The role of the Nimrod protein and gene family in the phagocytosis of microorganisms

PhD thesis

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1. Introduction

The process of phagocytosis has evolved early in life, in unicellular eukaryotic organisms, where it is utilized for feeding. In multicellular organisms phagocytosis plays a key role in the tissue reorganizations during ontogenesis and in the defense against pathogenic microbes. Professional phagocytes, the effector cells of the innate immune response sense the foreign or altered self particles appearing in their environment by their cell surface receptors. The ligand binding of these receptors induces signal transduction pathways which reorganize the actin cytoskeleton, and the cell grows pseudopodia, to engulf the target particle, which is lysed in the hydrolytic enzymes of the phagosome.

We chose *Drosophila melanogaster* to study the molecular processes of phagocytosis, because the fruit fly relies only on innate immune processes to maintain its homeostasis. The phagocytes of *Drosophila*, the plasmatocytes differentiate early in ontogenesis, in the embryo, where their main function is the elimination of apoptotic cells in the developing nervous system. Plasmatocytes recognize and engulf foreign particles in the larva and in the adult, contributing to the immune defense.

In our work group we have created monoclonal antibodies, which recognize hemocytes of *D. melanogaster*. The first transmembrane protein expressed exclusively on the surface of plasmatocytes is the NimC1 which we identified by monoclonal antibodies as the P1 antigen.

We identified eleven genes in the *D. melanogaster* genome coding proteins with domain structures similar to NimC1. All eleven NimC1 structural homologue proteins harbor at least one NIM domain, and a CCxGY motif preceding the first NIM domain. Nine from these eleven genes are located in the direct genomic vicinity of the *nimC1* gene, constituting the *nimrod* gene family located on the second chromosome. We have classified the proteins encoded by the *nimrod* gene family into three different cathegories. The NimA protein harbors a single NIM domain and a transmembrane domain. The NimB-type proteins harbor 1-7 NIM domains, but lack a transmembrane domain, and the NimC-type proteins harbor 2-16 NIM domains and a transmembrane domain.

The fruit fly genome encodes additional NIM domain containing proteins: The Draper which plays a role in the phagocytosis embryonic macrophages and the Eater protein involved in the bacterium binding of phagocytic cells.

2. Aims

In the mass spectrometric analysis of the P1 antigen we detected a peptide which identified a transmembrane protein, which we named NimC1. We intended to verify by loss-of-function and gain-of-function studies, that the p1 antigen recognized by our antibody is encoded by the *nimC1* gene.

We intended to measure the transcriptional activity of *nimrod* genes, constituting the *nimrod* gene cluster identified *in silico*, to exclude any hypothetical pseudogenes, not transcribed into RNA from further studies.

We intended to identify the function of NimC1 in phagocytosis. We conducted bacterium binding experiments to measure the binding of NimC1 to different bacterial strains.

To assess the function of different *nimrod* genes in the immune response we expressed recombinant Nimrod proteins and have studied their bacterium binding capacity.

3. Methods

-We studied the expression of the *nimrod* genes using reverse transcription coupled polymerase chain reaction.

-For the loss-of-function studies of NimC1 we created a UAS-*nimC1*-IR construct from the *nimC1* gene containing a section of the gene in both orientations, under the control of an inducible promoter.

- For the gain-of-function studies of NimC1 we cloned the *nimC1* gene into an expression vector under the control of an inducible promoter, and have expressed the NimC1 protein in the Schneider-2 *D. melanogaster* cell line.

-We measured the bacterial binding capacity of the native NimC1 protein in an *in vitro* bacterium binding assay. We stained fluorescently labeled bacteria by indirect immunfluorescence, and visualized NimC1 bound to bacteria by flow cytometry.

-To study the bacterium binding of the NimC1, NimA, NimB1, NimB2 and Draper, we expressed these proteins in an eukaryotic expression system. We tagged the recombinant proteins with FLAG tag, and used the antibody recognizing the FLAG epitope for bacterium binding experiments.

4. Results

We have identified the first plasmatocyte-specific transmembrane protein, the P1 antigen. We isolated the P1 antigen by immuneprecipitation, using monoclonal antibodies, and have analyzed it by mass spectrometry. We identified a peptide which is present in a single protein in the *D. melanogaster* proteome, the NimC1, encoded by the CG8942 predicted gene, which we named *nimrod* (*nimC1*). The silencing of *nimC1* with RNAi resulted in a dramatic decrease of the P1 antigen on the surface of plasmatocytes. The Schneider-2 cell line does not express the P1 antigen, however after expressing recombinant NimC1, the P1 molecule can be detected on the surface of the cells showing, that the *nimC1* gene encodes the P1 antigen.

Inferring from the sequence of the *nimC1* gene the predicted NimC1 protein shows a domain structure typical of a transmembrane protein, harboring a signal peptide, extracellular, transmembrane and intracellular regions. In the extracellular domain of NimC1 we identified a new subclass of the EGF-receptor domain, which can be characterized by a stricter consensus sequence (CxPxCxxxCxNGxCxxPxxCxCxxGY). We named this new domain the NIM domain.

We studied the expression of the *nimrod* gene family by reverse transcription coupled polymerase chain reaction. We found that the *nimB1, nimB2, nimB3, nimB4, nimB5, nimC1, nimC2, nimC3* and *nimC4* genes are transcribed in all three larval stages, in the imago and in isolated hemocytes. The *nimA* gene is transcribed in the larva and the adult, however it is not expressed in hemocytes.

We studied the function of the NimC1 protein in loss-of-function and gain-of-function genetic tests. In the loss-of-function studies we inhibited the expression of NimC1 by RNAi, and by decreasing the amount of NimC1 on the surface of hemocytes, their *Staphylococcus aureus* phagocytic capacity also decreased. For the gain-of-function studies we expressed the NimC1 protein in the Schneider-2 cell line which does not express NimC1. The occurrence of NimC1 in the membrane of Schneider-2 cells the *S. aureus* and *E. coli* phagocytic capacity of the cells increased.

To study the function of the NimC1 protein we conducted bacterium binding experiments. According to our results NimC1 binds *E. coli, Serratia marcescens, Xanthomonas campestris, Pseudomonas aeruginosa* Gram-negative and the *Bacillus cereus* var. *mycoides* Gram-positive bacteria, while does not show binding to Gram-positive *Staphylococcus epidermidis, Micrococcus luteus* and *Bacillus subtilis.*

We intended to identify the ligand of NimC1 on bacterial cells by competition experiments. Lipopolysaccharide and peptidoglican does not

inhibit NimC1 binding to *E. coli* bacteria, these molecules can be excluded as the ligands of NimC1.

In our experiments we found that the lysate of *E. coli* cells effectively inhibits NimC1 binding, however protein-free *E. coli* lysate does not inhibit NimC1 binding. We concluded that the ligand of NimC1 expressed by *E. coli* cells is a protein.

We measured the bacterium binding of several Nimrod proteins. We expressed the extracellular domains of NimA, NimB1, NimB2 and NimC1 as FLAG-tagged recombinant proteins in a baculovirus expression system. According to our results the recombinant NimA, NimB1, NimB2, NimC1 bound *E. coli* similarly to the Draper protein used as positive control, however only NimB1 bound *S. epidermidis*.

5. Summary

-We identified the NImC1 protein as the P1 antigen.

-We studied the expression of the *nimrod* gene cluster. The *nimB* and *nimC* genes are expressed in hemocytes and the *nimA* is expressed in some other tissue of the larva and the adult.

-The NimC1 protein plays a role in the phagocytosis of bacteria.

-The NimC1 protein binds bacteria.

-The ligand of NimC1 is a protein.

-The recombinant NimA, NimB1, NimB2, NimC1 and Draper bind the Gram-negative *E. coli*, however only NimB2 binds the Gram-positive *S. epidermidis*.

6. Publications

Publications which the thesis is based on:

Zsámboki János, Csordás Gábor, Honti Viktor, Pintér Lajos, Bajusz Izabella, Galgóczy László, Andó István, Kurucz Éva (2013) *Drosophila* Nimrod proteins bind bacteria. Central European Journal of Biology. 8: 633-645

Kurucz Éva, Márkus Róbert, Zsámboki János, Folkl-Medzihradszky Katalin, Darula Zsuzsanna, Vilmos Péter, Udvardy Andor, Krausz Ildikó, Lukacsovich Tamás, Gateff Elisabeth, Zettervall Carl-Johan, Hultmark Dan, Andó István (2007) Nimrod, a putative phagocytosis receptor with EGF repeats in *Drosophila melanogaster.* Current Biology. 17: 1-6

Other publications:

Andó István, Laurinyecz Barbara, Nagy István, Márkus Róbert, Florentina Rus, Váczi Balázs, Zsámboki János, Fehér László, Elisabeth Gateff, Dan Hultmark, Kurucz Éva (2003) Ősi örökségünk: a veleszületett immunitás A *Drosophila* sejtes immunitása. Magyar Immunológia 4: 39-46.

Andó István, Laurinyecz Barbara, Márkus Róbert, Florentina Rus, Váczi Balázs, Zsámboki János, Kurucz Éva (2004) Ősi örökségünk, a veleszületett immunitás: A *Drosophila* sejtes immunitása; Magyar Tudomány. 111: 1080-1089.

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Somogyi Kálmán, Sipos Botond, Pénzes Zsolt, Kurucz Éva, Zsámboki János, Hultmark Dan, Andó István (2008) Evolution of genes and repeats in the Nimrod superfamily. Molecular Biology and Evolution 25: 2337-47

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