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A Structural Perspective on the Interaction between Lipopolysaccharide and Factor C, a Receptor Involved in Recognition of Gram-negative Bacteria*[S]

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The recognition of broadly conserved microorganism components known as pathogen-associated molecular patterns is an essential step in initiating the innate immune response. In the horseshoe crab, stimulation of hemocytes with lipopolysaccharide (LPS) causes the activation of its innate immune response, and Factor C, a serine protease zymogen, plays an important role in this event. Here, we report that Factor C associates with LPS on the hemocyte surface and directly recognizes Gram-negative bacteria. Structure-function analyses reveal that the LPS binding site is present in the N-terminal cysteine-rich (Cys-rich) region of the molecule and that it contains a tripeptide sequence consisting of an aromatic residue flanked by two basic residues that is conserved in other mammalian LPS-recognizing proteins. Moreover, we have demonstrated that the Cys-rich region specifically binds to LPS on Gram-negative bacteria and that mutations in the tripeptide motif abrogate its association with both LPS and Gram-negative bacteria, underscoring the importance of the tripeptide in LPS interaction. Although the innate immune response to LPS in the horseshoe crab is distinct from that of mammals, it appears to rely on structural features that are conserved among LPS-recognizing proteins from diverse species.

Innate immunity, which defends the host against infectious microorganisms, is an ancient and ubiquitous system in both vertebrates and invertebrates. Although different organisms employ a variety of environment-specific adaptations to ensure host defense, several generalized features underlie the innate immune response. First, germline-encoded pattern recognition receptors (PRRs)[2] of the host recognize broadly conserved microbial components known as pathogen-associated molecular patterns, including lipopolysaccharide (LPS) of Gram-negative bacteria, β-1,3-glucans of fungi, and peptidoglycans of Gram-positive bacteria (1–3). For example, the *Drosophila* peptidoglycan recognition protein family members and vertebrate Toll-like receptors have been well characterized as PRRs (4, 5). Second, the stimulation of PRRs by pathogen-associated molecular patterns activates intracellular signaling cascades that result in transcriptional activation (6). Finally, phagocytosis and the production of antimicrobial peptides by the host are essential for the clearance and killing of infectious microbes (7, 8).

Hemocytes play a fundamental role in the invertebrate innate immune system both as the initial determinants of non-self-recognition and subsequently as the mediators of phagocytosis, encapsulation, and melanization (9). Granular hemocytes predominate in the hemolymph of the horseshoe crab (accounting for >99% of total hemocytes) and constitute a key component of the host defense against infectious microorganisms, as they contain an array of defense molecules that are deployed to protect against infectious microorganisms (10). Horseshoe crab granular hemocytes are especially sensitive to LPS, a major cell wall component of Gram-negative bacteria. Stimulation of the granular hemocytes with LPS causes the activation of hemolymph coagulation within a few seconds, leading to the immobilization and engulfment invading microorganisms. Hemocytes concurrently release defense molecules by LPS-induced exocytosis, thereby killing infectious microbes (10, 11).

Because of their sensitivity to LPS, it has been thought that granular hemocytes express LPS receptors on their surfaces and that binding of LPS to these receptors could initiate a series of signaling processes important for the innate immunity, as has been observed in vertebrate. A close homolog of vertebrate Toll-like receptors, designated *t*Toll, was identified in the horseshoe crab *Tachypleus tridentatus*. However, the apparent absence of a pathogen-associated molecular pattern binding domain in *t*Toll as well as its relatively nonspecific tissue expression pattern (12) cast doubt upon the idea that *t*Toll itself functions as an LPS receptor in horseshoe crab hemocytes. Another candidate for the putative LPS receptor is Factor C, a serine protease zymogen predominantly stored in hemocyte intracellular granules that initiates the hemolymph coagulation cascade following proteolytic activation. Features of Factor C that are consistent with its potential role as an LPS receptor include its high sensitivity toward LPS (13, 14) and its restricted hemocyte-specific expression pattern (see supplemental Fig. S1). We recently proposed that a portion of Factor C might

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: PRR, pattern recognition receptor; ALF, anti-LPS factor; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; HEK, human embryonic kidney; CCP, complement control protein.

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localize to the hemocyte surface and undergo autocatalytic activation upon association with LPS, thereby initiating signal transduction via protease-activated receptors coupled to heterotrimeric GTP-binding proteins (15).

In this study, we have established the essential involvement of the N-terminal Cys-rich region of Factor C in LPS recognition and determined the importance of a tripeptide motif contained within this region of the molecule. Based on these findings, we propose a mode of LPS recognition by Factor C that employs this motif.

EXPERIMENTAL PROCEDURES

Materials—Salmonella minnesota Re595 LPS and biotinylated ultrapure *Escherichia coli* O111:B4 LPS were obtained from List Biological Laboratories (Campbell, CA) and InvivoGen (San Diego, CA), respectively. Fluorescein isothiocyanate (FITC)-conjugated LPS (*E. coli* O111:B4) was supplied by Sigma-Aldrich. Biotinylation of *S. minnesota* Re595 LPS was performed with EZ-Link Sulfo-NHS-LC-Biotin reagent (Pierce).

Immunofluorescence Microscopy—One milliliter of *T. tridentatus* hemolymph was collected into 50 ml of pyrogen-free 10 mM HEPES buffer (pH 7.0) containing 0.5 M NaCl, and the diluted hemolymph (200 μl) was plated on coverslips. After a 20-min incubation to allow attachment, hemocytes were fixed with 3.7% formaldehyde for 10 min and washed twice with phosphate-buffered saline (PBS). For LPS treatment, the attached cells were incubated with 50 μg/ml FITC-conjugated LPS for 30 min and then fixed with 3.7% formaldehyde. The cells were blocked with 5% bovine calf serum and incubated for 1 h with anti-Factor C monoclonal antibody 2C12 (16) diluted in blocking buffer. For detection, Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used. Cells were imaged with an Olympus BX-FLA fluorescence microscope (Tokyo, Japan).

Cloning and Mutagenesis—To construct Factor C variants with Myc epitope tags, plasmid pMT/FC725, which contains a cDNA encoding the heavy chain of *T. tridentatus* Factor C, or plasmid pMT/FC410–994, which contains a cDNA encoding the light chain of *T. tridentatus* Factor C, was used as a template to amplify regions of cDNAs encoding residues 1–723 (heavy chain), 1–296 (Cys/EGF/CCP123), 1–116 (Cys/EGF), 117–296 (CCP123), 300–543 (LCCL/Lectin), 551–609 (CCP4), 669–723 (CCP5), and 738–994 (Ser protease domain) by PCR by using 5′ primers containing a 5′ AscI site and 3′ primers containing a 3′ KpnI site. The amplified fragments were subcloned into the vector pSecTag2A (Invitrogen). Three tandem copies of the Myc epitope tag derived from p5-Helix/3Myc (17) were then inserted into the 5′ KpnI site (3′ XhoI site) to generate constructs pFC-(1–723)/3Myc, pFC-(1–296)/3Myc, pFC-(1–116)/3Myc, pFC-(117–296)/3Myc, pFC-(300–543)/3Myc, pFC-(551–609)/3Myc, pFC-(669–723)/3Myc, and pFC-(738–994)/3Myc. Mutations (R36E/R38E, K55E/K57E, and W37A) in pFC-(1–116)/3Myc were introduced by standard site-directed mutagenesis (18). The *T. tridentatus ALF* gene was amplified from a hemocyte cDNA library using the oligonucleotides TK260 (5′-aaaaaGGCGCGCCcaaggtggtatatggactcaacttgc-3′) and TK261 (5′-tttttGGTACCttgatattgagagatgaacgtttgtgc-3′) to generate an AscI/KpnI fragment that was used to produce a Myc epitope-tagged construct corresponding to those of Factor C.

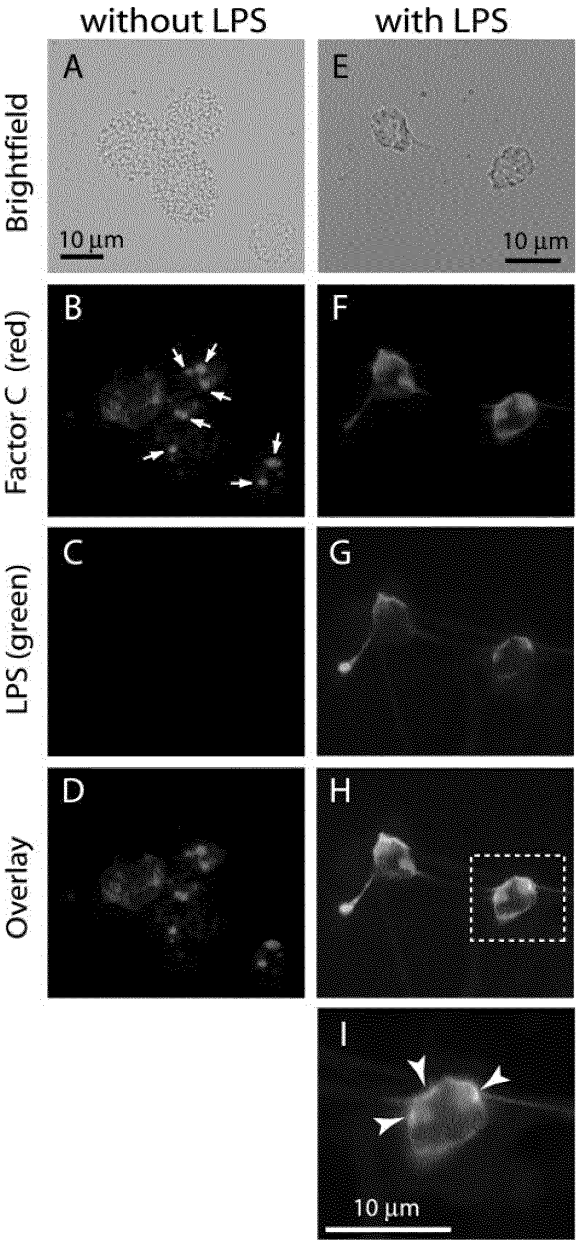

FIGURE 1. **Factor C is a membrane-bound LPS receptor on hemocytes.** The hemocytes from *T. tridentatus* were treated without (*A–D*) or with (*E–H*) FITC-labeled LPS, stained with a monoclonal antibody (2C12) against Factor C (*B* and *F*), and identified by immunofluorescence. *I*, higher magnification image of the *boxed area* in *H*. *Arrowheads* indicate LPS co-localized with membrane-bound Factor C. Note that non-permeabilized hemocytes were used in these experiments. *Scale bar*, 10 μm.

Co-immunoprecipitation—3Myc-tagged Factor C variants and anti-LPS factor (ALF) proteins were transiently expressed in HEK293 cells and secreted into the culture medium. HEK293

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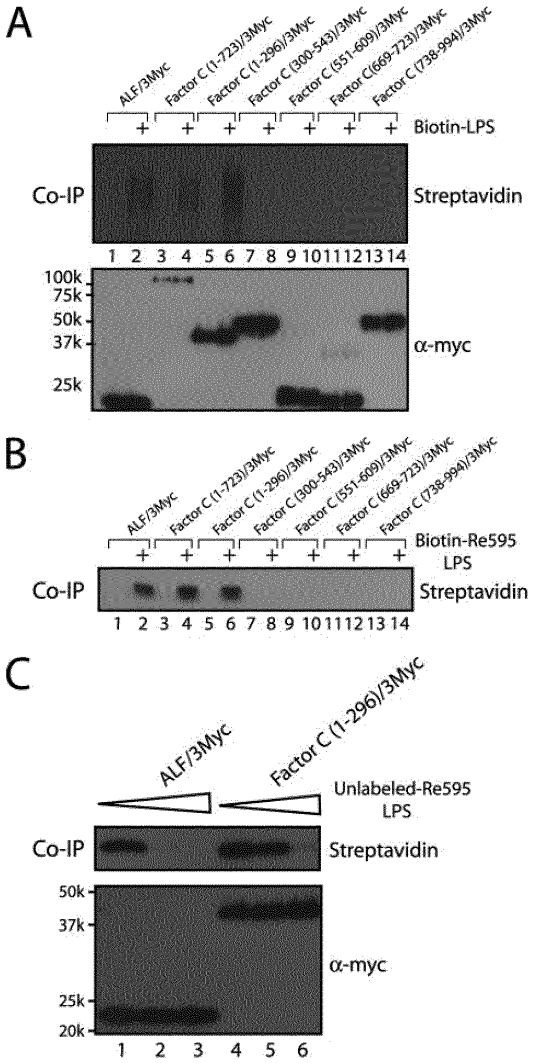

FIGURE 2. **LPS binding assay of Factor C variants.** ALF (positive control) and Factor C variants were incubated with biotinylated *E. coli* O111:B4 LPS (*A*) or *S. minnesota* Re595 LPS (*B*) and subjected to co-immunoprecipitation (*Co-IP*) with an anti-Myc antibody. *C*, binding of Myc-tagged Factor C-(1–296) to biotinylated *S. minnesota* Re595 LPS was specific and was competed by an excess amount of unlabeled Re595 LPS (*lanes 4–6*) as seen in the positive control (*lanes 1–3*). *Lanes 1* and *4*, no competitor; *lanes 2* and *5*, an equal amount of competitor; and *lanes 3* and *6*, 10-fold excess of competitor. Binding to biotinylated LPSs were determined by Western blot analysis with a streptavidin-horseradish peroxidase conjugate (*top*) and anti-Myc monoclonal antibody 9E10 (*bottom*). The positions of protein molecular mass markers are indicated at the *left*.

cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1% glutamine, 1% penicillin-streptomycin, and 10% bovine calf serum, respectively, at 5% CO_2 and 37° C. Four micrograms of the 3Myc-tagged Factor C variants and ALF expression plasmids were used to transfect HEK293 cells at 50% confluence in a six-well plate using the calcium phosphate method. Conditioned media were collected 2 days after transfection, and the protein expres-

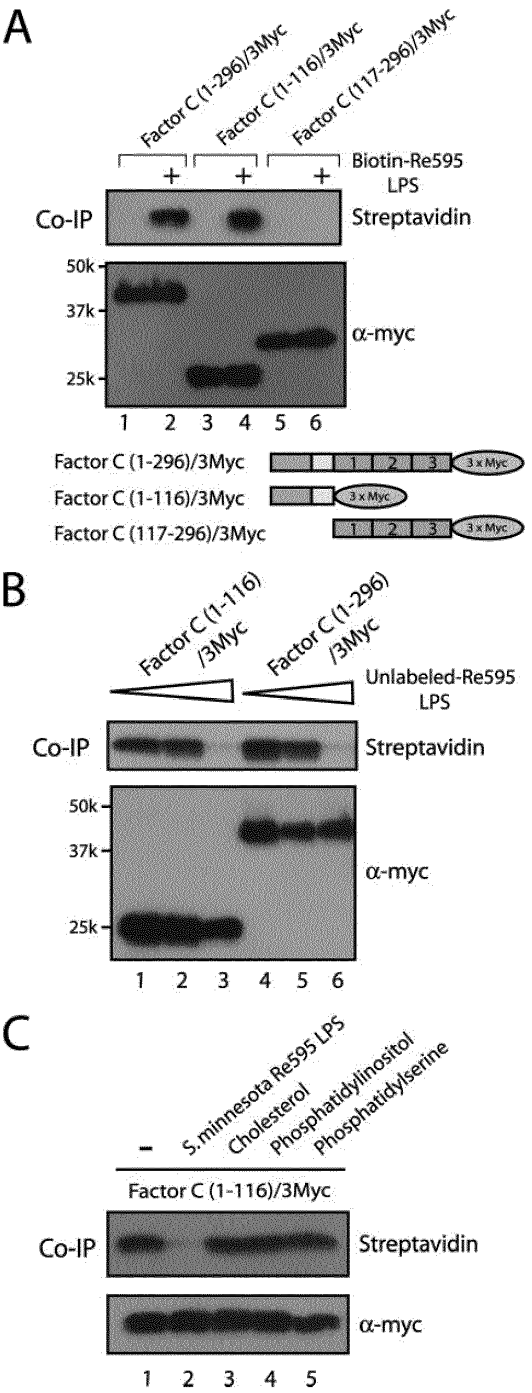

FIGURE 3. **The Cys-rich/EGF-like domain is a potential LPS binding site in Factor C.** *A* and *B*, the LPS binding site was located in the Cys-rich/EGF-like domains of Factor C. The amounts of competitor in *B* were ordered the same as in Fig. 2*C*, and all LPS binding experiments were performed as in Fig. 2. *C*, an excess amount (5-fold excess) of cholesterol (*lane 3*) or acidic phospholipids (*lanes 4* and *5*) had no effect on Factor C-(1–116)/3Myc-LPS complex formation. However, *S. minnesota* Re595 LPS (*lane 2*) effectively disrupted the complex. *Lane 1*, without competitor. *Co-IP*, co-immunoprecipitation.

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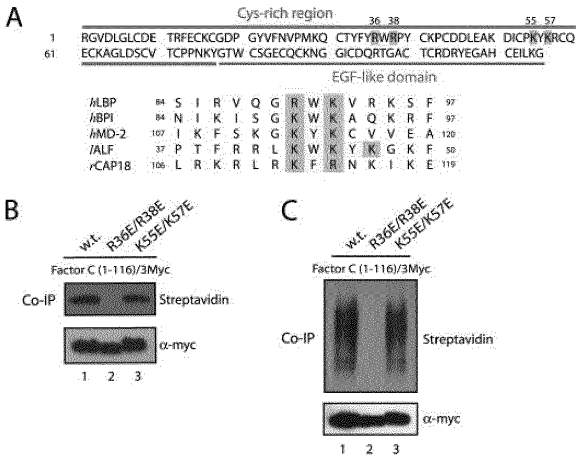

FIGURE 4. **A conserved tripeptide motif in Factor C-(1–116) is critical for LPS interaction.** *A*, the sequence of Factor C-(1–116) with Cys-rich region and EGF-like domain are shown (*top panel*). Two of the tripeptide motif in the Cys-rich region (36–38 and 55–57) are *highlighted* by *cyan* (basic residues) and *yellow* (aromatic residues) and aligned with the LPS-recognizing sites of five different proteins (*bottom panel*). The amino acid sequences are *numbered* according to positions in the mature proteins. *h*LBP, human LPS-binding protein; *h*BPI, human bactericidal permeability-increasing protein; *h*MD-2, human MD-2; *l*ALF, *Limulus* anti-LPS factor; and *r*CAP18, rabbit cationic antimicrobial protein. *B* and *C*, mutational analysis in Factor C-(1–116). Two Factor C-(1–116)/3Myc mutants (R36E/R38E and K55E/K57E) were evaluated for the ability to bind *S. minnesota* Re595 LPS (*B*) and *E. coli* O111:B4 LPS (*C*). Co-immunoprecipitation experiments were performed as in Fig. 2. *w.t.*, wild type.

sion was evaluated by Western blotting with the anti-Myc monoclonal antibody 9E10 (Covance, Berkeley, CA). The clarified supernatants were incubated with 3 μg of biotinylated-Re595 LPS (for the *E. coli* O111:B4 LPS co-immunoprecipitation experiment, 5 μg of biotinylated-LPS was used) at 4 °C for 30 min and then incubated 6 h with 20 μl of agarose beads coupled to the anti-c-Myc polyclonal antibody (Sigma-Aldrich). After four washes with PBS, the immunoprecipitates were separated by SDS-PAGE and immunoblotted with streptavidin-horseradish peroxidase conjugate (Amersham Biosciences) or anti-Myc monoclonal antibody 9E10.

Microbial Organisms and Bacteria Binding Assay—Staphylococcus aureus, Enterococcus hirae, E. coli B, and K12 were cultured in 3% tryptosoy broth at 37 °C overnight. For bacterial immunoprecipitation experiments, 150 μl of the overnight cultures were centrifuged at 12,000 revolutions/min for 2 min and washed twice with PBS. The washed cells were resuspended in conditioned medium from HEK293 cells expressing Factor C variants and incubated at 4 °C for 2 h. After four washes with PBS, the bacteria were directly resuspended in SDS-PAGE loading buffer. Associated proteins were resolved by SDS-PAGE, immunoblotted with the anti-Myc monoclonal antibody 9E10, and subsequently developed with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad). For flow cytometric analysis, Myc-tagged protein bound to bacteria (*E. coli* K12) were incubated with FITC-conjugated anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 1 h and washed twice with PBS, and the FITC-labeled bacteria were analyzed using a FACScan flow cytometer (BD Biosciences).

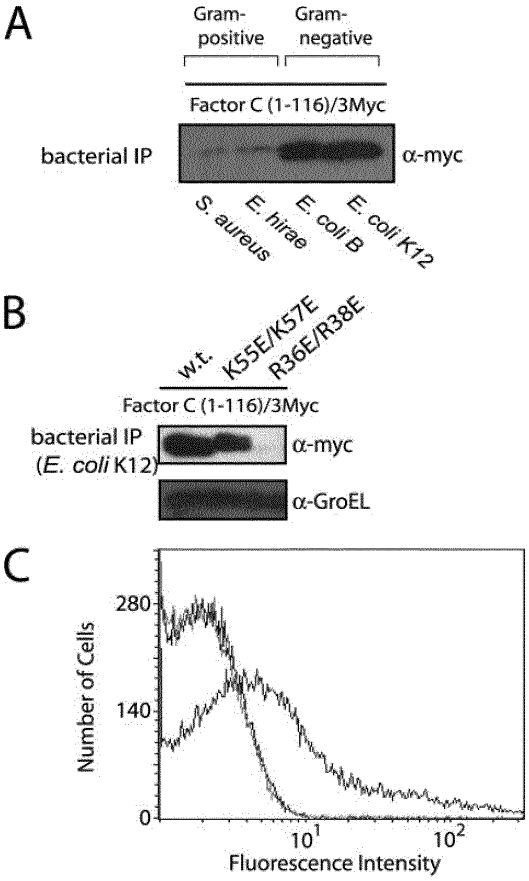

FIGURE 5. **Direct binding of Factor C-(1–116) to Gram-negative bacteria.** *A*, Myc-tagged Factor C-(1–116) specifically bound to Gram-negative bacteria *E. coli* B and K12 but not to Gram-positive bacteria *S. aureus* and *E. hirae*. Factor C-(1–116)/3Myc was subjected to bacterial cell surface immunoprecipitation with the anti-Myc monoclonal antibody 9E10. *B*, bacterial (*E. coli* K12) immunoprecipitation with Factor C-(1–116)/3Myc and its mutants. The R36E/R38E mutant completely lacked the ability to bind bacteria. *C*, flow cytometric analysis of Factor C-(1–116)/3Myc (*blue line*) and its R36E/R38E mutant (*green line*) binding to *E. coli* K12. For detection of surface-bound protein, FITC-labeled anti-myc monoclonal antibody 9E10 was used. As a negative control, untreated bacteria were detected with FITC antibody (*black line*). *w.t.*, wild type.

RESULTS AND DISCUSSION

Factor C Is a Membrane-bound LPS Receptor on the Hemocyte Surface—At least two proteins, ALF and Factor C, have been determined to associate with LPS in the horseshoe crab. We recently reported that Factor C, an LPS-sensitive serine protease zymogen, exists on the surface of hemocytes and initiates signaling through heterotrimeric GTP-binding proteins (15). We reasoned that hemocyte surface-bound Factor C could serve as an LPS receptor, and if so, it would be a likely candidate for the LPS-responsive PRR that induces exocytosis of granular components.

To address this issue, we morphologically investigated whether Factor C and LPS co-localize on the hemocyte surface (Fig. 1). Immunostaining of hemocytes with an antibody to Factor C (red) demonstrated that endogenous Factor C was pres-

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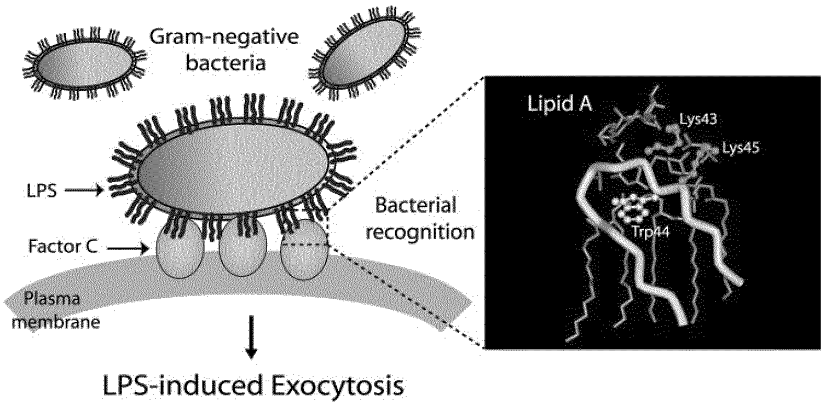

FIGURE 6. **A model of bacterial recognition by Factor C.** The recognition of LPS on Gram-negative bacteria by membrane-bound Factor C initiates the horseshoe crab innate immune response. We propose that the conserved tripeptide motif (Arg^{36}, Trp^{37}, and Arg^{38}) in the Cys-rich region of Factor C interacts with the lipid A portion of LPS. Formation of the Factor C-LPS complex could result in tethering of bacteria to the hemocyte surface and ultimately lead to LPS-induced exocytosis. The modeled structure of the ALF-LPS complex (25) is shown in the *right panel* and may resemble that of the complex between N-terminal elements of Factor C and LPS.

ent in a punctate distribution on the hemocyte surface (Fig. 1*B*, *arrows*). We next tested whether the surface-localized Factor C could bind to LPS. Hemocytes were incubated with FITC-labeled LPS (*E. coli* O111:B4). FITC-LPS accumulated on the cell surface of hemocyte (Fig. 1*G*) and co-localized with Factor C (Fig. 1, *H* and *I*, *arrowheads*). Interestingly, FITC-LPS accumulation was not observed in fixed hemocytes (supplemental Fig. S2), which would be expected to undergo chemical modification and inactivation of surface proteins. These results suggest that Factor C is a membrane-bound LPS receptor in horseshoe crab hemocytes. Factor C can bind to cholesterol and acidic phospholipids with high affinity (14, 15) likely through a different site (19), suggesting that it might associate with plasma membrane lipid rafts enriched with cholesterol and glycosphingolipids (20) or microdomains and subsequently initiate signaling processes through protease-activated receptors following LPS-induced autocatalytic activation.

Identification of the Factor C Domain Essential for LPS Recognition—We therefore attempted to identify the region of Factor C responsible for LPS recognition. Factor C is a multidomain glycoprotein with an estimated molecular mass of ~120 kDa. In addition to the typical serine protease domain at its C terminus, it also contains a Cys-rich region, an epidermal growth factor (EGF)-like domain, five complement control protein (CCP) modules, an LCCL module (derived from a conserved domain of *Limulus* Factor C, Coch-5b2, and Lg11 (21)), and lectin-like domains (supplemental Fig. S3) (22).

We expressed five Factor C variants in HEK293 cells, each containing three copies of the Myc epitope tag (EQKLISEEDL) at its C termini, and tested whether these proteins could form a complex with LPS (Fig. 2*A*). As a positive control, we constructed Myc-tagged ALF, which is known to be an LPS-recognizing protein (23). In co-immunoprecipitation assays, both ALF/3Myc and Factor C-(1–723)/3Myc bound to biotinylated *E. coli* O111:B4 LPS as expected (Fig. 2*A*, *lanes 2* and *4*). The N-terminal fragment, Factor C-(1–296)/3Myc, also bound to LPS (*lane 6*). In contrast, constructs that lacked this N-terminal region, namely Factor C-(300–543)/3Myc, Factor C-(551–609)/3Myc, Factor C-(669–723)/3Myc, and Factor C-(738–994)/3Myc, did not bind to LPS (*lanes 8*, 10, 12, and 14). Consistent with these results, Factor C-(1–296)/3Myc also showed significant binding to biotinylated *S. minnesota* Re595 LPS to an extent that was similar to that of positive controls (Fig. 2*B*, *lanes 2*, *4*, and *6*).

We also investigated the specificity of the Factor C-(1–296)/3Myc-LPS complex (Fig. 2*C*). For both ALF/3Myc and Factor C-(1–296)/3Myc, complex formation with biotinylated LPS was diminished with increasing amounts of unlabeled Re595 LPS, with complex formation completely disrupted by the addition of a 10-fold excess of unlabeled competitor (Fig. 2*C*, *lanes 3* and *6*). Taken together, these results indicate that the N-terminal region of Factor C is sufficient for LPS recognition.

The Cys-rich/EGF-like Domain Encompasses the LPS-recognizing Domain—We performed structure-function analyses to further define the LPS-recognizing sequence within the N-terminal region of Factor C. Two fragments of the previously identified LPS-recognizing domain, Factor C-(1–116)/3Myc and Factor C-(117–296)/3Myc, were generated and tested for their ability to bind to LPS (Fig. 3*A*). Of these two, only the most N-terminal fragment (Factor C-(1–116)/3Myc), which contains the tandem Cys-rich region and EGF-like domain, was capable of forming a complex with biotinylated *S. minnesota* Re595 LPS (Fig. 3*A*, *lane 4*). The interaction between Factor C-(1–116)/3Myc and LPS was determined to be specific, because the complex was disrupted by the addition of excess competing LPS (unlabeled Re595 LPS), as was previously observed for Factor C-(1–296)/3Myc (Fig. 3*B*). The specific interaction between Factor C-(1–116)/3Myc and LPS was further demonstrated by the inability of either cholesterol or acidic phospholipids to disrupt the complex (Fig. 3*C*, *lanes 3–5*). Our results contrast with those presented in a previous study of the Factor C ortholog from the Southeast Asian horseshoe crab *Carcinoscorpius rotundicauda*, which defined a region within the tandem CCP domains as important for LPS binding (24). However, these authors did not specifically investigate the role of the N-terminal region identified in the present study, nor did they include negative controls similar to those described in the following sections.

Mutations within the Cys-rich Region Abolish LPS Binding—From the present study, it is clear that the potential LPS recognition domain in Factor C is located in its N-terminal region, which consists of a Cys-rich region and an EGF-like domain. An interesting feature of the Cys-rich region is the presence of a unique conserved tripeptide motif, namely an aromatic residue immediately flanked by two basic residues, which appears in

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five other known LPS-recognizing proteins (Fig. 4*A*, *bottom*). This sequence motif is present in two copies within the LPS-recognizing domain of Factor C from Arg^{36} to Arg^{38} and from Lys^{55} to Lys^{57} (Fig. 4*A*, *top*).

To test whether these tripeptide motifs are essential for the LPS binding ability, we introduced paired glutamate substitutions into Factor C-(1–116)/3Myc at locations corresponding to basic residue pairs (Arg^{36}/Arg^{38} and Lys^{55}/Lys^{57}). The results of co-immunoprecipitation experiments indicate that the R36E/R38E mutant is incapable of binding to LPS (Fig. 4, *B* and *C*, *lanes 2*), whereas the K55E/K57E mutant retains the ability to bind LPS (Fig. 4, *B* and *C*, *lanes 3*). We also substituted an alanine residue for Trp^{37} to assess the importance of an aromatic residue at this position and found that the resulting W37A mutant also lacked the ability to bind LPS (supplemental Fig. S4). These results establish the essential nature of the Cys-rich region for LPS recognition by Factor C and further highlight the importance of the Arg^{36}-Trp^{37}-Arg^{38} motif.

The Cys-rich/EGF-like Domain of Factor C Binds to Gram-negative Bacteria—The preservation of LPS binding activity in Factor C-(1–116)/3Myc allowed us to explore the direct LPS-dependent recognition of live bacteria. Using bacterial immunoprecipitation, we investigated whether Factor C-(1–116)/3Myc could selectively recognize Gram-negative bacteria, which are rich in LPS on their surface. Four types of live bacteria (two Gram-positive bacteria, *S. aureus* and *E. hirae*, and two Gram-negative bacteria, *E. coli* B and K12) were incubated with Factor C-(1–116)/3Myc, and the association of the protein with the bacteria was assessed by Western blotting with the anti-Myc monoclonal antibody 9E10. Factor C-(1–116)/3Myc was specific for Gram-negative bacteria, showing significant binding to *E. coli* B and K12 but no binding to Gram-positive bacteria (Fig. 5*A*). In addition, the binding of Factor C-(1–116)/3Myc to Gram-negative bacteria (*E. coli*) was confirmed by flow cytometry (Fig. 5*C*). Finally, the R36E/R38E mutant was incapable of binding to *E. coli*, as judged by both bacterial immunoprecipitation (Fig. 5*B*) and flow cytometry (Fig. 5*C*), indicating that the association between Factor C-(1–116)/3Myc and bacteria is LPS-dependent.

Implications for Innate Immunity—Factor C associates with LPS on the hemocyte surface, and the N-terminal Cys-rich region can selectively bind Gram-negative bacteria in an LPS-dependent manner. Taken together, these findings suggest that Factor C may serve as a membrane-bound LPS receptor capable of tethering invading bacteria to the hemocyte surface (Fig. 6), ultimately leading to hemolymph coagulation by Factor C secreted from granules. We propose that the first conserved tripeptide motif (Arg^{36}-Trp^{37}-Arg^{38}) in the Cys-rich region of Factor C plays an important role in the recognition of LPS, an assumption that is consistent with a modeling study of ALF that predicts direct docking of the corresponding tripeptide motif with LPS (25). In this model, two lysine residues that correspond to Arg^{36} and Arg^{38} of Factor C interact with glucosamine (GlcN) I-1-phosphate in lipid A, and a tryptophan residue, which corresponds to Trp^{37} of Factor C, interacts with a hydrophobic portion of lipid A (Fig. 6, *right panel*). Our results are consistent with such a model and underscore the importance of these elements within the Cys-rich region of Factor C for mediating its association with LPS, an essential structural characteristic that appears to be conserved among other LPS-recognizing proteins. Finally, these findings imply that Factor C acts as a PRR for Gram-negative bacteria in the horseshoe crab and lay the groundwork for future studies to elucidate the mechanism whereby hemocyte-bound Factor C potentiates LPS-induced exocytosis degranulation.

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