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Report

Nimrod, a Putative Phagocytosis Receptor with EGF Repeats in *Drosophila* Plasmatocytes

Éva Kurucz,[1] Róbert Márkus,[1] János Zsámboki,[1] Katalin Folkl-Medzihradszky,[1,2] Zsuzsanna Darula,[1] Péter Vilmos,[1] Andor Udvardy,[1] Ildikó Krausz,[1] Tamás Lukacsovich,[3] Elisabeth Gateff,[4] Carl-Johan Zettervall,[5] Dan Hultmark,[5,*] and István Andó[1,*]

[1] Institute of Genetics
Biological Research Center of the Hungarian Academy of Sciences
P.O. Box 521
H-6701 Szeged
Hungary

[2] Department of Pharmaceutical Chemistry
University of California San Francisco
San Francisco, California 94143

[3] Department of Developmental and Cellular Biology
Developmental Biology Center
University of California Irvine
Irvine, California 92697

[4] Institut für Genetik
Johannes Gutenberg Universität
Saarstrasse 2100
D-65099 Mainz
Germany

[5] Umeå Centre for Molecular Pathogenesis
By. 6L
Umeå University
S-901 87 Umeå
Sweden

Summary

The hemocytes, the blood cells of *Drosophila*, participate in the humoral and cellular immune defense reactions against microbes and parasites [1–8]. The plasmatocytes, one class of hemocytes, are phagocytically active and play an important role in immunity and development by removing microorganisms as well as apoptotic cells. On the surface of circulating and sessile plasmatocytes, we have now identified a protein, Nimrod C1 (NimC1), which is involved in the phagocytosis of bacteria. Suppression of NimC1 expression in plasmatocytes inhibited the phagocytosis of *Staphylococcus aureus*. Conversely, overexpression of NimC1 in S2 cells stimulated the phagocytosis of both *S. aureus* and *Escherichia coli*. NimC1 is a 90–100 kDa single-pass transmembrane protein with ten characteristic EGF-like repeats (NIM repeats). The *nimC1* gene is part of a cluster of ten related *nimrod* genes at 34E on chromosome 2, and similar clusters of *nimrod*-like genes are conserved in other insects such as *Anopheles* and *Apis*. The Nimrod proteins are related to other putative phagocytosis receptors such as Eater and Draper from *D. melanogaster* and CED-1 from *C. elegans*. Together, they form a superfamily that also includes proteins that are encoded in the human genome.

*Correspondence: dan.hultmark@ucmp.umu.se (D.H.), ando@brc.hu (I.A.)

Results and Discussion

Identification of P1 as a Plasmatocyte-Specific Antigen

We have previously generated a set of monoclonal antibodies that define hemocyte subsets and identify hemocyte-specific molecules [9, 10], putative regulators of hemocyte development and function. Here, we have used antibodies for a plasmatocyte-specific antigen, P1, to identify a novel transmembrane protein. P1 is defined by two monoclonal antibodies, P1a [10] and P1b, which recognize two different epitopes on the same antigen (see Figure S1 in the Supplemental Data available online). This antigen is present in a subpopulation of circulating hemocytes in the *l(3)mbn-1* hemocyte-overproducing mutant [11]. Indirect immunofluorescence analysis of live larval hemocytes shows that both epitopes are expressed on the cell surface on the majority of larval hemocytes, all with plasmatocyte morphology, and are absent on the lamellocytes and the crystal cells, the two other major classes of larval hemocytes (Figure 1A). FACS analysis showed that P1 is present on approximately 80% of the circulating hemocytes in *l(3)mbn-1* larvae, corresponding to the plasmatocyte fraction in this mutant, which spontaneously produces large numbers of all three classes of hemocytes (Figure 1B). It is found on approximately 90% of the hemocytes in first instar Oregon-R larvae immediately after hatching, 94% in second instar, and 97% in late third instar larvae (Figure 1C). It is also expressed in the adult, although it is essentially absent from the sessile population at that stage. We did not detect P1 in embryonic hemocytes (data not shown). The P1-positive cells phagocytose bacteria (Figure 1G) and produce antimicrobial peptides (data not shown), suggesting that they are involved in the antimicrobial defense. Cells with lamellocyte morphology, lacking P1, are phagocytically inactive ("L" in Figure 1G).

Most larval hemocytes are believed to originate directly from a population of embryonic hemocytes [12], although evidence has been presented that a second population of hemocytes develops in a specialized hematopoietic tissue, the lymph glands. In late third instar larvae, we found P1 expressed in islands of cells in the anterior lobes of the lymph glands (Figure 1D) as well as in islands of sessile subepidermal hemocytes (Figure 1E). Because actively dividing P1-positive cells were also found among the circulating hemocytes (Figure 1F), we conclude that plasmatocytes, defined as P1-positive cells, are produced in both populations of larval hemocytes.

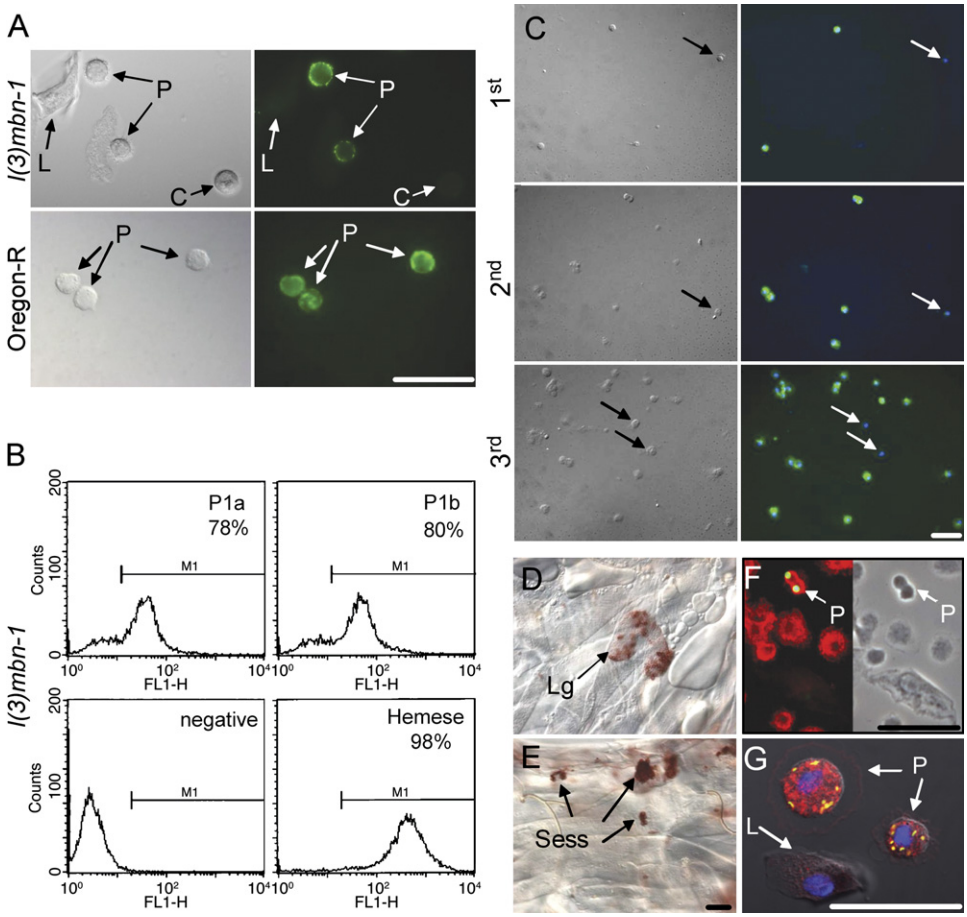

Figure 1. Tissue Localization of the P1 Antigen

(A) Immunofluorescent staining of P1 on circulating native hemocytes with plasmatocyte morphology, isolated from larvae of the *l(3)mbn-1* hemocyte-overproducing mutant or Oregon-R. Hemocytes were stained with the mixed P1a and P1b antibodies and anti-mouse FITC conjugate.

(B) FACS analysis of larval hemocytes stained as in (A), with either the P1a and P1b antibodies separately or with a negative hybridoma tissue culture supernatant or Hemese antibody [9] as controls.

(C) Immunofluorescent analysis of acetone-fixed circulating hemocytes derived from first, second, and third instar Oregon-R larvae. Staining was performed as in (A) except that nuclei were stained with DAPI. Arrows indicate nuclei of P1-negative cells.

(D–E) Immunohistochemical staining of the P1 antigen in fixed lymph glands (in [D], Lg) and sessile hemocytes (in [E], Sess) of Oregon-R larvae. Tissues were treated with the P1 antibodies and visualized by immunohistochemistry [10].

(F) Mitotically active hemocytes expressing the P1 antigen (indicated by arrows). Hemocytes of *l(3)mbn-1* larvae were adhered to microscopic slides, fixed, and reacted with P1 and phosphohistone H3 (PhH3) antibodies. The reactions were visualized with anti-rabbit FITC conjugate for PhH3 (shown in green) and with biotinylated anti-mouse/Streptavidin-Cy3 for P1 (shown in red).

(G) Phagocytically active hemocytes express the P1 antigen. Hemocytes of *l(3)mbn-1* mutant larvae were incubated with FITC-labeled *S. aureus* bacteria, extensively washed, and then adhered, fixed, and reacted with P1 antibodies; this was followed by incubation with biotinylated anti-mouse/Streptavidin-Cy3 (shown in red). Nuclei were stained with DAPI (shown in blue). The panel shows a merge of Nomarski and confocal images. The bacteria appear yellow when they overlap with P1 staining. Under these conditions, essentially all bacteria associated with the hemocytes are internalized, as shown in the confocal section. Abbreviations are as follows: P, plasmatocytes; L, lamellocytes; and C, crystal cells. Scale bars represent 20 μm.

The P1 Antigen Is Encoded by the *nimrod C1* Gene

We used a mixture of the two P1-specific antibodies to isolate the P1 antigen by immunoprecipitation (Figure 2A). A 90–100 kDa silver-stained protein band, corresponding to the P1 antigen, was excised, digested with trypsin, and analyzed with MALDI-TOF (matrix assisted laser desorption ionization time-of-flight) mass spectrometry. A peptide with MH^+ at m/z 912.54 (data not shown) identified a sequence, VIPYQHR from a predicted *Drosophila* gene, *CG8942*, hereafter called *nimrod C1* (*nimC1*). A single sequenced cDNA clone, LP05465 (accession AY119029), defines a 2040 nucleotide *nimC1* transcript. It encodes a 620 amino acid open reading frame, NimC1, with an N-terminal signal peptide and then a putative single-pass transmembrane protein of 65 kDa. The larger size observed on the western blot indicates that NimC1 may be glycosylated.

To verify that the *nimC1* gene encodes P1, we expressed it in the P1-negative *Drosophila* cell line, Schneider-2 (S2), under the control of an inducible promoter. The NimC1 protein could be detected on the plasma membrane of live transfected S2 cells with the antibodies P1a and P1b (Figures 2C and 2D and Figure S1A). The overexpressed protein is slightly smaller (Figure 2B, lanes 1 and 3) than the antigen found in l(3)mbn-1 hemocytes (Figure 2A), presumably because of differences in glycosylation. No signal was detected in cells transfected with the empty vector (Figure 2B,

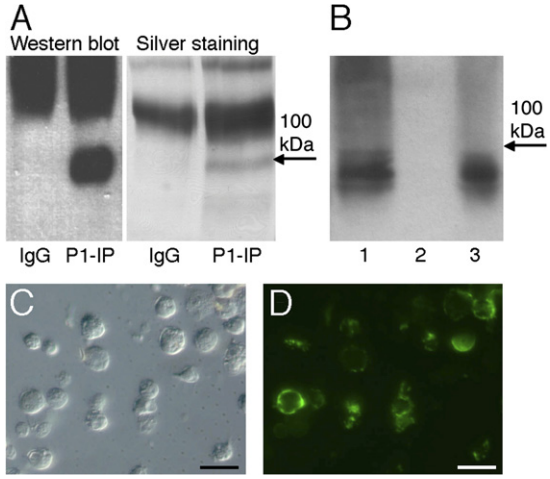

Figure 2. Isolation of the P1 Antigen and Confirmation that It Is a Product of the *nimC1* Gene

(A) Western-blot analysis and silver staining of the P1 antigen, immunoprecipitated from a hemocyte extract of third instar *l(3)mbn-1* larvae. The blotted samples were stained with a P1 antibody mixture and anti-mouse-immunoglobulin/horseradish peroxidase conjugate and visualized by chemoluminescence.

(B) Detection of expressed NimC1 protein in the transfected S2 cell line by western-blot analysis with a P1a + P1b antibody mixture. Lanes 1 and 3 are cell extracts from two independent pMT-*nimC1*/V5-HisA vector transfectants, and lane 2 is an extract from cells transfected with the pMT-V5-HisA empty vector.

(C) Nomarski image of live S2 cells transfected with the pMT-*nimC1*/V5-HisA vector.

(D) Detection of the transiently expressed NimC1 protein by indirect immunofluorescence of the same cells with a mixture of P1a and P1b antibodies and anti-mouse FITC conjugate. Scale bars represent 20 μm.

lane 2; Figure S1A). Overexpression of NimC1 made the S2 cells highly adherent. They formed aggregates, and their growth was retarded (data not shown).

We further verified the identity of the P1 antigen by silencing the *nimC1* gene product by RNAi. We expressed a UAS-*nimC1*-IR hairpin construct in transgenic larvae by using the hemocyte-specific *Hemese*-Gal4 (He-Gal4) driver [13]. This resulted in a significant decrease of the P1 expression on the plasmatocytes (Figure 3A). Two other independent transgenic lines gave similar results (data not shown). However, P1 is still expressed in a minor population of cells, probably corresponding to the approximately 20% of the hemocytes that do not express the He-Gal4 driver [13]. FACS analysis confirms that P1 expression on the cell surface is correspondingly reduced in the He-GAL4xUAS-*nimC1*-IR cross compared to the parental He-GAL4 or UAS-*nimC1*-IR stocks (Figure 3B). Hemese antigen expression was normal (data not shown). The results of these loss-of-function and gain-of-function experiments clearly show that the P1 antigen is a product of the *nimC1* gene.

NimC1 Is Involved in Phagocytosis

Because NimC1 is expressed exclusively in phagocytic cells, we tested its possible role in phagocytosis. Initial attempts to block phagocytosis in Oregon-R hemocytes with P1 antibodies were negative (data not shown), but when we suppressed NimC1 expression with the hairpin construct, we saw a dramatic effect on the phagocytic capacity of the plasmatocytes. The phagocytic index for *Staphylococcus aureus* bacteria decreased to approximately one-third of the controls (Figure 3C). However, the phagocytosis of *Escherichia coli* was not significantly affected in these experiments (data not shown). NimC1 suppression had no effect on the gross binding of bacteria at 4°C, showing that NimC1 is involved in phagocytosis but contributes little to the overall adhesion of bacteria to the cells. Further evidence for the role of NimC1 in phagocytosis was obtained by $CuSO_4$-induced overexpression of NimC1 in Schneider-2 cells, a cell line that does not express NimC1 (Figure 2B, lane 2; Figure S1). This stimulated the uptake of both *S. aureus* and *E. coli* (Figure 3D, top panels). The mean fluorescence intensity of the whole (M3 gated) population increased by 1.91-fold for *E. coli* and by 2.45-fold for *S. aureus*. From the experiments in Figures 3C and 3D, we conclude that NimC1 is a major factor in the phagocytosis of *S. aureus* by plasmatocytes and that it may also play a redundant role in the phagocytosis of *E. coli*. The fact that NimC1 is not expressed in S2 cells may explain why it was not detected in previous screens for phagocytosis receptors.

Like in the suppression experiment described above, NimC1 overexpression did not significantly affect the total binding of bacteria to the cell surface (Figure 3D, bottom panels). The background of nonspecific adhesion of bacteria to the cell surface may have obscured the binding to NimC1. Alternatively, it is possible that NimC1 may act indirectly, as a coreceptor or at a later stage in the phagocytic process.

A Family of *nimrod* Genes Encode Proteins with Characteristic EGF-like Repeats

The extracellular region of the NimC1 protein has ten repeats of an EGF-like motif with six cystein residues; the motif is followed by a nonrepetitive cystein-rich domain, a predicted transmembrane domain, and a short intracellular domain (Figure 4B). The EGF-like repeats, here called NIM repeats, have a well-conserved consensus sequence CxPxCxxxCxNGxCxxPxxCxCxxGY and are separated by variable loops of typically 6–11 residues (see Table S1). This motif differs significantly from the typical EGF repeat, $xxxxCx_{2\text{-}7}Cx_{1\text{-}4}(G/A)xCx_{1\text{-}13}ttaxCx$-$CxxGax_{1\text{-}6}GxxCx$ [14, 15], and is shifted by one cystein unit compared to the latter.

The *nimC1* gene is located at 34E5 on chromosome 2, immediately 5′ of the hemocyte-specific *Hemese* gene, and within a cluster of ten genes that all have NIM repeats (Figure 4A). These *nimrod* genes also share with *nimC1* a short conserved motif, CCxGY, immediately preceding the first NIM repeat. Similar sequences are also present in the *Drosophila* genes *draper* at 62B, *CG7447* at 64B, and *eater* at 97E.

The ten *nimrod* genes encode three different classes of proteins (Figure 4B). The *nimA* gene encodes a protein with an EMI domain, a possible protein-protein interaction module that was first named after its presence in proteins of the EMILIN family [16]. The EMI domain is followed by a single NIM repeat, two copies of another atypical EGF-like repeat with eight cysteins, a putative transmembrane region, and a relatively large intracellular domain. A similar arrangement is found in the products of the *ced-1* gene in *C. elegans* and *draper* in

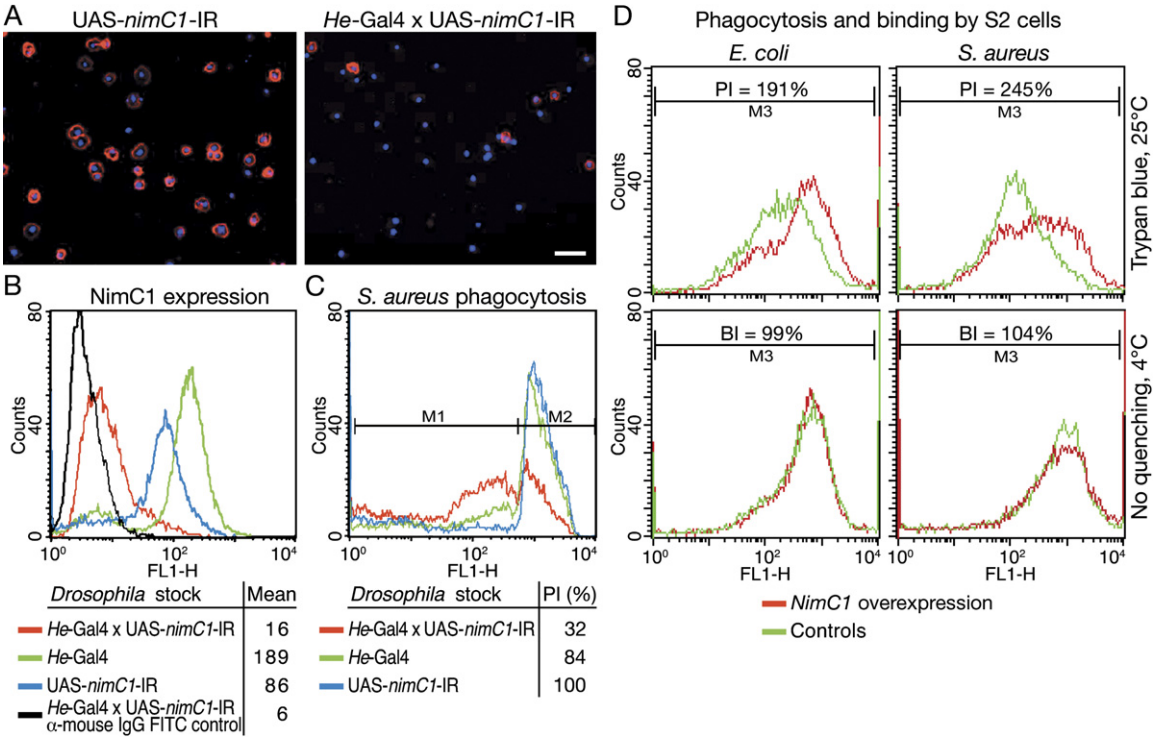

Figure 3. NimC1 Is Required for Phagocytosis

(A) Fluorescence microscopy and (B) FACS analysis of hemocytes isolated from He-GAL4xUAS-*nimC1*-IR#102 larvae and of the parental strain containing the transgene UAS-*nimC1*-IR#102; these analyses show that NimC1 expression is suppressed by this hairpin construct. The cells were incubated with the P1a and P1b antibodies and then stained with (A) biotinylated anti-mouse/Streptavidin-Cy3 or (B) FITC anti-mouse IgG, respectively. Scale bars represent 20 μm. In the FACS analysis (B), the mean fluorescence intensity values were compared for quantification of the difference of NimC1 expression of parental stocks and the He-GAL4xUAS-*nimC1*-IR#102 strain. (C) shows phagocytosis of FITC-labeled *S. aureus* bacteria by the NimC1-deficient RNAi strain and the parental strains. Hemocytes were incubated with FITC-labeled bacteria and analyzed by FACS. The phagocytic index (PI, see [19]) was calculated as the fraction of cells that phagocytose (the gated M2 population), multiplied by the mean intensity of that population. The value for the UAS-*nimC1*-IR control is set to 100%. (D) shows phagocytosis (top panels) and total binding (bottom panels) of fluorescently labeled *E. coli* and *S. aureus* bacteria by Schneider S2 cells that overexpress NimC1 (shown in red). The controls were *NimC1*-transfected but uninduced cells (shown in green). The phagocytic index (PI) or binding index (BI) was calculated as above from the whole population (M3) because essentially all cells had phagocytosed or bound bacteria.

Drosophila. By contrast, the *nimB1-nimB5* gene products lack membrane anchors and are probably exported. They have one to eight NIM repeats and share a weakly conserved sequence at the amino terminal (see Figure S2), but they have no other known motifs. Finally, *nimC1-nimC4* gene products represent a third class. They are transmembrane proteins, with the possible exception of *nimC3* for which we have not yet identified a 3′ exon. They have 2–16 NIM repeats and also show additional sequence conservation at the amino terminal (Figure S2). RT-PCR assays indicate that all *nimrod* genes except *nimA* are transcribed in hemocytes, both in wild-type larvae and in the hemocyte-overproducing *l(3)mbn-1* mutant (data not shown).

Homologous *nimrod* gene clusters with similar genomic organization can also be identified in the sequenced genomes of the mosquito, *Anopheles gambiae* [17] and the honeybee, *Apis mellifera* [18] (see Figure S3). However, the number of *nimrod* homologs is smaller in *Anopheles* and *Apis*: one *nimA*-like, one *nimB*-like, and two *nimC*-like genes, but no *Hemese* homolog. The class-specific N-terminal motifs are also present, except in the *Anopheles nimC*-like genes (Figure S2). Homologs of *draper* and *CG7447* can also be found in *Anopheles* and *Apis*, and there is a possible *eater* homolog in the mosquito but not in the honeybee (Figure S3).

A Superfamily of Proteins Implicated in Phagocytosis and Microbial Binding

A database search shows that proteins with a CCxGY motif, followed by one or several NIM repeats, can be found in many organisms, including man. Their functions are known in a few cases, all of them related to phagocytosis, microbial binding, or both. Recently, Kocks et al. [19] showed that the *Drosophila* protein Eater is directly involved in the phagocytosis of bacteria. Eater is very similar to the NimC class of proteins, with a CCxGY motif, 28–32 NIM repeats, and a transmembrane region, but it lacks the conserved region at the amino terminus. Furthermore, the NimA-like proteins CED-1 in *C. elegans* and Draper in *D. melanogaster* are both receptors for phagocytosis of apoptotic cells [20, 21]. A series of *ced-1*-like genes in mammals [16], such as MEGF10 in humans and Jedi in mouse, may have the same function. Close relatives of the *nimB* genes have been described from the silkmoth, *Bombyx mori*, and from a beetle, *Holotrichia diomphalia* [22, 23]. The latter encodes an LPS-binding protein that agglutinates bacteria in the hemolymph [23]. Furthermore, a likely

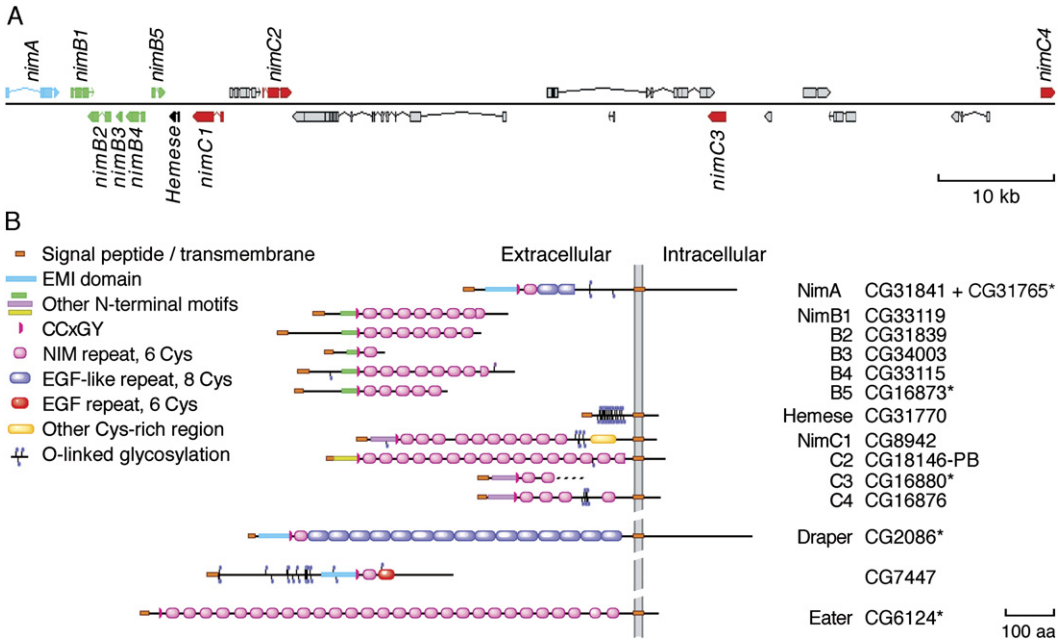

Figure 4. The *nimrod* Genes

(A) Ten related *nimrod* genes are clustered near the *Hemese* gene at 34E. The *nimA* gene is shown in blue, *nimB*-like genes are shown in green, and *nimC*-like genes are shown in red.

(B) The predicted protein domains of the corresponding proteins and three other *Drosophila* homologs. For the *nimA*, *nimB5*, *nimC3*, *draper*, and *eater* genes (indicated by asterisks) we have used new or modified gene models, described in Table S1. The conserved N-terminal motifs are described in Figure S2 (see the Supplemental Data).

ortholog to the *nimC2* gene in flesh fly, *Sarcophaga peregrina* (see Figure S2), encodes a 120 kDa protein that was proposed to act as a scavenger receptor in hemocytes of this fly [24]. Thus, Nimrod C1 belongs to a diverse class of proteins, many of which are phagocytosis receptors or bacteria-binding factors.

Conclusions

Nimrod C1 and the related Eater protein represent a novel class of putative phagocytosis receptors, characterized by a unique variant of the EGF repeat: the NIM repeat. It remains to be seen whether NimC1, like Eater [19], binds directly to bacteria, but our experiments show that it is a major factor in the phagocytosis of *S. aureus* and that it can also contribute to the phagocytosis of *E. coli*. Two other potential receptors for phagocytosis of bacteria have previously been described from *Drosophila*, and they are PGRP-LC and SR-CI [25–27], although the main role for PGRP-LC is to mediate induction of antimicrobial peptide genes [27–29]. There is probably much redundancy among the factors involved in the phagocytosis of bacteria. Proteins with EGF-like repeats play an important role in this process, in insects and perhaps in man.

Supplemental Data

Supplemental Data include Experimental Procedures, three figures, and one table and are available with this article online at http://www.current-biology.com/cgi/content/full/17/7/649/DC1/.

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