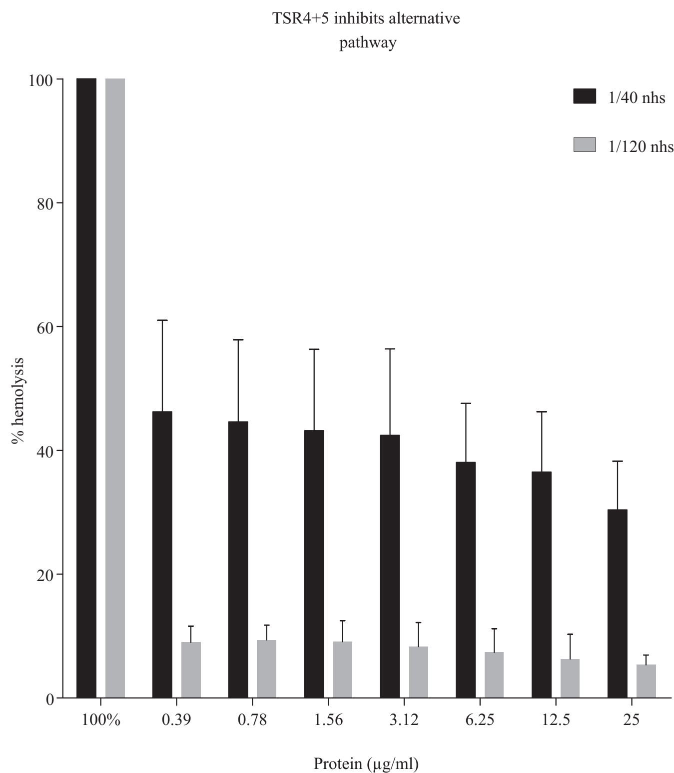

Fig. 6. Inhibition of alternative pathway haemolysis. To examine effects of TSR4 + 5 in an alternative pathway haemolytic assay, different concentrations of MBP-TSR4 + 5 (final concentration starting at 25 μg/ml) were incubated with normal human serum at 1/40 or 1/120 dilution. The amount of red blood cell lysis was determined by absorbance 541 nm. MBP-TSR4 + 5 inhibits the pathway by about 90% at all concentrations tested at 1/120 serum dilution. At 1/40 dilution, MBP-TSR4 + 5 shows a dose dependent inhibition. At 1/120 serum dilution there is about 0.16 μg/ml of properdin in the serum, suggesting that 0.39 μg/ml of MBP-TSR4 + 5 may have been able to almost completely occupy the binding sites for properdin. All experiments were done in triplicate and error bars represent standard deviation.

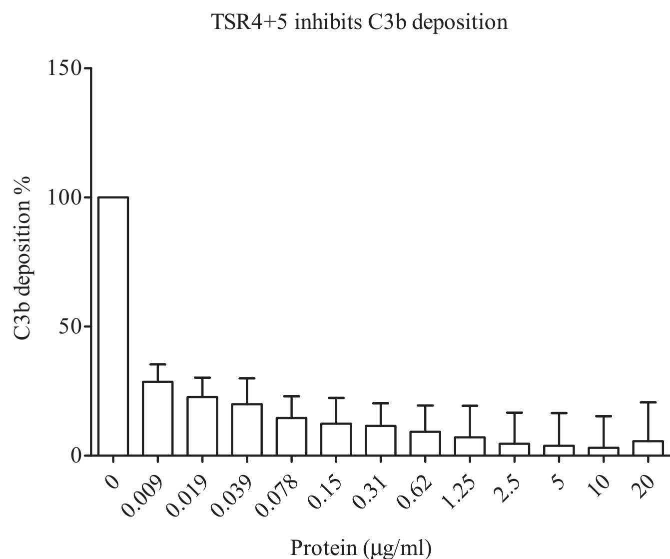

Fig. 7. Inhibition of C3b deposition. To determine the interaction of TSR4 + 5 in a C3b deposition assay, TSR4 + 5 was incubated with 1/100 dilution of normal human serum in zymosan-coated microtitre plates, using the alternative pathway buffer DGVB-Mg-EGTA. The 100% control was 1/100 normal human serum only without any added protein. MBP or TSR4 or TSR5 did not show any significant inhibition (results not shown).

TSR5 deletion completely abrogated binding to C3b and sulfatides whereas the TSR4 deletion mutant bound C3b (and sulfatides) but failed to stabilise the C3bBb complex (Higgins et al., 1995). These results clearly implicated TSR5 as the principal module involved in C3b binding and C3bBb stabilisation, with a subsidiary contribution from the TSR4 module. To follow this study, individual TSR modules, as defined by exon-intron boundaries within the properdin gene, were expressed in *E. coli* as fusions to MBP and characterised for binding to C3b (and sulfatides) and stabilising C3bBb (Perdikoulis et al., 2001). None of the modules including TSR4 and TSR5 showed biological functions associated with properdin. The notion that individual modules were not correctly folded was addressed by 1D and 2D-NMR spectra of TSR3 module, which showed a stable and correctly folded structure. Thus, polyclonal antibodies were raised against each TSR module in rabbit. Purified antibodies (and their Fab portion) against recombinant TSR5 were as able to inhibit binding of properdin to solid-phase C3b and sulfatides, in addition to inhibiting properdin-dependent haemolysis of rabbit erythrocytes (via complement alternative pathway) (Perdikoulis et al., 2001). These results reaffirmed the earlier evidence (Higgins et al., 1995) that TSR5 is the principal C3b binding site for properdin that receives a co-operative contribution from TSR4. Therefore, in the current study, we expressed TSR4 and TSR5 modules together (TSR4 + 5) in tandem. The recombinant MBP-TSR4 + 5 bound C3b, sulfatides, heparin, and CSA in a dose-dependent manner and competed with native human properdin to interact with these ligands to varying degrees (Figs. 2–5). Consistent with the earlier report (Perdikoulis et al., 2001), individual TSR 4 and TSR5 modules did not show binding to these ligands, again implying that both TSR4 and TSR5 are required for the binding. Competitive inhibition ELISA results suggest that the binding of human properdin to C3b, GAGs and sulfatides could be inhibited by the double module (Figs. 2b, 3b, 4b, 5b). In the presence of MBP-TSR4 + 5, the binding of properdin to these ligands decreased. In addition, MBP-TSR4 + 5 was able to inhibit alternative pathway activation quite potently (Figs. 6 and 7). This raises the possibility that the two-module protein can be used to down-regulate the complement alternative pathway prophylactically and therapeutically.

Alcorlo et al. (2013) have obtained structural data for the complex of properdin with C3bBb, by electron microscopy. They suggest a range of possible structures for the tetrameric form of Properdin, and in one of these, TSRs 4 and 5 are exposed at the junction ("vertex") between properdin monomers. They show that the "vertex" region binds to C3bBb, and that a properdin oligomer can bind a C3bBb at each vertex. From this observation, they state that "the structure assembled by the oligomerization of two properdin monomers was essential for C3bBb convertase recognition" However, our data indicate that TSR4 + 5, in monomeric form, is sufficient for C3b binding (Fig. 2), but TSR4 + 5 does not stabilise C3bBb, as suggested by the data on inhibition of the alternative pathway (Figs. 6 and 7).

Alternative pathway dysregulation in dense deposit disease occurs due to autoantibodies (C3 nephritic factors) to C3 convertase. The C3 nephritic factors elevate the half-life of C3 convertase up to 60 min (Zhang et al., 2012). Impaired or dysfunctional regulation of alternative pathway has been associated with atypical haemolytic uremic syndrome, dense deposit disease, age-related macular degeneration, neuroinflammation and other neuropathologies (Mache et al., 2009). Recent studies demonstrate that in human kidneys with anti-GBM disease, both complement classical pathway and alternative pathway are activated. Renal damage may be due to the involvement of alternative pathway activation, as renal biopsy tissues of patients with anti-GBM disease showed deposition of complement components C1q, factor B, properdin, C3d, C4d and C5b-9. Both components of the alternative pathway factor B and properdin co-localized with the C5b-9

membrane attack complex and properdin also co-localized with C3d, causing activation of complement alternative pathway (Ma et al., 2014).

Inhibition of alternative pathway has been previously reported to be beneficial in various conditions. For example, mAb 1379, an anti-Factor B antibody, which inhibits formation of the convertase C3bBb, has been shown to provide protection against anti-phospholipid antibody-induced complement activation and fetal loss (Thurman et al., 2005). A definite protection, by the same antibody against traumatic brain injury leading to complement-mediated neuroinflammation and neuropathology has also been reported (Leinhase et al., 2007). Anti-C5 monoclonal antibody eculizumab has been used as a therapeutic for paroxysmal nocturnal hemoglobinuria. Hemolysis is caused by the spontaneous tick over of the alternative pathway, which increases at night due to the blood pH being lowered. Further therapeutics may be required to inhibit haemolysis (Holers, 2008). The TSR4 + 5 recombinant modules also form an excellent model for structure-function studies since it is capable of binding to C3b and GAGs. The therapeutic implications can be further explored.

To our knowledge this is the first properdin sequence-derived recombinant inhibitor of the alternative pathway. This paves the way for further testing of its prophylactic and therapeutic values in murine models where properdin deficiency renders the mice susceptible to a range of infections. This also offers a unique opportunity for raising a panel of monoclonal antibodies against TSR4 + 5, which can potentially be inhibitors of the alternative pathway. With the availability of properdin deficient mice, it should be possible to raise polyclonal or monoclonal antibodies against the two-module recombinant protein without having to worry about cross- reactivity with mouse properdin. In addition, the recombinant TSR4 + 5 is ripe for three-dimensional crystallographic studies. Its 13 kDa size also makes it amenable to NMR spectral studies for solving its three dimensional solution structure. An affinity matrix made up of TSR4 + 5 maybe useful in purifying C3b from plasma.

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