C-reactive protein, CRP, is a predominant pattern-recognition receptor (PRR) in the plasma of the horseshoe crab, which recognizes lipopolysaccharide (LPS). Native CRP2 has previously been shown to exhibit agglutination activity against the polysialic capsule of *Escherichia coli* K1 but its role in bacterial clearance is not well characterized. In this work, the antimicrobial activity of a recombinant CRP2 isoform (rCRP2) was tested against *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. rCRP2 agglutinates bacteria and exhibits bactericidal activity against Gram-negative bacteria. In addition, the antimicrobial activity of rCRP2 is calcium-independent. GST pulldown experiments suggest that in the naïve physiological state, CRP2 interacts with hemocyanin, native CRPs, a 35-kDa plasma lectin and an as yet unidentified 40-kDa protein. This interaction was enhanced upon *Pseudomonas* infection. We propose that rCRP2 is a PRR with potent antimicrobial activity and its interacting partners contribute to effective bacterial clearance.

Keywords: C-reactive protein, horseshoe crab, endotoxin, lipopolysaccharide, antimicrobial activity

Received 17 December 2004
Revised 21 February 2005
Accepted 21 February 2005

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regulated following infection. In vitro, recombinant CRP2 (rCRP2) causes agglutination and is bactericidal towards Gram-negative bacteria. The bactericidal activity of rCRP2 is Ca\textsuperscript{2+}-independent. Additionally, rCRP2 recruits plasma proteins and this interaction is enhanced during infection. This complex probably boosts bacterial clearance by mediating other antimicrobial mechanisms.

**MATERIALS AND METHODS**

**Collection of horseshoe crab hemolymph**

The C. rotundicauda were collected from the Kranji River estuary, Singapore and acclimatised overnight in minimal levels of 30% sea water. Hemolymph was obtained by cardiac puncture and cells were removed by centrifugation before the plasma was used for further analysis. For infection, 10\textsuperscript{6} CFU of Pseudomonas aeruginosa was resuspended in 200 µl of 0.9% saline and injected intracardially. At 1 h post-infection (hpi), the infected hemolymph was similarly collected and processed.

**Cloning, expression and purification of rCRP2**

A DNA fragment encoding the mature sequence of CRP2 was cloned into the pGEX-4T-3 plasmid and transformed into Escherichia coli Top10-competent cells. Sequence-verified plasmids were then transformed into the E. coli BL21 strain for expression. To obtain rCRP2 protein, E. coli BL21 transformants were inoculated into 2x YTA media containing 100 µg/ml of ampicillin and cultured overnight at 37°C with shaking at 230 rpm. An aliquot of 100 µl of overnight culture was added to 1 l of 2x YTA media.

The bacteria were grown at room temperature with shaking at 230 rpm until OD\textsubscript{600} ~0.6–0.8 was achieved. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM. At 3 h post-induction, bacteria were harvested and washed by centrifugation at 5000 g for 5 min at 4°C. The cells were resuspended in 50 ml ice-cold Tris-buffered saline, pH 7.4 (TBS) and French pressed at 15 kPa. The cell debris was removed by centrifuging the lysate at 9000 g for 1 h at 4°C. Bacterial supernatants containing GST-CRP2 were repeatedly passaged through a 50% glutathione–Sepharose 4B (Amersham Biosciences) column at 4°C overnight. Following washing with TBS, target proteins were eluted with 20 mM reduced glutathione and dialysed overnight against TBS containing 10 mM EDTA at 4°C. A supernatant containing GST was similarly treated as a control.

For purification of untagged CRP2, the GST domain of the fusion protein was cleaved on-column with 2 units 250 Tan, Ng, Ho, Ding

Table 1. Purification of rCRP2

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Total (mg)</th>
<th>Recovery Units</th>
<th>Purification Units</th>
<th>Purification (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell lysate</td>
<td>1.54</td>
<td>92.4</td>
<td>1</td>
<td>1</td>
<td>92.4</td>
</tr>
<tr>
<td>Bacterial supernatant</td>
<td>1.24</td>
<td>62</td>
<td>1.02</td>
<td>0.066</td>
<td>3.03</td>
</tr>
<tr>
<td>Soluble supernatant</td>
<td>0.128</td>
<td>1.92</td>
<td>1</td>
<td>0.132</td>
<td>14.42</td>
</tr>
<tr>
<td>Glutathione-Sepharose</td>
<td>0.148</td>
<td>2.23</td>
<td>1</td>
<td>0.148</td>
<td>15.2</td>
</tr>
<tr>
<td>Dialysis</td>
<td>0.121</td>
<td>1.82</td>
<td>0.15</td>
<td>0.121</td>
<td>1</td>
</tr>
<tr>
<td>Thrombin-antimouse</td>
<td>0.128</td>
<td>1.92</td>
<td>0.15</td>
<td>0.128</td>
<td>1</td>
</tr>
<tr>
<td>LPS removal</td>
<td>0.147</td>
<td>1.06</td>
<td>0.15</td>
<td>0.147</td>
<td>1</td>
</tr>
<tr>
<td>Purification volume (ml)</td>
<td>100</td>
<td>100%</td>
<td>0.75</td>
<td>14.42</td>
<td></td>
</tr>
<tr>
<td>Purification volume (mg/ml)</td>
<td>100</td>
<td>100%</td>
<td>0.75</td>
<td>14.42</td>
<td></td>
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<tr>
<td>Purification volume (mg)</td>
<td>100</td>
<td>100%</td>
<td>0.75</td>
<td>14.42</td>
<td></td>
</tr>
</tbody>
</table>

The amount of rCRP2 was determined by densitometric measurements on SDS-PAGE and Bradford assay quantification.
GST pulldown assay to determine the protein–protein interactions of rCRP2

Purified GST and GST-CRP2 fusion proteins were incubated overnight with freshly-prepared glutathione–Sepharose 4B. Plasma samples were introduced to aliquots of the recombinant protein-bound slurry. Bound proteins were washed with TBS, 0.35% Tween-20 and eluted with 50 mM Tris-Cl, 10 mM reduced glutathione, pH 8.0. The eluants were analysed on 12% SDS-PAGE.

In-gel digestion and protein identification by mass spectrometry

Protein bands of interest were excised from SDS-PAGE and dehydrated with acetonitrile. Proteins were then reduced with 10 mM DTT at 57°C for 1 h, and alkylated by 55 mM iodoacetamide at room temperature for 1 h. In-gel digestion was carried out with 12.5 ng/µl trypsin at 37°C for 15 h. The resultant peptide fragments were identified by MALDI-TOF MS/MS analysis (4700 Proteomics Analysis, ABI). Peptide mass fingerprints (pmfs) of the digested proteins were analysed by Mascot (<http://www.matrixscience.com>) against the Mass...
Spectrometry protein sequence Data Base (MSDB). Peaks of pmfs were also matched to known proteins following in-silico analysis.9

Antimicrobial assays

*E. coli* ATCC25922, *P. aeruginosa* ATCC27853 and *Staphylococcus aureus* ATCC25923 were used for antimicrobial activity assays. Bacteria were cultured, washed twice with TBS and adjusted to final concentrations of $1 \times 10^5$–$9 \times 10^9$ cells/ml with half-strength Muller-Hinton broth (MHB; Becton Dickinson). Aliquots of culture were separately incubated with 1.25–10 µM of rCRP2 in 96-well ELISA plates at 37°C, with shaking at 180 rpm. OD$_{600nm}$ readings were taken at 0, 2, 3, 6, 12 and 24 h to monitor bacterial density. Additionally, cultures were serially diluted with TBS, plated onto tryptone soy agar (TSA; Oxoid) and incubated overnight at 37°C for colony enumeration.

The density of the bacteria following incubation with rCRP2 was also visualized by the method of Miles and Misra.10 As a control, TBS was added to the bacterial suspensions and the mixture was similarly treated.

Bacterial agglutination was performed using *P. aeruginosa* according to the method of Lanyi and Bergan.11 The culture was grown to mid-log phase and the concentration adjusted to $5 \times 10^6$ CFU/ml. Varying concentrations of rCRP2 were separately added to aliquots of bacteria. The results were observed on glass slides after

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**Fig. 3.** rCRP2 causes bacterial agglutination and is bactericidal. rCRP2 (10 µM) causes visible bacterial agglutination of $5 \times 10^6$ CFU/ml of *P. aeruginosa* (A, bottom panel). Bacterial clumps are indicated by brackets/arrows. This effect was immediately observable. In contrast, bacteria suspended in TBS did not agglutinate (A, top panel). Both photomicrographs were taken at x400 magnification. (B) Residual colony counts reveal that 2.5 µM rCRP2 is capable of reducing viable bacteria densities by up to 10³-fold. This result was confirmed by (C) the Miles and Misra method10 of serial dilutions to monitor viable bacteria after the antimicrobial assault by rCRP2.
3–5 min. Agglutination of live cells is characterized by a coarse granular bacterial clumping.

**RESULTS AND DISCUSSION**

Purified rCRP2 (Table 1) was separately incubated with 1 x 10^6 CFU/ml of *P. aeruginosa*, *E. coli* and *S. aureus*. At 3 h, the *E. coli* and *P. aeruginosa* cultures showed no increase in OD_{600nm} (Fig. 1A,B). In contrast, *S. aureus* cultures incubated with rCRP2 continued to exhibit increases in bacterial density (Fig. 1C). The results suggest that rCRP2 possesses antibacterial activity selectively against Gram-negative bacterial species.

Contrary to earlier reports, the bactericidal activity of rCRP2 appears to be calcium-independent (Fig. 2). This observation is supported by our recent demonstration that *Carcinoscorpius* CRPs bind LPS in a calcium-independent manner. This calcium independence is not due to high pre-existing levels of Ca^{2+} in the medium (MHB). The background level of Ca^{2+} in MHB has been investigated by atomic absorption spectrometry and shown to be much lower than the naturally occurring levels in eukaryotic hosts. The calcium independence of rCRP2 is in contrast to the activities of known aminoglycosides; these become progressively less effective with increasing concentrations of calcium. Previous studies have established that interaction between Ca^{2+} and rCRP2 interacts with plasma proteins. rCRP2 does not act in isolation but interacts with plasma proteins, forming a formidable antimicrobial complex. (A) Members of this complex include hemocyanin (solid arrow), native CRPs (dashed arrow) and a mixture of other plasma lectins, including CLs (dotted arrows). (B) Peaks of pmfs derived from 28-kDa and 70-kDa protein bands from different samples identified the proteins to be CRPs and hemocyanin (flagged in white and black), respectively. That rCRP2 is able to recruit a complex from naive plasma suggests that it is a pivotal pre-existing PRR that mediates pathogen-recognition and recruitment of downstream immune molecules (continued on next page).
ions and the bacterial membrane is responsible for the bacteria becoming less susceptible, although the exact mechanism of this resistance remains unknown.\textsuperscript{16} rCRP2 is thus capable of potent antimicrobial activity, even as calcium is recruited to strengthen the survival of the pathogen, and appears to circumvent the problem of calcium-mediated bacterial resistance.

To determine the minimum antimicrobial concentration of rCRP2, bacterial suspensions were incubated with decreasing concentrations from 10 \( \mu \text{M} \) to 1.25 \( \mu \text{M} \) rCRP.
Inspection by light microscopy revealed that at concentrations of 2.5 µM or higher, rCRP2 is able to agglutinate Gram-negative bacteria (Fig. 3A). Previously, Tachypleus CRP2 (tCRP2) was reported to be unable to agglutinate or show antibacterial activity against a range of Gram-negative and Gram-positive bacteria with the exception of *E. coli* K117. However, the highest protein concentration tested was apparently only up to 1.64 µM.\(^{17,18}\) Here, we show that rCRP2 can agglutinate *P. aeruginosa* at a concentration of 2.5 µM. Taken together, these results suggest that horseshoe crab CRP2 exhibits higher affinity for *E. coli* K1 than other Gram-negative bacteria, but can nevertheless act on other Gram-negative bacteria at concentrations of above 2.5 µM.

We thus continued to investigate the bactericidal activity of rCRP2 at 2.5 µM by enumeration of viable colonies following incubation. Results show that at 2.5 µM, rCRP2 is capable of causing up to 10\(^2\)-fold reduction in bacterial density rapidly within 1 h (Fig. 3B), which is 4-fold lower than that initially tested (Fig. 1). Visualization of bacterial colonies following their incubation with rCRP2 (Fig. 3C) confirms that rCRP2 possesses potent bactericidal activity.

While rCRP2 on its own is a potent PRR, it does not act in isolation. GST pulldown experiments show that
GST-CRP2 is able to recruit hemocyanin, native CRP isotypes and a mixture of plasma lectins of 35 kDa and 40 kDa (Fig. 4) that includes homologues of tachylectins (data not shown). The ability of rCRP2 to form homo- and hetero-oligomers with other CRP isotypes and other plasma lectins serves to enhance the avidity of CRPs to the PAMP-bearing surfaces. Such ensemble of plasma lectins may enhance frontline pathogen recognition and binding, as well as bacterial agglutination as an early pathogen-neutralization strategy.

Hemocyanin is known principally as an oxygen carrier, and the abundance of hemocyanin in the plasma has long been reported.19 However, more recently, hemocyanin has been demonstrated to exhibit phenol oxidase activity when induced by proteolytic enzymes and detergents such as SDS.20–22 This is necessary for melanization of pathogen and is a key innate immune defence mechanism.25 The high level of circulating hemocyanin suggests that tight control must be maintained to prevent autol melanization of the blood during infection. The recruitment and adhesion of hemocyanin to the Gram-negative bacteria-bound CRP may provide such a regulation. This process ensures that the defence mechanism is activated only at the vicinity of pathogens.

CONCLUSIONS

Overall, differential activities of members of the CRP2-recruited complex contribute towards effective bacterial clearance. In the presence of calcium, the formation of this complex becomes enhanced following infection. The effect is specific, since addition of divalent magnesium does not enhance this complex formation. Taken together with other evidence of CRP2 binding to LPS on Gram-negative bacteria, this finding strongly suggests that CRP2 is a crucial frontline defence molecule that mediates pathogen binding as well as being the pivotal plasma lectin that recruits downstream immune-related molecules to form a formidable protein-complex for antimicrobial activity and effective bacterial clearance.

Work is currently underway to elucidate the structure of rCRP2. This will pave the way towards greater understanding of the interactions of CRP2 and LPS and to determining the antimicrobial centre of the molecule.

REFERENCES