Distinct Carbohydrate Recognition Domains of an Invertebrate Defense Molecule Recognize Gram-negative and Gram-positive Bacteria*

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Martin Bilej†, Patrick De Baetselier§, Els Van Dijck§, Benoit Stijlemans§, Alain Colige¶, and Alain Beschin§§

From the †Department of Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague 4, Czech Republic, the ‡Department of Immunology, Parasitology, and Ultrastructure, Flemish Interuniversity Institute for Biotechnology, Free University of Brussels, Sint-Genesius-Rode 1640, Belgium, and the ¶Laboratory of Connective Tissue Biology, University of Liège, Liège 4000, Belgium

Coelomic fluid of Eisenia foetida earthworms (Oligochaeta, Annelida) contains a 42-kDa defense molecule named CCF for coelomic cytolytic factor. By binding microbial antigens, namely the O-antigen of lipopolysaccharide (LPS), β-1,3-glucans, or N,N'-diacetylchitobiose present, respectively, on Gram-negative bacteria or yeast cell walls, CCF triggers the prophenoloxidase activating pathway. We report that CCF recognizes lysosome-predigested Gram-positive bacteria or the peptidoglycan constituent muramyl dipeptide as well as muramic acid. To identify the pattern recognition domains of CCF, deletion mutants were tested for their ability to reconstitute the prophenoloxidase cascade in E. foetida coelomic fluid depleted of endogenous CCF in the presence of LPS, β-1,3-glucans, N,N'-diacetylchitobiose, and muramic acid. In addition, affinity chromatography of CCF peptides was performed on immobilized β-1,3-glucans or N,N'-diacetylchitobiose. We found that the broad specificity of CCF for pathogen-associated molecular patterns results from the presence of two distinct pattern recognition domains. One domain, which shows homology with the polysaccharide and glucanase motifs of β-1,3-glucanases and invertebrate defense molecules located in the central part of the CCF polypeptide chain, interacts with LPS and β-1,3-glucans. The C-terminal tryphtophan-rich domain mediates interactions of CCF with N,N'-diacetylchitobiose and muramic acid. These data provide evidence for the presence of spatially distinct carbohydrate recognition domains within this invertebrate defense molecule.

Invertebrate innate defense strategies are based on pattern recognition receptors that do not discriminate between individual antigens but recognize surface determinants common to potential pathogens (1). Pattern recognition receptors interacting with different saccharide moieties of microbial lipopolysaccharides (LPS),1 peptidoglycans, and β-1,3-glucans were described in numerous invertebrate and vertebrate species. We have characterized a 42-kDa lectin named coelomic cytolytic factor 1 (CCF) from the coelomic fluid of Eisenia foetida earthworms (Oligochaeta, Annelida (2, 3)). CCF displays significant amino acid sequence homology with bacterial and animal β-1,3-glucanases but does not exhibit such enzymatic activity (3–5). More interestingly, CCF shows homology with the α subunit of the β-1,3-glucan sensitive factor G from the horseshoe crab Tachypleus tridentatus (6) and with the Gram-negative bacteria-binding proteins of various insects (7–10). More recent reports concern the sequence homology of CCF with LPS and β-1,3-glucan recognition proteins from arthropods (11–13, for review see Ref. 14). All these invertebrate homologs have been suggested to play a role in invertebrate innate immunity acting as pattern recognition receptors. Accordingly, CCF binds with cell wall components of Gram-negative bacteria or yeast (the O-antigen of LPS, β-1,3-glucans, N,N'-diacetylchitobiose), triggering the activation of the prophenoloxidase (pro-PO) cascade (3, 15). This pathway results in the formation of cytotoxic and antimicrobial compounds and thus represents an important defense mechanism in a variety of invertebrates (for review see Refs. 16–18).

Pro-PO cascade is activated within 6 h in E. foetida coelomic fluid by Gram-negative but not Gram-positive bacteria (3). However, E. foetida coelomic fluid incubated for 6 h with Gram-negative or Gram-positive bacteria exhibits a similar antibacterial effect (19). Whether the failure of Gram-positive bacteria to induce the pro-PO cascade resulted from insufficient activation is not clear.

The cell wall of virtually all bacteria consists of a rigid peptidoglycan layer that is either overlaid by an outer LPS layer in Gram-negative bacteria or remains exposed on the surface of Gram-positive bacteria. Peptidoglycan is a polymer of alternating N-acetylmuramyl and N-acetylmuramic acid units connected by short pentapeptides. The β-1,4-glycosidic bond of the N-acetylmuramyl-N-acetylmuramic acid pepti-

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1 The abbreviations used are: LPS, lipopolysaccharide; CCF, coelomic cytolytic factor; ECF, E. foetida CCF; E1–E5, E. foetida CCF fragments 1–5; LCCF, L. terrestris CCF; L2 or L4, L. terrestris CCF fragments 2 or 4; pro-PO, prophenoloxidase; PO, phenoloxidase; MDP, muramyl dipeptide; CF, coelomic fluid proteins; kb, kilobase(s); RACE, rapid amplification of cDNA ends; GSP, generated specific primer; PCR, polymerase chain reaction; 1-DOPA, 3,4-dihydroxyphenyl)1-t-alanine.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF030028 and AF395805.

† To whom correspondence should be addressed: Dept. of Immunology, Parasitology, and Ultrastructure, Flemish Interuniversity Institute for Biotechnology, Free University of Brussels, 65 Paardenstraat, Sint-Genesius-Rode B-1640, Belgium. Tel.: 32-2-359-0302; Fax: 32-2-359-0359; E-mail: abeschin@vub.ac.be.
doglycan backbone can be hydrolyzed by lysozyme (muramidase; mucopeptide N-acetylmuramoylhydrolase; EC 3.2.1.17), a ubiquitous enzyme involved in innate immune reaction of numerous animal species, including annelids (20, 21).

In the present work, we examined whether Gram-positive bacteria trigger the pro-PO cascade in the earthworm coelomic fluid. We report that digestion of the Gram-positive bacteria or peptidoglycan by lysozyme-like activity makes it possible to induce the pro-PO cascade through the recognition of muramic acid by CCF. We also show that the broad specificity of CCF results from the presence of two distinct lectin-like domains within the molecule, a domain located in the central part of CCF implicated in interactions with LPS and β-1,3-glucans, and a C-terminal tryptophan-rich domain interacting with N,N'-diacetylchitobiose and muramic acid. Finally we observed that the CCF homolog in the E. foetida-related species Lumbricus terrestris displays distinct saccharide specificities and does not recognize N,N'-diacetylchitobiose and muramic acid. E. foetida CCF is, to our knowledge, the first invertebrate defense molecule acting as pattern recognition molecule for cell wall components of yeast and Gram-negative and Gram-positive bacteria.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell-free coelomic fluid was collected from adult specimens of E. foetida foetida (common earthworm) and L. terrestris (common earthworm) in the absence of serine protease inhibitor as described previously (2). Micrococcus lysodeikticus was purchased from Sigma Chemical Co. Dr. J. Felsberg (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague) kindly provided Escherichia coli and Bacillus subtilis. Soluble peptidoglycan from M. lysodeikticus was a kind gift of Dr. J. Coyette (Department of Bacteriology, University of Liège, Belgium). Synthetic analogs of peptidoglycan fragments (muramic acid, muramyl dipeptide (MurNAc-L-Ala-D-isoglutamine, MDP), disaccharide dipeptide (GlcNAc-β-1,4-N,N-diacetylchitobiose and muramic acid. Finally we observed that the CCF homolog in the E. foetida-related species Lumbricus terrestris displays distinct saccharide specificities and does not recognize N,N'-diacetylchitobiose and muramic acid. E. foetida CCF is, to our knowledge, the first invertebrate defense molecule acting as pattern recognition molecule for cell wall components of yeast and Gram-negative and Gram-positive bacteria.

**Cloning of L. terrestris CCF**—An E. foetida CCF (ECCF) homolog was detected in L. terrestris by Northern blot using total ECCF cDNA as a probe; a 1.6-kb mRNA was observed. L. terrestris CCF (LCCF) cDNA was cloned using 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE (Life Technologies, Inc.) with generated specific primers (GSP, Table I). For 5'-RACE, GSP-1 was based on ECCF and glucanase motif homologous sequences (3, 14). PCR products were gel-purified and sequenced (Thermo Sequenase radiolabeled terminator sequencing kit, Amersham Pharmacia Biotech). The complete cDNA encoding LCCF was amplified (Titan One tube RT-PCR system, Roche Molecular Biochemicals) using RNA as template and primer pairs (LCCF, Table I) containing a BamHI or a SmaI site at the 5'-N-terminal or 3'-C-terminal ends, respectively.

**E. foetida and L. terrestris CCF Vector Constructions and Bacterial Expressions**—The cDNA encoding E. foetida (ECCF, E1–E5) or L. terrestris (LCCF, L2, L4) CCF peptides were amplified (Titan One tube RT-PCR system) using RNA as templates and primer pairs (Table I)
FIG. 1. Activation of the pro-PO cascade in E. foetida coelomic fluid. A. M. lysodeikticus or E. coli were used as such or were pretreated with coelomic fluid proteins (CF), lysozyme, or trypsin for the indicated times before testing their ability to trigger pro-PO activation in E. foetida coelomic fluid. PO activity elicited in the absence of bacteria in coelomic fluid either untreated or treated with lysozyme or trypsin is indicated by black bars. *, p < 0.05 as compared with background values. B, M. lysodeikticus peptidoglycan was used as such or was pretreated with lysozyme or CF for the indicated times before testing the pro-PO activation in E. foetida coelomic fluid. Black bars indicate PO activity in coelomic fluid in the absence of triggering agents. *, p < 0.05 as compared with background values.

containing a BamHI or a Smal site at the 5′-N-terminal or 3′-C-terminal ends, respectively. After digestion, PCR products were ligated in QIAexpress PQE-30 vector containing a N-terminal 6× His-affinity tag (Qiagen). The TOP10F′ E. coli strain was transformed, grown in the presence of ampicillin (100 μg/ml), and induced for 2 h at 37 °C in the presence of isopropyl-1-thio-β-D-galactopyranoside (2 mM). CCF peptides were purified and resuspended in TN buffer as described previously (3). LPS contamination was <15 pg/mg CCF peptides (QCL LAL test, Bio-Whittaker Europe).

Prophenoloxidase-activating Assay—The level of pro-PO cascade activation was assessed as described previously (3). Briefly, 50 μl of the coelomic fluid (without or with 1 mM Pefabloc (serine proteinase inhibitor, Roche Molecular Biochemicals)), 25 μl of 0.1 mM Tris, pH 8, containing 50 mM CaCl₂ and 10 μl of 1-DOPA (3-(3,4-dihydroxyphenyl)-l-alanine, Fluka, final concentration 1.5 mM) was incubated at room temperature for 6 h in the absence or presence of tested compounds (1 μg/ml). The oxidation of 1-DOPA was measured at 492 nm and expressed as the difference between the values without and with Pefabloc.

In some experiments, M. lysodeikticus and E. coli suspensions or M. lysodeikticus peptidoglycan were preincubated with hen egg lysozyme or trypsin (1 μg/ml bacteria or 1 μg/ml peptidoglycan, up to 5 h), or with coelomic fluid proteins (1 mg/ml bacteria or 1 mg/ml peptidoglycan, up to 12 h) before testing the activation of the pro-PO cascade.

CCF-depleted coelomic fluid was obtained by incubating the coelomic fluid with an anti-CCF immunoaffinity column for 1 h at 4 °C (2, 3). After centrifugation, CCF-depleted coelomic fluid was used in L-DOPA oxidation test as described above. To reconstitute the pro-PO-activating cascade, equivalent concentrations of recombinant CCF or CCF peptides (25 pmol/ml) were added to CCF-depleted coelomic fluid before testing 1-DOPA oxidation.

Glucan and N,N′-Diacytethylchitobiose Binding Assay—CCF and CCF peptides in TN buffer (25 mM in 500 μl) were incubated for 1 h at 4 °C with curdian particles (10 mg/ml; 10 μl) or with agarose-insolubilized N,N′-diacytethylchitobiose (50 μl). After washings in phosphate-buffered saline (1 ml, 13 000 × g, 10× for 2 min) the bound material was eluted in 50 μl of 0.0625 M Tris-HCl buffer, pH 6.8, containing 2% SDS, 20% glycerol, and 5% β-mercaptoethanol, boiled (6 min) and subjected to SDS-polyacrylamide gel electrophoresis (12%). To investigate the specificity of the interaction with curdian or N,N′-diacytethylchitobiose, CCF peptides were preincubated with soluble laminarin or N,N′-diacytethylchitobiose (100 μg/100 μl; 30 min at 4 °C) before testing the ability to bind curdian or agarose-insolubilized N,N′-diacytethylchitobiose as described above. Coomasie Blue-stained proteins were quantified densitometrically using Analytical Imaging Station software (Imaging Research Inc., St. Catharines, Ontario, Canada).

Data Analysis—Data (mean of triplicate ± S.D.) are representative of three independent experiments performed. The validity of the results was assessed by Student’s t test.
**RESULTS**

**Pattern Recognition Specificity of E. foetida CCF**—The coelomic fluid of *E. foetida* earthworms displays phenoloxidase (PO) activity within 6 h upon recognition of yeast or Gram-negative bacteria (3, 15). With a similar experimental procedure, Gram-positive bacteria did not trigger the coelomic fluid PO activity. However, the coelomic fluid of *E. foetida* was described to display lysozyme-like activity, with 50 µg of coelomic fluid proteins exhibiting an activity comparable with 1 µg of hen egg lysozyme in *M. lysodeikticus* bacteriolytic assay (20, 21). Therefore, we tested whether PO activity could be triggered in *E. foetida* coelomic fluid by *M. lysodeikticus* pretreated with coelomic fluid or hen egg lysozyme. As shown in Fig. 1A, whereas intact *M. lysodeikticus* did not trigger detectable PO activity within 6 h of incubation in coelomic fluid, bacteria pretreated for 12 h did so with considerable efficiency. In addition, bacteria pretreated for 5 h with lysozyme, but not with trypsin (up to 12 h), activated the pro-PO cascade in *E. foetida* coelomic fluid. Similar results were observed with *B. subtilis.*

In contrast, Gram-negative *E. coli* suspension triggered similar levels of pro-PO activity whether or not predigested with lysozyme or coelomic fluid proteins (Fig. 1A). On the other hand, peptidoglycan purified from the *M. lysodeikticus* cell wall did not induce PO activity unless preincubated with hen egg lysozyme for 5 h or with coelomic fluid for 12 h (Fig. 1B). Because lysozyme hydrolyzes the β-1,4-glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid of peptidoglycan, we tested the ability of synthetic peptidoglycan analogs (muramyl dipeptide, disaccharide dipeptide, muramic acid, pentapeptide, and *N*-acetylmuramic acid) to trigger the pro-PO activation in *E. foetida* coelomic fluid. Muramyl residue-containing analogs and muramic acid efficiently activated the pro-PO cascade, whereas pentapeptide and *N*-acetylmuramic acid did not (Fig. 2). The PO-inducing activity of Gram-positive bacteria, or peptidoglycan digested with lysozyme or coelomic fluid proteins, of muramyl dipeptide, disaccharide dipeptide, and muramic acid (Fig. 2) was abolished in CCF-depleted coelomic fluid and reconstituted by adding recombiant CCF.

Together, these data indicate that *E. foetida* CCF displays a broad pattern recognition specificity for yeast as well as Gram-negative and Gram-positive bacteria.

**Pattern Recognition Specificity of L. terrestris CCF** —The coelomic fluid of another oligochaete species, *L. terrestris,* contains a 42-kDa protein cross-reacting with monoclonal antibody elicited against *E. foetida* CCF, indicating the existence of a CCF-like molecule. PO activity was induced by LPS and laminarin (β-1,3-glucan) in *L. terrestris* coelomic fluid (Fig. 3). However, in contrast to *E. foetida,* neither *N*,*N*-diacetylmalto-

biose (GlcNAc-β-1,4-GlcNAc), nor peptidoglycan hydrolyzed with lysozyme or coelomic fluid proteins, nor synthetic peptidoglycan analogs (muramic acid, muramyl dipeptide, disaccharide dipeptide, pentapeptide, or *N*-acetylmuramic acid) activated the PO cascade in *L. terrestris* coelomic fluid. These results suggest that *L. terrestris* CCF has a narrower pattern recognition specificity than *E. foetida* CCF.

**Comparison of the Primary Structure of E. foetida and L. terrestris CCF Molecules**— *L. terrestris* CCF-like molecule was cloned, and the amino acid sequence was compared with the sequence of *E. foetida* CCF (Fig. 4). The two molecules displayed 91% of homology, with identity in the putative polysaccharide-binding motif of bacterial glucanase (7, 22) and in the putative catalytic site of bacterial endo-1,3(4)-β-glucosidase (23). Both *E. foetida* and *L. terrestris* CCF molecules contained three conserved cysteine residues and a remarkably high number of tryptophan residues (7%), the majority of which are located in the C-terminal part of the molecule.

Peptide constructs covering the two putative functional regions of CCF, as well as the tryptophan-rich domain, were expressed in bacteria to identify the carbohydrate recognition
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| Binding to curdian | Inhibition with laminarin | Binding to N,N'-diacetylchitobiose | Inhibition with N,N'-diacetylchitobiose |
|---------------------|---------------------------|------------------------------------|----------------------------------------|
| E. foetida          | L. terrestris             | E. foetida                          | L. terrestris                          |
| CCF                 | +                         | +                                   | +                                     |
| Peptide 1           | +                         | ND*                                | ND*                                   |
| Peptide 2           | +                         | ND                                  | ND*                                   |
| Peptide 3           | –                         | +                                   | +                                     |
| Peptide 4           | –                         | +                                   | +                                     |
| Peptide 5           | –                         | ND                                  | ND*                                   |

* Mean inhibition of triplicates, representative of three independent experiments; S.D. were <10% of the mean inhibition.

ND, not done.

TABLE II: Binding of CCF peptides to insoluble β-1,3-glucans or insolubilized N,N'-diacetylchitobiose

E. foetida or L. terrestris CCF and CCF peptides, used as such or preincubated with soluble laminarin or N,N'-diacetylchitobiose, were incubated with curdian (insoluble β-1,3-glucan) or with agarose-insolubilized N,N'-diacetylchitobiose. Bound material was eluted, subjected to SDS-polyacrylamide gel electrophoresis, and Coomassie Blue-stained. The intensity of proteins eluted without or after preincubation with laminarin or N,N'-diacetylchitobiose were compared to determine the percent binding inhibition.

Identification of CCF Pattern Recognition Domains Involved in Prophenoloxidase Activation

The binding experiments indicated that distinct domains of CCF recognized different saccharide moieties. This assumption was tested in the pro-PO activation test.

E. foetida coelomic fluid, but not CCF-depleted coelomic fluid, exhibited PO activity upon activation with LPS, laminarin, N,N'-diacetylchitobiose, and muramic acid (Table III). In CCF-depleted coelomic fluid, the PO activity was completely reconstituted in the presence of the four triggering agents by adding ECCF or E2. Peptides E1 and E4 containing polysaccharide-binding and glucanase motifs reconstituted the pro-PO cascade only in the presence of LPS and laminarin, whereas E5 covering the C-terminal part of ECCF restored the PO activity triggered by N,N'-diacetylchitobiose and muramic acid. The CCF N-terminal part E3 did not restore the PO activity assessed by any of the triggering agents in CCF-depleted coelomic fluid.

As mentioned above (Fig. 3), the pro-PO cascade was activated in the coelomic fluid of L. terrestris by LPS and laminarin but not by N,N'-diacetylchitobiose or muramic acid (Table IV). LCCF, L2, and L4 reconstituted the sensitivity to LPS and laminarin in L. terrestris CCF-depleted coelomic fluid. Interestingly, ECCF, E2, and E5 conferred the ability to activate the pro-PO cascade in L. terrestris CCF-depleted coelomic fluid by N,N'-diacetylchitobiose and muramic acid.

Collectively, these data suggest that CCF consists of two distinct carbohydrate recognition domains. The first (amino acids 149–227), surrounding the polysaccharide and glucanase motifs, mediates interaction of CCF with β-1,3-glucans and LPS. The second domain located in the C-terminal region of CCF recognizes β-1,4-N-acetylglucosamine-linked saccharides and muramic acid in E. foetida CCF only. The reconstitution of PO activity in CCF-depleted L. terrestris coelomic fluid by E. foetida CCF peptides suggests that all components triggering the pro-PO cascade except the pattern recognition specificity of CCF are common to both earthworm species.

DISCUSSION

CCF was previously described to recognize cell wall components of yeast and Gram-negative bacteria that trigger the activation of pro-PO cascade in the coelomic fluid of E. foetida earthworm (3). Under similar experimential conditions, we did not detect activation of pro-PO with intact Gram-positive bacteria. Furthermore, it was reported that CCF agglutinated smooth Gram-negative bacteria, but not rough Gram-negative bacteria or Gram-positive bacteria (3). Here we show that preincubation of Gram-positive bacteria with lysozyme triggers the pro-PO cascade in E. foetida coelomic fluid. Lysozyme treatment does not affect the pro-PO activation by Gram-negative bacteria. The coelomic fluid of E. foetida earthworm exhibits a lysozyme-like activity mediated by a 13-kDa protein displaying considerable homology to other invertebrate lysozymes (20, 21). Accordingly, preincubation of Gram-positive bacteria with E. foetida coelomic fluid for 12 h resulted in pro-PO activation comparable with 5-h lysozyme pretreatment.

Trypsin pretreatment has no effect on the inability of Gram-positive bacteria to induce pro-PO cascade suggesting that the β-1,4-N-acetylmuramidase activity of lysozyme rather than proteolysis allows the recognition of Gram-positive bacteria cell wall components by earthworm defense molecule(s). Similarly, peptidoglycan, but not the synthetic peptidoglycan analogs muramyl dipeptide, disaccharide dipeptide, or muramic acid, required lysozyme pretreatment to trigger l-DOPA oxidation in E. foetida coelomic fluid. Moreover, synthetic pentapeptide did not exhibit significant PO-inducing activity. Hence, it can be suggested that recognition of peptidoglycan saccharide moi-
TABLE III
Reconstitution of pro-PO cascade in E. foetida CCF-depleted coelomic fluid by E. foetida CCF peptides

Pro-PO activation triggered by LPS, laminarin, N,N'-diacetylchitobiose, and muramic acid was determined in E. foetida coelomic fluid (CF) or in CCF-depleted coelomic fluid reconstituted with E. foetida CCF peptides (see Fig. 5). Data are shown as mean of triplicates ± S.D., representative of three independent experiments.

| E. foetida | CF | Depleted CF | Depleted CF + E1 | Depleted CF + E2 | Depleted CF + E3 | Depleted CF + E4 | Depleted CF + E5 |
|------------|----|-------------|------------------|------------------|------------------|------------------|------------------|
| LPS        | 0.618 ± 0.086 | 0.058 ± 0.016$^a$ | 0.426 ± 0.063 | 0.406 ± 0.048 | 0.406 ± 0.051 | 0.044 ± 0.015$^a$ | 0.372 ± 0.073 | 0.074 ± 0.023$^a$ |
| Laminarin  | 0.564 ± 0.061 | 0.048 ± 0.013$^a$ | 0.395 ± 0.071 | 0.392 ± 0.055 | 0.448 ± 0.073 | 0.059 ± 0.019$^a$ | 0.381 ± 0.059 | 0.085 ± 0.031$^a$ |
| N,N'-Diacetylchitobiose | 0.486 ± 0.054 | 0.044 ± 0.022$^a$ | 0.420 ± 0.054 | 0.046 ± 0.017$^a$ | 0.405 ± 0.063 | 0.052 ± 0.017$^a$ | 0.057 ± 0.019$^a$ | 0.379 ± 0.055 |
| Muramic acid | 0.631 ± 0.089 | 0.054 ± 0.021$^a$ | 0.431 ± 0.059 | 0.077 ± 0.021$^a$ | 0.448 ± 0.077 | 0.061 ± 0.027$^a$ | 0.061 ± 0.021$^a$ | 0.409 ± 0.067 |

$^a$ Lower than the CF values ($p < 0.01$).

TABLE IV
Reconstitution of pro-PO cascade in L. terrestris CCF-depleted coelomic fluid by L. terrestris or E. foetida CCF peptides

Pro-PO activation triggered by LPS, laminarin, N,N'-diacetylchitobiose, and muramic acid was determined in L. terrestris coelomic fluid (CF) or in CCF-depleted coelomic fluid reconstituted with L. terrestris or E. foetida CCF (see Fig. 5). Data are shown as mean of triplicates ± S.D., representative of three independent experiments.

| L. terrestris | CF | Depleted CF | Depleted CF + LCCF | Depleted CF + E1 | Depleted CF + E2 | Depleted CF + L4 | Depleted CF + E5 |
|---------------|----|-------------|-------------------|------------------|------------------|------------------|------------------|
| LPS           | 0.468 ± 0.088 | 0.073 ± 0.019$^a$ | 0.402 ± 0.099 | 0.366 ± 0.057 | 0.461 ± 0.089 | 0.385 ± 0.101 | 0.389 ± 0.057 | 0.081 ± 0.019$^a$ |
| Laminarin     | 0.414 ± 0.073 | 0.069 ± 0.015$^a$ | 0.441 ± 0.071 | 0.392 ± 0.084 | 0.421 ± 0.077 | 0.386 ± 0.079 | 0.407 ± 0.095 | 0.096 ± 0.025$^a$ |
| N,N'-Diacetylchitobiose | 0.081 ± 0.023 | 0.074 ± 0.015 | 0.087 ± 0.019 | 0.355 ± 0.057$^b$ | 0.082 ± 0.019 | 0.372 ± 0.073$^b$ | 0.076 ± 0.019 | 0.475 ± 0.102$^b$ |
| Muramic acid   | 0.075 ± 0.021 | 0.079 ± 0.027 | 0.092 ± 0.023 | 0.348 ± 0.078$^b$ | 0.091 ± 0.027 | 0.468 ± 0.103$^b$ | 0.091 ± 0.021 | 0.401 ± 0.078$^b$ |

$^a$ Lower than the CF values ($p < 0.01$).

$^b$ Higher than the CF values ($p < 0.01$).
eties rather than recognition of amino acid determinants by earthworm defense molecule(s) is required to elicit pro-PO activation. The PO-inducing activity of muramyl acid, muramyl dipeptide, and disaccharide dipeptide was abolished in CCF-depleted coelomic fluid and was recovered by recombinant CCF. Hence, CCF plays a central role in the activation of the pro-PO cascade in E. foetida coelomic fluid, recognizing, besides yeast and Gram-negative bacteria cell wall components (3, 15), Gram-positive bacteria cell wall components, at least muramyl acid.

The coelomic fluid of the related earthworm species L. terrestris displays distinct biochemical properties as compared with E. foetida coelomic fluid reflecting most likely the antigenicity of the biotope where they live (soil versus compost). For example, proteolytic and hemolytic activities are considerably lower in L. terrestris than in E. foetida coelomic fluid (24, 25). Moreover, in contrast to the E. foetida coelomic fluid (2, 15), the coelomic fluid of L. terrestris does not display lytic activity against murine cell lines or African trypanosomes.3 Furthermore, here we report that the pro-PO cascade in L. terrestris coelomic fluid can be triggered by β-1,3-glucans and LPS but not by peptidoglycan treated with lysozyme, muramyl acid, or N,N′-diacetylchitobiose. These results indicate that the specificity and/or function of pattern recognition molecules in E. foetida and L. terrestris differ. The comparison of the amino acid sequence of CCF from E. foetida and L. terrestris revealed 91% homology with a complete identity in the putative polysaccharide-binding motif and in the catalytic region of bacterial and animal endo-1,3(4)-β-endoglucosidasidases (7, 22, 23). These two regions are highly conserved in invertebrate pattern recognition molecules homologous to CCF that were reported to bind LPS or β-1,3-glucans (14). The remarkable number of tryptophan residues located mainly in the C-terminal part of CCF is of particular interest, because tryptophan residues were described to be important for the recognition of N-acetyl saccharides such as N,N′-diacetylchitobiose by tachylectin-2, a defense molecule from the horseshoe crab T. tridentatus (26).

The crucial role of polysaccharide-binding and glucanase motifs in the recognition of LPS and β-1,3-glucans, and of the C-terminal part of CCF for the recognition of β-1,4-linked N-acetyl saccharides was confirmed in binding experiments and in the pro-PO activating assay using recombinant CCF-derived molecules from both E. foetida and L. terrestris. Based on these data, we propose the existence of distinct functional carbohydrate recognition domains within the CCF. The domain comprising amino acids 149–227, present in both E. foetida and L. terrestris, binds LPS and β-1,3-glucans from Gram-negative bacteria and yeast, respectively. The C-terminal domain (amino acids 227–384), showing high divergence in E. foetida and L. terrestris CCF, displays specificity for the structurally related β-1,4-linked N-acetyl saccharides N,N′-diacetylchitobiose, muramyl dipeptide, and muramyl acid. Thus, this tryptophan-rich domain allows the recognition of cell wall components of yeast or Gram-positive bacteria by E. foetida but not by L. terrestris CCF. Mutations in the C-terminal part may be responsible for the inability of L. terrestris CCF to bind muramyl acid or N,N′-diacetylchitobiose. The existence of two distinct carbohydrate recognition domains may extend the protection of E. foetida against a variety of microorganisms or facilitate the interaction of immunocytes with the pathogen and subsequent induction of cellular defense. Indeed, one domain of CCF may interact with the pathogen, while the second domain may interact with a saccharide moiety on a putative immuneocyte receptor (27–30). Alternatively, the N-terminal part of CCF (amino acids 1–148) could allow the binding to a putative receptor mediating its interaction with immunocytes upon pathogen recognition.

Peptidoglycan recognition proteins have been described from insects to vertebrates, and the level of homology points to their common origin (31). Vertebrate peptidoglycan recognition proteins inhibit the growth of Gram-positive bacteria (32). In insects, peptidoglycan recognition proteins are often involved in activation of the pro-PO cascade (33, 34). Despite its analogous function, CCF does not show primary structure homology with any described peptidoglycan recognition proteins. In addition, CCF displays no amino acid homology with lysozymes, Toll-like receptors, or vertebrate CD14. Although it recognizes LPS and β-1,3-glucans through the polysaccharide-binding and glucanase motifs as other invertebrate LPS- and β-1,3-glucan-binding proteins, CCF may recognize peptidoglycan by a different mechanism from the described peptidoglycan recognition proteins. The latter bind peptidoglycan in the absence of lysozyme treatment, whereas CCF requires lysozyme-like activity to recognize peptidoglycan. CCF also differs from other peptidoglycan recognition proteins in the specificity for peptidoglycan constituents. The minimal peptidoglycan structure required to induce silkworm antibacterial defenses consists of two N-acetylmuramoyl-N-acetylmuramic acid (muramyl-dipeptide) units with peptide side chains (35). Similarly, CD14 binds specifically to (muramyl-dipeptide), as a minimal structure of peptidoglycan (36, 37). CCF recognizes soluble muramyl-dipeptide initiating innate defense reactions against Gram-positive bacteria in E. foetida coelomic fluid.

In conclusion, we have provided evidence for the presence of two distinct functional domains in an invertebrate defense molecule, allowing the recognition of yeast and Gram-negative and Gram-positive bacteria.

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**Defense Molecule Recognizes Gram-negative and -positive Bacteria**

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Martin Bilej, Patrick De Baetselier, Els Van Dijck, Benoit Stijlemans, Alain Colige and Alain Beschin

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