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C-reactive protein collaborates with plasma lectins to boost immune response against bacteria

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Although human C-reactive protein (CRP) becomes upregulated during septicemia, its role remains unclear, since purified CRP showed no binding to many common pathogens. Contrary to previous findings, we show that purified human CRP (hCRP) binds to *Salmonella enterica*, and that binding is enhanced in the presence of plasma factors. In the horseshoe crab, *Carcinoscorpius rotundicauda*, CRP is a major hemolymph protein. Incubation of hemolymph with a range of bacteria resulted in CRP binding to all the bacteria tested. Lipopolysaccharide-affinity chromatography of the hemolymph co-purified CRP, galactose-binding protein (GBP) and carcinolectin-5 (CL5). Yeast two-hybrid and pull-down assays suggested that these pattern recognition receptors (PRRs) form pathogen recognition complexes. We show the conservation of PRR crosstalk in humans, whereby hCRP interacts with ficolin (CL5 homologue). This interaction stabilizes CRP binding to bacteria and activates the lectin-mediated complement pathway. We propose that CRP does not act alone but collaborates with other plasma PRRs to form stable pathogen recognition complexes when targeting a wide range of bacteria for destruction.

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Introduction

C-reactive protein, CRP (Tillett and Francis, 1930; Macleod and Avery, 1941; Kaplan and Volanakis, 1974), is a plasma protein that is highly upregulated during acute phase response. CRP is an indicator of acute infection and inflammation (Pepys and Hirschfield, 2003; Marnell *et al*, 2005). Systemic infection by a broad range of Gram-positive and -negative bacteria raises CRP level by up to 1000-fold (Hengst, 2003; Black *et al*, 2004; Sierra *et al*, 2004), suggesting its involvement in the immune response against most pathogens.

Direct binding of purified CRP to *Streptococcus pneumoniae* (Volanakis and Kaplan, 1971), *Neisseriae lactamica* (Serino and Virji, 2000) and *Haemophilus influenzae* (Weiser *et al*, 1998) via repetitive phosphorylcholine moieties on the lipoteichoic acid or the lipopolysaccharide (LPS) of these pathogens has been shown. Binding of CRP recruits phagocytic cells, and activates the classical complement pathway (Kaplan and Volanakis, 1974; Siegel *et al*, 1974), causing bacterial clearance. However, purified CRP failed to bind *Escherichia coli*, *Salmonella enterica* (This includes *S. enterica* Group D serotype and *S. enterica* subsp enterica serovar Typhimurium, previously named *Salmonella typhimurium* (Truper, 2005)), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Campylobacter fetus jejuni*, *Neisseria meningitidis* and *Neisseria gonorrhoeae* (Mold *et al*, 1982; Szalai *et al*, 2000), when tested *in vitro*, although increased CRP was observed in systemic infection by many of these bacteria (Sierra *et al*, 2004). Thus, the *in vivo* role of CRP during systemic infection is not completely understood.

Mice administered with human CRP (hCRP) and/or hCRP-transgenic mice that were infected with *S. pneumoniae* (Mold *et al*, 1981; Szalai *et al*, 1995) or *S. enterica* previously named *S. typhimurium* (Szalai *et al*, 2000) showed decreased bacteremia and increased survival, reflecting the efficacy of hCRP *in vivo*. However, conclusions from these findings are problematic because: (1) purified hCRP showed no binding to *S. enterica* (Szalai *et al*, 2000), (2) hCRP did not activate the classical complement pathway in mice during infection by *S. pneumoniae* (Suresh *et al*, 2006) and (3) the major acute phase protein in mice is the serum amyloid P component and not CRP (Ku and Mortensen, 1993).

The discrepancy between *in vitro* and *in vivo* findings and problems with research using mice have prompted us to explore the *in vivo* role of CRP against infection, using the horseshoe crab, which harbors no adaptive immune system and naturally relies on CRP as a potentially important front-line defense protein (Ng *et al*, 2004; Iwanaga and Lee, 2005). Many pattern recognition receptors (PRRs) are evolutionarily conserved (Shrive *et al*, 1999; Kairies *et al*, 2001) from horseshoe crab to human, suggesting that these proteins play essential roles in innate immunity. Horseshoe crab CRP exists in families of multiple isoforms (Nguyen *et al*, 1986; Iwaki *et al*, 1999; Iwanaga, 2002). In the Japanese species, *Tachypleus tridentatus*, the CRP families are designated CRP-1, CRP-2 and CRP-3 (Iwaki *et al*, 1999; Iwanaga, 2002). In the American species, *Limulus polyphemus*, CRP-2 is known as limulin (Roche and Monsigny, 1974; Kaplan *et al*, 1977; Robey and Liu, 1981; Armstrong *et al*, 1996). CRP-1

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and CRP-2 bind phosphorylcholine in a calcium-dependent manner (Robey and Liu, 1981; Iwaki *et al*, 1999), and thus fulfill the functional definition of CRP (Kaplan and Volanakis, 1974).

Studies using purified horseshoe crab CRP-1, CRP-2 and CRP-3 against *E. coli* K12, *Enterococcus hirae, Micrococcus luteus* and *Staphylococcus aureus* 209P showed neither agglutination nor growth inhibition activity (Iwaki *et al*, 1999). These unexpected observations raised doubts on the role of horseshoe crab CRP as a potentially important frontline immune response protein. However, our study on the horseshoe crab showed its remarkable ability to clear *P. aeruginosa in vivo*, and suggested that CRP plays an effective role in frontline defense against infection (Ng *et al*, 2004). We found that in the presence of plasma or hemolymph, CRP binds to a wider range of bacteria than when CRP was tested in isolation (Mold *et al*, 1982; Iwaki *et al*, 1999; Szalai *et al*, 2000). Co-purification results and yeast two-hybrid analysis suggest that CRP collaborates with other PRRs to form stable complexes as they bind to the pathogens. Significantly, such PRR–PRR crosstalk is conserved from horseshoe crab to human, where the collaboration between hCRP and ficolin (another PRR) was found to consequently activate downstream components in the lectin-mediated complement pathway.

Results

Plasma factors enhance the binding of CRP onto bacteria

As hCRP does not bind *S. enterica in vitro*, but protects against blood infection by this pathogen (Mold *et al*, 1982; Szalai *et al*, 2000), we reasoned that *in vivo*, other plasma factors probably enable hCRP to interact with the pathogen effectively. To test this, we incubated *S. enterica* with hCRP in the presence of either 10% human plasma or human serum albumin (HSA, as control). In the presence of HSA, hCRP binds to bacteria (Figure 1A, lane 1). This binding is enhanced in the presence of other plasma proteins (lane 2). The enhancement is not due to endogenous CRP in the plasma, since control using plasma alone showed negligible binding of CRP (lane 3). Although calcium is required for hCRP binding, controls with and without ethylenediaminetetraacetic acid (EDTA) showed that endogenous calcium in the plasma is not the cause of the enhancement (Figure 1B). These results suggest that there are plasma factors other than calcium ions that enhance the binding of hCRP to bacteria.

To test if plasma factors enhance the binding of hCRP, we pretreated, co-treated or post-treated the *S. enterica* with plasma before, during or after addition of hCRP, respectively. Addition of plasma in any order to hCRP enhanced its binding

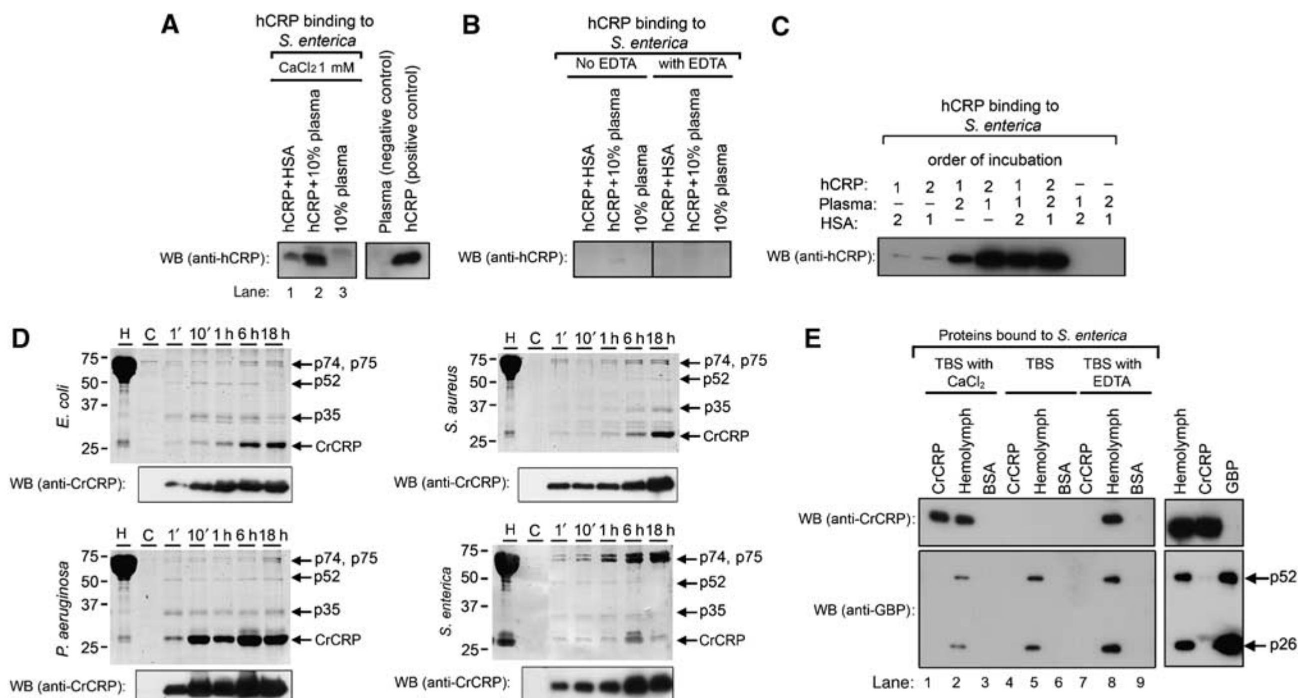

Figure 1 Plasma factors enhance the binding of CRP onto bacteria. (**A**) Immunodetection of hCRP bound to *S. enterica*. *S. enterica* were incubated with (1) purified hCRP in human serum albumin (HSA, control), (2) purified hCRP in 10% plasma or (3) directly with 10% plasma. The experiment was performed in the presence of 1 mM $CaCl_2$. Proteins bound to bacteria were eluted (lanes 1–3) and analyzed by Western blot using anti-hCRP antibody. Purified hCRP (50 ng) and plasma (100 μg) were loaded as controls. (**B**) Immunodetection of hCRP that was bound to *S. enterica* (in VBS), with and without 2 mM EDTA, showed no difference in that both lacked hCRP binding, confirming that the purified hCRP and the plasma samples did not have any calcium that would have interfered with the study of hCRP binding in these experiments. (**C**) Immunodetection of hCRP bound to *S. enterica* during pretreatment, co-treatment or post-treatment with plasma and/or HSA in the indicated order of incubation. (**D**) SDS–PAGE of hemolymph proteins (e.g., 35, 52, 74 and 75 kDa) binding to bacteria and immunodetection of CrCRP (lower panels). Hemolymph was incubated with *E. coli, P. aeruginosa*, *S. aureus* and *S. enterica*. The bound proteins were eluted after various time periods of incubation with the bacteria. Lane H shows the relative abundance of hemocyanin and CRP in untreated hemolymph. Lane C shows proteins eluted from bacteria after incubation with buffer alone. (**E**) Immunodetection of CrCRP and GBP bound to *S. enterica*. Purified CrCRP, 'hemolymph CrCRP' with an equal amount of CrCRP, or BSA alone (negative control) was incubated with *S. enterica*. In each treatment, either 1 mM $CaCl_2$, 2 mM EDTA or neither was included in the buffer. Proteins bound to the bacteria were eluted and analyzed by Western blot using anti-CrCRP and anti-GBP antibodies. Hemolymph, purified CrCRP and GBP were loaded as controls.

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to bacteria (Figure 1C). However, pretreatment and co-treatment of *S. enterica* with plasma gave the most effective hCRP binding. These results suggest that plasma enhances the ability of hCRP to bind bacteria. This is possibly due to either (a) interaction of hCRP with other pathogen-bound plasma PRRs or (b) exposure of hCRP-binding sites on the bacteria by the plasma treatment.

To study the role of CRP in an innate immune model, we incubated horseshoe crab hemolymph with *E. coli* K12, *P. aeruginosa*, *S. aureus* and *S. enterica* R595, and examined the proteins that associated with the bacteria. Figure 1D shows that from 1 min to 18 h, the *C. rotundicauda* CRP (CrCRP) binds rapidly and incrementally to all the bacteria tested, confirming that CrCRP is a PRR that recognizes different bacteria. Differences in the accumulation of CrCRP are probably due to different chemostructures of the bacterial outer membrane. To ascertain that the increase in CrCRP binding is not due to bacterial growth over time, the bacteria were pretreated with 5% acetic acid. The fixation process did not affect CrCRP binding to bacteria (Supplementary Figure 1). Addition of the serine protease inhibitor, phenylmethylsulfonylfluoride (PMSF) also did not inhibit the deposition of CrCRP (Supplementary Figure 2), suggesting that the binding and accumulation of CrCRP is independent of a serine protease. Although it is unclear how the other components of the hemolymph might enhance binding of CrCRP to bacteria, various possible processes might be involved, including the formation of a protein complex that binds to the bacterial surface and/or the involvement of ancillary proteins that can modify the bacterial surface (e.g. via limited proteolysis) to promote binding of CrCRP.

To study the influence of hemolymph factors, purified CrCRP or endogenous CrCRP in hemolymph (henceforth named 'hemolymph CrCRP', present at the same level as the purified CrCRP) was added to *S. enterica*. In the presence of calcium, purified CrCRP and 'hemolymph CrCRP' bind the bacteria (Figure 1E, lanes 1 and 2). With EDTA, purified CrCRP does not bind (lane 7), but 'hemolymph CrCRP' still binds the bacteria (lane 8). This suggests that other hemolymph proteins can collaborate with CrCRP to bind bacteria under conditions where cations are chelated by EDTA. The lack of binding of purified CrCRP and 'hemolymph CrCRP' to bacteria in the absence of both calcium and EDTA (lanes 4 and 5) further indicates that the chelation effect of EDTA on other hemolymph proteins is necessary for these protein–protein interactions to occur. Figure 1E shows the difference in CrCRP binding to bacteria without the addition of calcium or EDTA (lane 5) and with the addition of EDTA (lane 8). Thus, like human plasma proteins, the horseshoe crab hemolymph proteins enhance CrCRP to bind to bacteria, although in the horseshoe crab, a 'chelation of cations from the hemolymph proteins' (which may occur in the micro-environment of pathogen invasion) seems to be necessary for hemolymph proteins to enable CrCRP bind bacteria. Supplementary Figure 3 illustrates and further explains the result of Figure 1E. Conceivably, infection that can result in the chelation of cations from hemolymph proteins due to cation uptake by the bacteria (Blackwell *et al*, 2000; Maguire, 2006; Ong *et al*, 2006; Papp-Wallace and Maguire, 2006) would have some effects on protein interactions and consequently, regulate the immune response. However, this mechanism remains to be confirmed.

Identification of hemolymph proteins that collaborate with CRP in LPS binding

To identify proteins that collaborate with CrCRP in the recognition of bacteria, we used LPS-affinity chromatography. ReLPS, a truncated form of LPS, containing only the conserved core elements of two 2-keto-3-deoxyoctonate (KDO) and the lipid A moiety (Figure 2A) was conjugated to the Sepharose. As ReLPS lacks the O-polysaccharide component and is expected to offer no cognate ligands for most of the other lectin PRRs, it is an ideal molecule to isolate PRRs, which may interact with each other via protein–protein rather than ligand–protein interactions. Previously, we have isolated a repertoire of calcium-independent proteins bound to ReLPS (Ng *et al*, 2004), and identified CrCRP among these proteins. Since 'hemolymph CrCRP' and other proteins bind bacteria when EDTA is present (Figure 1E), we washed the ReLPS-bound proteins with EDTA before elution to retrieve CrCRP-interacting proteins. Proteins associated to the ReLPS-Sepharose and not to the control Sepharose (Figure 2B) were identified by mass spectrometry (MS). Results show isoforms of CrCRP-1 and CrCRP-2 (Figure 2C), carcinolectin-5 (CL5) (Figure 2D and Supplementary Figure 4) and the 26 and 52 kDa forms of galactose-binding protein (GBP) also known as Tachypleus lectin (Figure 2E and F). Next, we determined whether GBP also binds bacteria when incubated with hemolymph. Figure 1E shows that GBP binds more strongly without addition of calcium (lane 5), and best in the presence of EDTA (lane 8). These data support the notion that GBP and possibly CL5 could potentially interact with CrCRP to enhance its binding to bacteria.

CRP interacts with GBP, which interacts with CL5

To test the interaction between CRP, GBP and CL5, we used the yeast two-hybrid system. CRP interacts with GBP but not CL5 (Figure 3A). However, GBP interacts with CL5. Hence, a bacteria-binding complex consisting of CRP, GBP and CL5 is mediated by direct interaction between CRP and GBP, and interaction of the latter with CL5. The relative growth rate of the transformed yeast suggests that interaction between CRP and GBP is stronger than that between GBP and CL5 (Supplementary Figure 5).

To test if GBP binds CRP and CL5 in native hemolymph conditions, we used CNBr-activated Tris-reacted Sepharose, which can bind GBP, to co-purify GBP-interacting proteins from the hemolymph. CL5 is co-purified with GBP when naïve or infected hemolymph is used, whereas CRP is only co-purified with GBP when infected hemolymph is used (Figure 3B). Under native conditions, GBP co-migrated with CL5, suggesting that GBP and CL5 is a complex (Figure 3C). Densitometric analysis of the CRP–GBP–CL5 complex resolved by two-dimensional electrophoresis gives an estimated stoichiometric ratio of 1 CRP:19 GBP:3 CL5 (Figure 3B). These results suggest that during infection, oligomers of GBP interact with CL5 and CRP to interlock the PRR network on the surface of the invading bacteria.

GBP is reciprocally co-immunoprecipitated with CRP when hemolymph is incubated with anti-CRP antibody. This is not seen in a control where an unrelated antibody (anti-rabbit IgG antibody) is used (Figure 3D and E). This confirms the specificity of interaction between CRP-GBP, despite the ability of GBP to bind unconjugated-Sepharose (Chen *et al*, 2001).

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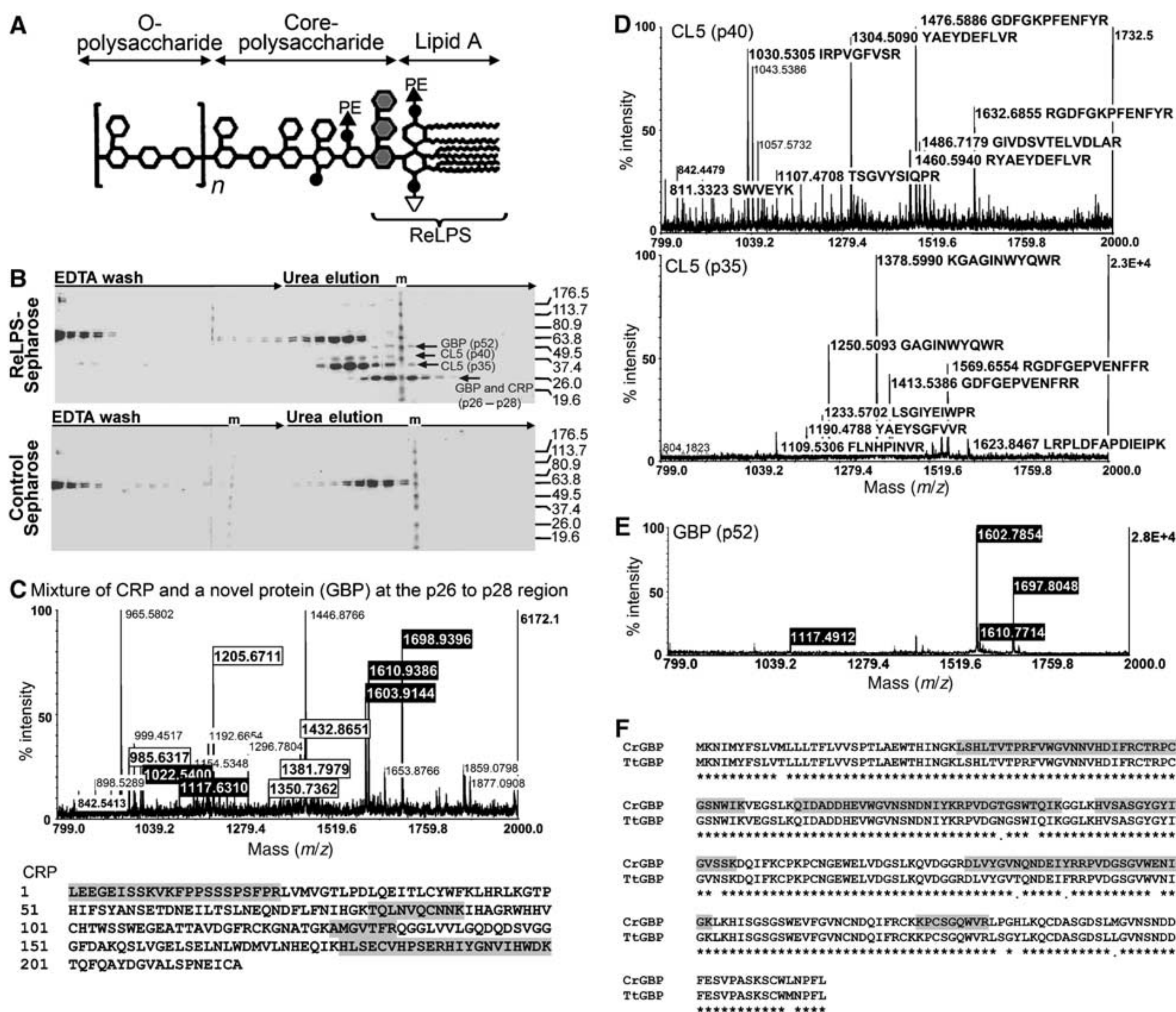

Figure 2 GBP and CL5 co-purified with CRP as an LPS-binding complex. (**A**) Schematic representation of LPS showing the O-polysaccharide and core polysaccharide and lipid A regions. Hexagons represent monosaccharides. KDO is shaded. The lipid A has acyl chains (wavy lines) attached to a disaccharide. Filled circles represent phosphate. The common substituents that are associated with the core region and lipid A moiety include phosphorylethanolamine (filled triangle) and 4-amino-4-dehydroarabinose (open triangles). The O-polysaccharide has a variable number (n) of the repeating units (in parentheses). ReLPS has only the lipid A region and two KDO residues. (**B**) SDS–PAGE of the plasma proteins purified with ReLPS-conjugated Sepharose or control Sepharose. Fractions from the EDTA wash and urea elution are shown. The proteins eluted from the ReLPS column (arrowed) are CRP, GBP and isoforms of CL5. Lanes 'm' represent protein markers. (**C**) Peptide mass fingerprint (PMF) of trypsin-digested proteins from the p26–28 shows peaks corresponding to CRP (white box) and GBP (black box). The 1205 and 1432 peaks were common to CRP-1 and CRP-2. The 1350 and 1381 peaks were from CRP-1 and the 985 was from CRP-2. The m/z values of peaks that are unidentified are set in smaller font. A representative CrCRP sequence (CrCRP-1 hp1, GenBank accession no. AAV65022) is shown. Shaded regions represent parts of the sequences detected by peptide sequencing via ESI-Q-TOF. (**D**) PMF of the CL5 isoforms p35 and p40 and the peptide sequences obtained by MS sequencing of the peptide fragments show that they belong to CL5s. The CL5 peaks are set in bigger and bolder fonts. The unidentified peaks are set in smaller font. (**E**) PMF of p52 is similar to the fingerprint of the p26, which was confirmed to be GBP by peptide sequencing and cDNA cloning. (**F**) Alignment of the cloned *C. rotundicauda* GBP sequence against *T. tridentatus* GBP (TtGBP) sequence. Shaded sequences represent the fragments detectable by MS (via m/z peaks and/or peptide sequencing). *In silico* trypsin digestion of the CrGBP protein sequence yielded m/z values corresponding to the peaks ((C) and (E), black box) in p26–p28 and p52.

Interestingly, GBP interacts with CRP immobilized via anti-CRP even when naïve hemolymph is used. This suggests that immobilization of CRP can facilitate its interaction with GBP, although conversely, immobilized GBP requires additional infection-triggered factors before it could bind soluble CRP. These findings reveal intricate protein–protein interactions between three PRRs that have been previously shown to individually bind certain pathogen-associated molecular patterns (PAMPs) or pathogens on their own (Ng *et al*, 2004; Kuo *et al*, 2006; Zhu *et al*, 2006).

hCRP collaborates with ficolin

The human ficolins (L-, M- and H-isoforms) share 35% homology with horseshoe crab TL5s and CL5s (Supplementary Figure 6), and both harbor a fibrinogen-like (FBG) domain (Holmskov *et al*, 2003). Although CrCRP cannot directly bind CL5, hCRP has been shown to bind fibrinogen (Salonen *et al*, 1984). We therefore reasoned that hCRP could possibly interact with ficolin.

As hCRP is mainly expressed by the liver and secreted into the plasma, we tested for interaction of hCRP with L-ficolin,

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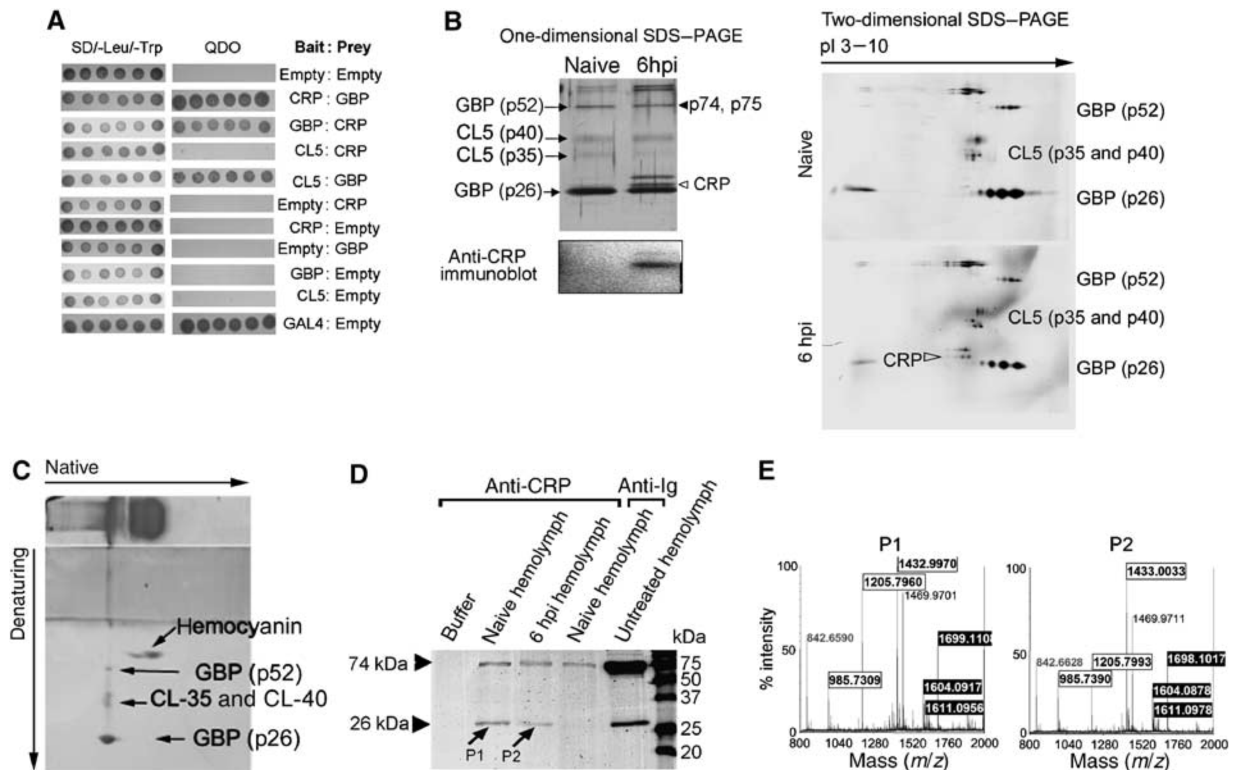

Figure 3 CRP interacts with GBP, which interacts with CL5. (**A**) Yeast two-hybrid analysis shows that CRP1 interacts with GBP, which in turn interacts with CL5. As the dominant isoform on the p35 spectrum from ReLPS-affinity chromatography, CL5c, was used for analysis (see Figure 2B). Growth on SC-Trp-Leu (Trp- and Leu-dropout) agar indicates the presence of both plasmids. Growth on QDO (quadruple dropout lacking Trp, Leu, His and Ade) agar indicates interaction. Empty denotes AH109 yeast strain co-transformed with either pGBKT7 or pGADT7-Rec vector without cDNA inserts. (**B**) One- and two-dimensional SDS–PAGE analyses show that CRP and CL5 co-purified with GBP from hemolymph. Western analysis using anti-CrCRP antibody showed that co-purification of CrCRP and GBP occurred only with 6 hpi hemolymph. The p74 and p75 (not identified) are probably hemocyanin, which, due to its sheer abundance in the hemolymph, commonly associates non-specifically onto Sepharose. (**C**) A first-dimensional native PAGE followed by second-dimensional denaturing SDS–PAGE of the complex co-purified with GBP from naïve hemolymph. (**D**) SDS–PAGE of proteins co-immunoprecipitated by anti-CRP antibody from naïve hemolymph, 6 hpi hemolymph or buffer alone (negative control). Control using an unrelated antibody (anti-IgG) for co-immunoprecipitation showed that the 74 kDa protein was nonspecific. The untreated hemolymph (naive) used for co-immunoprecipitation is shown. The 26 kDa proteins (P1 and P2) were excised for MS. (**E**) Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) spectra of P1 and P2 show CRP (white boxes) and GBP (black boxes) peaks.

a ficolin isoform found in the plasma. L-ficolin shows binding to immobilized hCRP and acetylated bovine serum albumin (BSA) (positive control). The minimal binding to *L. polyphemus* CRP (LpCRP) (Figure 4A) indicates that the co-evolution of these proteins has ensured species-specific interaction. Interestingly, M-ficolin FBG domain can bind to hCRP better than to acetylated BSA (Figure 4B). M-ficolin is found in the secretory granules of neutrophils, monocytes and type II alveolar epithelial cells of the lung (Liu *et al*, 2005). Since expression of hCRP by alveolar macrophages has been reported (Dong and Wright, 1996), hCRP may interact with M-ficolin in the lung surfactant during infection. Affinity analysis suggests that M-ficolin FBG domain binds to immobilized hCRP with a k_D of 4×10^{-8} M (Figure 4C). It remains to be confirmed if such observations are consistent when full-length M-ficolin is used. In the reverse orientation, we observed preferential binding of soluble hCRP to immobilized L-ficolin, rather than to immobilized M-ficolin FBG domain (Figure 4D). It appears that binding of both the L- and M-ficolins to immobilized hCRP (Figure 4A and B) and the reverse binding of hCRP to immobilized L-ficolin (Figure 4D) could be more predominant than the binding of hCRP to immobilized M-ficolin FBG domain (Figure 4D). The differential binding of hCRP to the ficolin isoforms under different conditions reveals an intricacy of PRR–PRR interactions.

Crosstalk between hCRP and ficolin enhances the recognition of Salmonella and activates the lectin-mediated complement pathway

To test if M-ficolin FBG domain augments the binding of hCRP to bacteria, we incubated *S. enterica* (1) with hCRP, followed by M-ficolin FBG domain, (2) in the reverse order or (3) concurrently. M-ficolin FBG domain added before or concomitantly with hCRP enhanced the amount of hCRP bound to bacteria, compared with the reaction without M-ficolin FBG domain (Figure 5A). This suggests that M-ficolin FBG domain aids the deposition of hCRP on the bacteria.

In vivo, binding of ficolin and MASP-2 to immobilized ligand activates MASP-2 to trigger the lectin complement pathway (Fujita *et al*, 2004). Thus, we examined whether immobilized hCRP would interact with L-ficolin and activate the lectin pathway. Complement activation could be monitored by the cleavage of C4 (Moller-Kristensen *et al*, 2003). Figure 5B shows that immobilized hCRP activated the

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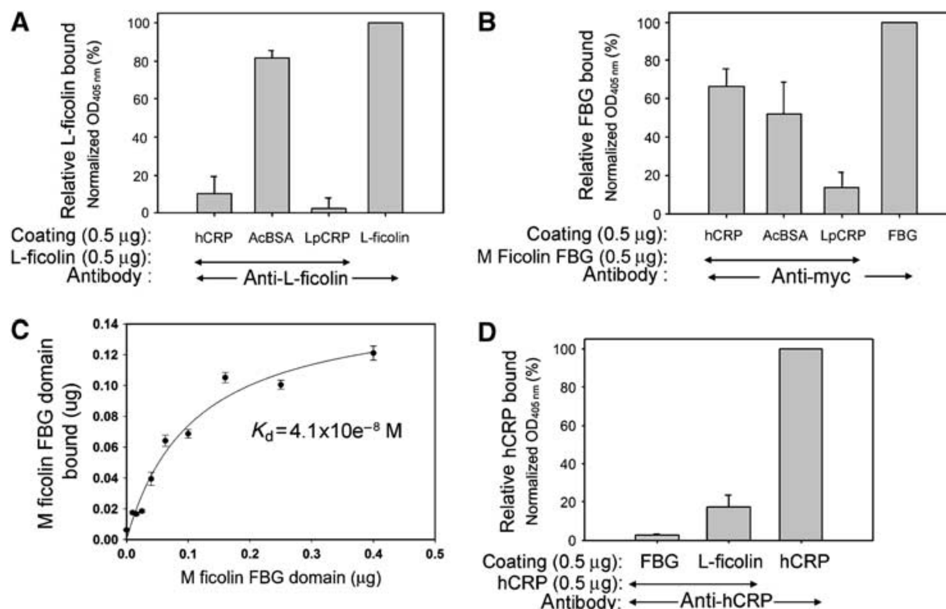

Figure 4 Interaction of hCRP with L-ficolin or myc-tagged M-ficolin FBG domain. (**A**) Relative binding of L-ficolin or (**B**) M-ficolin FBG domain to immobilized hCRP. L-ficolin or M-ficolin FBG domain bound to hCRP, acetylated BSA (AcBSA) or *L. polyphemus* CRP (LpCRP) that was coated on wells, was detected by anti-L-ficolin or anti-myc antibody, respectively. The positive control was directly coated L-ficolin or M-ficolin FBG domain. (**C**) Binding curve of M-ficolin FBG domain to immobilized hCRP. Addition of an increasing amount of M-ficolin FBG domain results in a saturable increase in its binding. The k_D of 4×10^{-8} M was determined from non-linear regression analysis of the binding curve using Sigma plot (version 8.0). (**D**) Relative binding of hCRP to immobilized ficolins. M-ficolin FBG domain (FBG) or L-ficolin was coated onto wells. hCRP binding was detected by goat anti-CRP antibody. Direct coating of hCRP was used as the positive control for 100% binding. For panels A, B and D, 0.5 μg of proteins was used for coating and adding of interaction partner. Readings were subtracted off negative controls (BSA-blocked wells with corresponding treatment) and expressed as a percentage of the corresponding positive control. The means ± s.e.m. of three independent experiments are plotted.

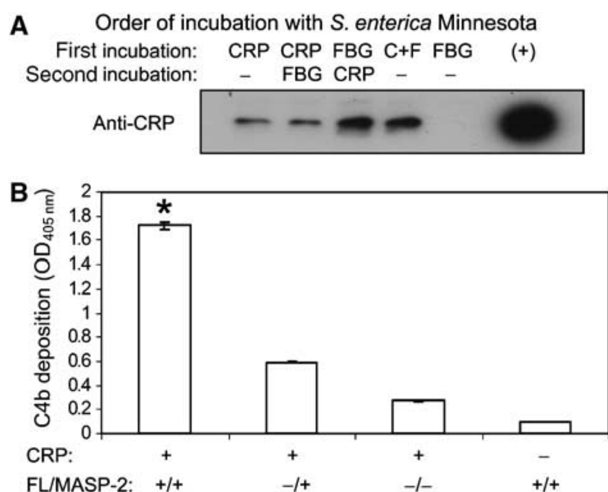

Figure 5 hCRP–ficolin crosstalk enhances pathogen recognition and activates the lectin-mediated complement pathway. (**A**) *S. enterica* incubated with CRP followed by M-ficolin FBG domain (FBG), in the reverse order or concurrently were analyzed by Western blot using anti-hCRP antibody. Addition of M-ficolin FBG domain (with myc tag) either before or together (C + F) with CRP enhanced the amount of pathogen-bound CRP. (**B**) CRP and L-ficolin (FL) collaboration triggered MASP-2 and C4b deposition. hCRP or BSA was coated onto the 96-well plate and incubated with L-ficolin/MASP-2 complex, followed by C4. C4b deposition was detected by anti-C4c antibody. Results are the means of triplicates. * indicates a significant difference of $P<0.05$ relative to the three controls.

L-ficolin–MASP-2 complex to cleave C4. Controls lacking either hCRP or L-ficolin show only partial C4 activation, indicating that the interaction between these two PRRs is essential and sufficient for augmenting >50% of the total C4 activation. These findings confirm that the collaboration between CRP and other PRRs is evolutionarily conserved in human. Moreover, collaboration between CRP and other plasma PRRs enables the recognition of a wider range of bacteria and the activation of the lectin complement pathway.

Discussion

The close association of CRP to septicemia makes it an important clinical marker (Sierra *et al*, 2004). Here, we show that CRP binds to *S. enterica* sub-species enterica Serovar Minnesota R595. This is contrary to findings with purified CRP, which was shown not to bind to several members of the Enterobacteriaceae family (Mold *et al*, 1982; Szalai *et al*, 2000). More importantly, we prove that this binding is enhanced in the presence of plasma (Figure 1A). Pretreatment or co-treatment of bacteria with human plasma resulted in the same level of enhancement of hCRP binding (Figure 1C), suggesting that other plasma PRRs on bacteria can help hCRP bind bacteria possibly by protein–protein interaction or by modifying the surfaces of bacteria and thus creating or exposing hCRP-binding sites on the bacteria. Our finding offers an explanation for the contradiction of *in vivo* studies where (1) hCRP-transgenic mice showed better survival against bacterial infection (Szalai *et al*, 2000) and (2) hCRP is upregulated during bacterial infection (Sierra *et al*, 2004) whereas purified hCRP did not show binding to most bacteria (Mold *et al*, 1982).

In the hemolymph, horseshoe crab CrCRP binds *E. coli*, *P. aeruginosa*, *S. enterica* and *S. aureus* (Figure 1D), contrary

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to earlier studies, which showed that purified CrCRP failed to agglutinate *E. coli* and *S. aureus* (Iwaki *et al*, 1999). Surprisingly, chelation of calcium and/or other divalent cations from hemolymph proteins triggered the binding of CrCRP, although the absence of calcium or the addition of EDTA prevented purified CrCRP from binding to bacteria (Figure 1E). Consistent with this observation, our earlier study also showed that in the presence of hemolymph, a repertoire of proteins including CrCRP binds ReLPS-Sepharose in the presence of EDTA (Ng *et al*, 2004). Here, we identified these proteins to be GBP and isoforms of CL5 (Figure 2), and showed that CRP-1 binds GBP, while GBP binds CL5 (Figure 3). CRP, GBP and CL5 exist at 1, 0.1 and 0.01%, respectively, of the total hemolymph proteins (Robey and Liu, 1981; Gokudan *et al*, 1999; Chen *et al*, 2001), and the levels of CrCRP and GBP remain constant during infection (Supplementary Figure 7). Presumably, during bacterial binding, a small fraction of CrCRP is sufficient to interact with most of the GBP, which may exist as oligomers. In view of the homo-oligomerization ability of these PRRs, we anticipate that CrCRP–GBP–CL complexes would have highly flexible stoichiometric ratio *in vivo*.

Interaction between CrCRP and GBP occurs during infection when either or both proteins are attached on the surface of the bacteria. Mechanisms mediating such PRR crosstalk are multiple and complex, and may involve factors that are released from hemocytes during infection. Since the interaction occurs in yeast two-hybrid analysis (Figure 3A), an inhibitor may be present in the naïve state of the host to prevent random interaction between these two PRRs. Immobilization of CrCRP and depletion of calcium or other divalent cations appear to mimic infection, which triggers interaction between the PRRs. Previously, when naïve CrCRP was purified with phosphorylcholine- or phosphorylethanolamine-conjugated Sepharose ,in the presence of calcium, GBP was not co-purified (Robey and Liu, 1981; Iwaki *et al*, 1999). Future studies should consider the effect of cations in the regulation of PRR–PRR interaction during infection. Although chelation of divalent cations inhibited hCRP binding to bacteria (Figure 1B), we have observed that M-ficolin FBG domain showed enhanced interaction with hCRP when both calcium levels and pH are lowered (unpublished data), suggesting that in human plasma, cations and pH levels may both be required to regulate hCRP collaboration with other proteins for bacteria binding. Taken together, the conditions that facilitate interaction amongst CRP and other PRRs are most probably brought about by infection. Understandably such tight regulations would be important for self–non-self recognition. In the human, the interaction between two frontline PRRs, hCRP and ficolins (Figure 4) augmented the binding of hCRP to bacteria (Figure 5A) and could conceivably widen the range of bacteria, which hCRP can bind to. PRR collaborations have been reported between the peptidoglycan recognition proteins (PGRPs) (Leclerc and Reichhart, 2004; Wang *et al*, 2006a, b) and between Toll-like receptors (TLRs) (Akira *et al*, 2001). Such collaborations were proposed to expand the immune repertoire and the defense network (Takehana *et al*, 2004).

Unlike other PRRs, hCRP initiates the classical pathway (Kaplan and Volanakis, 1974) instead of the lectin-mediated pathway (Fujita, 2002; Holmskov *et al*, 2003; Fujita *et al*, 2004) and hitherto, hCRP and ficolins (Fujita *et al*, 2004; Liu *et al*, 2005) are assumed to initiate different complement pathways. However, we show that immobilized hCRP can recruit L-ficolin, leading to MASP-2 activation and the deposition of C4. Significantly, this suggests that hCRP can activate both the lectin and the classical complement pathways (Figure 6). Congruent to our finding, hCRP was reported to activate the deposition of complement component, C3, via a pathway that is independent of the complement component, C1q (Suresh *et al*, 2006). Our finding that immobilized L-ficolin (Figure 4D) can recruit hCRP also implies that the classical pathway could potentially utilize ficolins, although this remains to be proven.

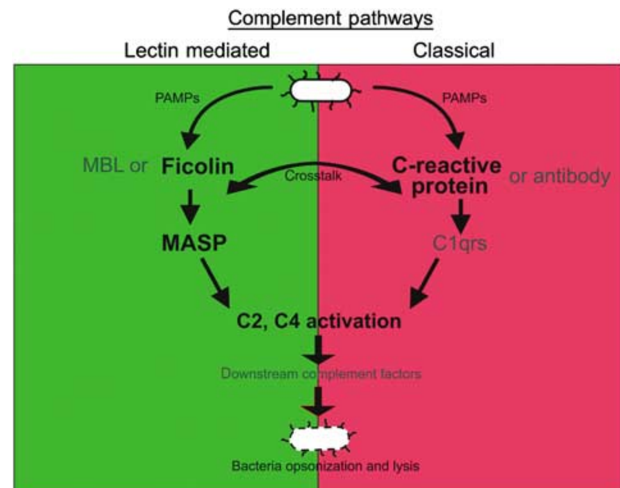

Figure 6 Interaction between two previously unlinked PRRs, CRP and ficolin suggests cross-activation of the classical and lectin-mediated complement pathways. The model shows that pathogen-bound CRP activates the classical pathway and cross-activates the lectin pathway via interaction with ficolin. Conversely, pathogen-bound ficolin, which activates the lectin pathway, may potentially activate the classical pathway via interaction with CRP. This ensures full activation of the complement armaments. The evolutionarily ancient status of CRP and CL5 (ficolin homologue) suggests that CRP:ficolin crosstalk represents part of an ancient complement activation pathway that is entrenched in the immune system of organisms that predated the divergence of the protostome and the deuterostome.

PRR crosstalk may represent an ancestral mode of complement activation that is crucial for survival. This hypothesis is supported by the conservation of hCRP and ficolins (homologues of CL5) from invertebrates to vertebrates, and the notable lack of genetic deficiency in CRP and ficolin (Holmskov *et al*, 2003). The discovery of the interaction between CRP and ficolins, and the consequential activation of the lectin complement pathway, will compel research on CRP-mediated inflammatory disease to consider the therapeutic potential of blocking ficolin. In studies where CRP is administered (Rodriguez *et al*, 2005, 2006) or inhibited (Pepys *et al*, 2006), monitoring of disease pathogenesis may include measuring the activation level of the lectin-mediated complement pathway.

In conclusion, our findings have unveiled the cryptic function of CRP, which has been overlooked, as it was investigated in isolation and not as part of an infection-triggered assembly of PRRs. Our findings show the need to re-evaluate the role of not only CRP but also other PRRs, and to search, through mechanistic approaches in invertebrates, for important phenomena possibly applicable in all life forms including the vertebrates.

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Materials and methods

Organisms

Horseshoe crabs (*Carcinoscorpius rotundicauda*) were from the Kranji estuary (30% seawater), Singapore. The animals were handled according to national and institutional guidelines (National Advisory Committee for Laboratory Animal Research, Singapore). Infection of horseshoe crabs and the bacteria strains used are described in the Supplementary data.

Biochemical reagents

S. enterica R595 ReLPS was from List Biologicals Laboratories Inc. (Campbell, CA, USA). hCRP, anti-hCRP goat antibody, *L. polyphemus* CRP were from Sigma. Anti-myc antibody was from Invitrogen. Biotinylated rabbit anti-human C4c, anti-mouse and anti-rabbit antibodies were from DAKO. Anti-goat antibody was from GE Healthcare. Biotin-GN5-anti-L-ficolin antibody was produced by biotinylation of antibodies from Hycult. Anti-CrCRP and anti-GBP antibodies were raised in New Zealand white rabbits. Human plasma was from healthy volunteers. Construction of clones used in the experiments, production of antibodies, expression and purification of the recombinant myc-tagged M-ficolin FBG domain are described in Supplementary data.

Binding of CRP and other plasma proteins to bacteria

For all treatments, bacteria from 100 μl of an OD 10.0/ml suspension were incubated with the proteins in a final volume of 500 μl. The human plasma used in all studies was pretreated by buffer exchanged into 10 mM veronal-buffered saline (VBS), pH 7.4 to remove the endogenous calcium and other ions. For sequential treatment studies, the bacteria were pelleted and supernatant removed between the first and second incubations.

For hCRP-in-plasma studies, *S. enterica* were incubated for 30 min with either 20 μg/ml hCRP in 10 mM VBS, pH 7.4, containing 10% (v/v) plasma or 0.8% (w/v) HSA, or incubated directly with 10% (v/v) plasma in VBS. In each set of experiment, 1 mM $CaCl_2$, 2 mM EDTA or neither was added.

For hCRP and ficolin studies, *S. enterica* were incubated with 20 μg/ml hCRP in 10% (v/v) human plasma, 50 mM Tris-buffered saline (TBS), pH 7.4, or 0.8% (w/v) HSA in the same buffer, concomitantly or after 1 h incubation, with 20 μg/ml M-ficolin FBG domain. The binding in the reverse order (incubation of bacteria with M-ficolin FBG domain before hCRP) was performed in the same conditions.

For horseshoe crab hemolymph studies, bacteria were incubated with 10% hemolymph in 10 mM Tris, pH 8.0. For protease inhibition studies, a final concentration of 1 mM PMSF was included into the hemolymph just before use. To analyze the binding to bacteria of purified CrCRP versus 'hemolymph CrCRP', the hemolymph was pretreated by buffer exchanged into TBS to remove the endogenous calcium ions. Bacteria were incubated for 30 min at room temperature with 80 μg/ml purified CrCRP in TBS, pH 7.4, or 8 mg/ml of hemolymph (which contains the same concentration of CrCRP) in TBS. Bound proteins were eluted with 0.5 M ammonium formate (pH 6.4) and visualized by SDS–PAGE and Western blot analysis. Western blot analysis and preparation of bacteria are described in Supplementary data.

ReLPS-Sepharose pull-down assay

ReLPS-conjugated or buffer-inactivated Sepharose was incubated with 1 ml of 6 hpi (hours-post-infection) hemolymph, at 4°C overnight, and washed with phosphate-buffered saline containing 5 mM EDTA. Bound proteins were eluted with 4 M urea in 50 mM Tris–Cl, pH 8.0. Elution fractions were analyzed on SDS–PAGE by Coomassie staining. Preparation of ReLPS-Sepharose and MS are described in Supplementary data.

Protein interaction studies

Immunoprecipitation was performed by incubating anti-CrCRP antiserum with protein-A Sepharose fast flow (GE Healthcare) for 1 h at room temperature with gentle rotation. Unbound proteins were washed away twice with TBS and then 50 μl of beads were incubated with 50 μl of hemolymph in a total volume of 300 μl TBS, for 1 h at room temperature. Captured proteins were eluted using 0.4 M *N*-acetyl-glucosamine (GlcNAc) in TBS, pH 7.4, and analyzed on SDS–PAGE. In the GBP pull down, 50 μl of hemolymph were directly incubated with 50 μl of 75% slurry of CNBr-activated, Tris-reacted Sepharose at 4°C. The beads were washed three times with ice-cold TBS. Captured proteins were eluted by 0.4 M GlcNAc in TBS and analyzed by one- or two-dimensional SDS–PAGE. For two-dimensional SDS–PAGE, either isoelectric focusing separation over pI 3–10, or native gel conditions at pH 8.8 was used as the first dimension. Yeast two-hybrid analysis is described in the Supplementary data.

Interaction between CRP and ficolin

For enzyme-linked immunosorbent assay (ELISA), 96-well Maxisorp plates (Nalgene) were coated with 0.5 μg of recombinant M-ficolin FBG domain, native purified L-ficolin, acetylated BSA, LpCRP (Sigma) or hCRP (Sigma). Blocking was with 1% BSA in TBS and washes were with TBS containing 0.05% Tween-20. Binding of either myc-tagged M-ficolin FBG domain, L-ficolin or hCRP was in TBS, and detection was by mouse anti-myc antibody (1:1000), biotin-GN5-anti-L-ficolin antibody at 0.1 μg/100 μl and goat anti-hCRP antibody (1:1000), respectively. Secondary detections were by HRP-anti-mouse antibody, HRP-conjugated streptavidin and HRP-anti-goat antibody (all 1:2000), respectively. For the biotinylated antibody and streptavidin-HRP, incubations and washes were performed in TBS with 0.02% BSA, instead of TBST to reduce nonspecific signal. The substrate, 2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid], (ABTS) from Roche was incubated for 30 min and $OD_{405\,nm}$ was read. OD readings were normalized by first subtracting with a corresponding experiment performed on BSA-blocked wells. The normalized readings were expressed as percentages of readings at 0.5 μg of the respective PRR directly coated.

C4 cleavage assay

The steps are similar to the aforementioned, except that after blocking, purified L-ficolin (Krarup *et al*, 2004) and recombinant MASP-2 supernatant (Stengaard-Pedersen *et al*, 2003) were incubated in TBST/Ca^{2+} overnight at 4°C. This was followed by the addition of 0.1 μg of human C4, 100 ng of biotinylated rabbit anti-human C4c, HRP-conjugated streptavidin (1:1000 dilution), and finally ABTS substrate solution.

Accession numbers

The GenBank accession numbers of the gene and proteins studied in this paper are GBP (AY647278), nine CrCRP isoforms (AY647269-AY647277), partial CL5b (AY956359-AY956361), full-length CL5b isoform (DQ841195) and CL5c (DQ250746).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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