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 categories. 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 \textit{nimC1} gene.

We intended to measure the transcriptional activity of \textit{nimrod} genes, constituting the \textit{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 \textit{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 immunofluorescence, 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 (CxPxCxxxCxNGxCxxPxxCxxCxGx). 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:


Other publications:


7. Acknowledgements

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