Florentina Rus

Expression and a novel function of Filamin-240 in lamellocyte development in *Drosophila*

Summary of Ph.D. thesis

Supervisor: Dr. István Andó, PhD, DSc

Biological Research Center of the
Hungarian Academy of Sciences
Institute of Genetics

University of Szeged
2006

Introduction

Drosophila has a very effective innate immune system with striking similarities to innate immunity in vertebrates. Cellular immune defense in *Drosophila* is mediated by three different classes of hemocytes: the plasmatocytes, the lamellocytes and the crystal cells. Lamellocytes represent a unique population of hemocytes, both in terms of morphology and function. The lamellocytes are large, flat, adherent cells, involved in encapsulation and neutralization of intruders or of abnormally developed tissues, too large to be engulfed by plasmatocytes. These cells are nonphagocytic and their cytoplasmic constituents are relatively sparse.

Lamellocytes are essentially absent in healthy uninfected larvae but they may appear in low numbers at the time of metamorphosis. However, immune induction by parasitization with the Hymenopteran wasp *Leptopilina boulardi* initiates the rapid differentiation of lamellocytes, which subsequently adhere to and surround the egg, which begin to melanize, thereby walling it off inside the larvae. To understand the signaling mechanisms and development of blood cells in *Drosophila* we have defined molecular markers expressed in blood cell subsets using monoclonal antibodies developed in our laboratory. In this work we have cloned and characterized a *Drosophila* blood cell antigen expressed in lamellocytes, and showed that it corresponds to *Drosophila* Filamin-240. Filamins are a family of high molecular mass cytoskeletal proteins that organize filamentous actin in networks and stress fibers, anchor various transmembrane proteins to the active cytoskeleton and provide a scaffold for a wide range of cytoplasmic signaling proteins. *Drosophila* filamin exists in four isoforms, as a result of alternative splicing, but available ESTs indicate

that still others may exist. It has been established that Filamin-240 associates with the plasma membrane of the ring canal in *Drosophila* ovaries and attaches the actin scaffold to the membrane. Also, it contributes to forming parallel bundles of actin filaments in the inner rim of the ring canals. The shorter form, Filamin-90, lacks the actin-binding domain and rod I, and is broadly expressed; the function of this variant has not been identified yet. So far it was found that *Drosophila* filamin interacts with Toll receptor, Tube and mutations in Toll can induce lamellocyte differentiation. Drosophila filamin interacts also with transmembrane endoprotease presenilin, showing that overexpression of filamin could supress dominant phenotypes produced by presenilin overexpression on notch signalling during development. The interactions of filamin with Toll, Tube, presenilin suggest that filamin provides a scaffold for a variety of signal transduction complexes.

Aim of study

In contrast to the well studied regulation of antimicrobial peptide production, the humoral responses, very little is known about the molecular mechanisms of the cellular immune response in *Drosophila* and the origin and differentiation of hemocytes. For an understanding of the recognition, signaling and development of hemocytes in the different cellular compartments of the *Drosophila* immune system, there is a need for molecular markers that are expressed in a cell type specific manner. Our laboratory created a library of hemocyte-specific monoclonal antibodies and used it to define specific marker molecules restricted to hemocytes. Some antibodies recognize antigens, expressed on all blood cells; others are restricted to a subset of blood cells, such as the plasmatocytes, the

lamellocytes or the crystal cells. The antibodies have been clustered on the basis of their reactivity with hemocytes and the biochemical features of the identified antigen.

We expect that identified molecules specifically expressed on different hemocyte subsets are involved either in the immune function of the corresponding blood cell type or may play a role in hematopoiesis. The definition and use of further hemocyte markers could help us to identify blood cell antigens, to define their genes and to study their structure and function. Finally, molecular and genetic analysis could elucidate the mechanisms of hemocyte development and activation in immune responses.

Materials and methods

- **Fly strains**

Flies were grown on cornmeal-yeast food at 25°C. We used Oregon-R wild-type stock and the *l(3)mbn-1* tumor suppressor, mutation with hemocyte overproduction. The *dom*[1] is a P-element induced *Drosophila* mutant devoid of circulating hemocytes and with melanized lymph glands in the third-instar larvae. The *cher*[1] is a loss of function mutation; Filamin-240 protein deficient females are sterile with defects in ring canal assembly and egg chamber morphology. The*P[hs-FLN1-20];cher*[1] rescue stock has a hsp 70 promoterdriven full length *cheerio* cDNA in a *cher*[1] homozygous mutant background. Heat shock rescue was accomplished by placing vials containing second stage larvae in a 37^0C incubator for half an hour, twice a day, for two days and then the larvae were dissected.

- **Preparation of antibodies**

Mouse monoclonal antibodies to lamellocytes, 4B8 and L1 were produced in our laboratory as described previously. The filamin specific polyclonal antibody was a gift from Dr. Lynn Cooley, Yale University, U.S. The singed, hts and kelch specific monoclonal antibodies were gift from Dr. Matyas Gorjanacz, BRC, Szeged.

- **Indirect immunofluorescence, double staining**

Hemocytes samples were incubated with the 4B8 antibody for 1 h at room. After the washing stepts the samples were incubated with polyclonal anti-C-Filamin antibody (dilution 1:100) for one hour and biotin coupled anti-mouse immunoglobulin was added for another one hour. After three washing steps the bound antibody was detected by Streptavidin-Cy3 complex and Cy2-conjugated anti-rat antibody. In the last staining step

DAPI was added to stain nuclei. Slides were analyzed as described in 4.5 Images were processed using Adobe Photoshop version 6.

- **Immunostaining of ovaries**

Ovaries isolated from wild type Oregon-R and *cher*[1] homozygous females in *Drosophila* Ringer solution and the samples were incubated with the primary antibody overnight, at 4^0C. The bound antibody was detected by Streptavidin-Cy3 complex (Amersham Pharmacia Biotech).

- **Western blot analysis**

Protein extracts were separated by SDS/PAGE. After electrophoresis the proteins were transferred onto nitrocellulose membrane (Hybond-C, Amersham Pharmacia) in transfer buffer. The blotted proteins were subjected to 4B8 mAb or the polyclonal anti-C-filamin (1:1000 dilution).

- **Screening of cDNA expression libraries**

We used the 4B8 antibody to screen *l(3)mbn-1* larval expression libraries About 8×10^4 plaques were transferred onto nitrocellulose filters (Amersham Pharmacia Hybond-C Extra). HRPO-conjugated secondary antibody (Amersham Pharmacia) was applied and the reaction was visualized with the ECL-Plus system (Amersham Pharmacia). The positive plaques were collected and re-screened twice.

- **Filamin cDNA clones and sequence analysis**

Plasmid DNA was prepared from positive clones of the pBK-CMV phagemid vector derived from the ZAP Express vector by *in vivo* excision using the ExAssist helper phage (Amersham Pharmacia) with the XLOR strain and sequenced on both strands. Database searches and sequence comparisons were performed with the BLAST servers at the Berkeley *Drosophila* Genome Project and the National Center for Biotechnology Information.

- **Bioassay for lamellocyte induction**

One-week-old females of the parasitic wasp, *Leptopilina boulardi*, strain G486 were used to infest second-instar larvae. Fifty larvae were exposed to five *Leptopilina boulardi* females overnight at 18°C and then kept at 25^0C. Two days after infestation the hemocytes isolated from the infested larvae were visualized with 4B8 or L1 antibody.

Results and discussion

By screening *Drosophila* cDNA expression libraries with 4B8, a monoclonal antibody reacting with the majority of lamellocytes in a hemocyte overproducing mutant stock, *l(3)mbn-1,* positive clones were obtained and 7DNA fragments were isolated. Partial sequencing of the five positive clones, identified regions from eight exons of the *Drosophila cheerio* gene, the gene coding for the *Drosophila* filamin. Mapping of the isolated cDNA sequences to the filamin domain showed that they corresponded to the "filamin-folding domain", a consensus motif profile generated from the 20 existing filamin repeats. So far, *Drosophila* Filamin-240 was found to be expressed in ring canals of the adult ovary in Drosophila. Our studies revealed that Filamin-240 is expressed in lamellocyte too.

Originally it was described that a loss of function allele of the *cheerio* gene, *cher*^{1}*,* due to the lack of Filamin-240, is defective in ring canal assembly, and as a consequence, has female sterility. As the initial results revealed that the isoforms have a cell-type specific expression among hemocytes, we have studied Filamin-240 expression upon lamellocyte-induction followed by infestation with *Leptopilina boulardi in vivo,* in *cher*^{1} homozygous larvae and also in *cher*^{1} heterozygous larvae as a control. Hemocytes prepared from the non infested and from wasp-infested *cher*^{1} homozygous larvae did not react with mAb 4B8 and the 240 kDa band was not detected by Western blot analysis of the hemocytes of non infested or infested larvae. In hemocytes of uninfested heterozygous larvae Filamin-240 is not expressed, however lamellocytes of wasp infested heterozygous larvae were stained and the staining correlated with expression of Filamin-240, as detected by Western blot analysis.

As the expression of Filamin-240 is restricted to lamellocytes among blood cells next we asked whether or not this isoform could be involved in regulation of lamellocyte development. Analysis of the blood cell phenotype of the loss-of-function, *cher*^{1} homozygous mutant revealed that lamellocytes appear in the circulation without any immune challenge, which is further increased following infestation with the parasitoid wasp. This phenotype is correlated with the lack of immunostaining with 4B8 antibody in lamellocytes of uninfested and infested and the lack of the 240 kDa band in larval extracts. In control experiments wild type Oregon-R larvae, which expressed the 240 kDa protein had no lamellocytes in the circulation and following infestation the number of lamellocytes was significantly less than that in the *cher*^{1} homozygotes.

On the basis of these data a specific enhancing effect of the mutation on the number of lamellocytes is observed therefore we propose that *Drosophila* Filamin-240 is a suppressor of lamellocyte development.

To confirm that the lamellocyte overproducing phenotype was the result of the specific mutation in the *cheerio* gene we rescued the mutant phenotype by introducing the full-length transgene (*P[hs-FLN1-20]*), encoding the *Drosophila* Filamin-240, into the *cher*^{1} homozygous mutant lacking the 240 kDa isoform. The rescued larvae expressed Filamin-240 as revealed by Western blotting, similarly to the induced Oregon-R wild type larvae. In the hemolymph of the rescued larvae, similarly to the wild type Oregon-R, we have found no lamellocytes; in addition after parasitic wasp infestation a comparable lamellocyte count was found in these two groups which however never reached the number of lamellocytes seen in the *cher*^{1} homozygotes. Comparison of the *cher*^{1} homozygotes with the rescue stock shows that there are 10 times more lamellocytes in the *cher*^{1} homozygotes (lamellocyte number: 2003) than in the *P[hs-FLN1-20];cher*^{1} stock (lamellocyte number: 227), while there is no significant difference between

the total cell numbers (*cher*[1] homozygotes total cell number: 3634 vs. *P[hs-FLN1-20];cher*[1] total cell number: 3292, p=0,3) showing that the effect is specific to lamellocytes. Also, we found the same pattern of Filamin-240 expression by immunofluorescence analysis in uninfested and wasp infested *P[hs-FLN1-20];cher1* hemocytes as in the wild type Oregon-R blood cells where fully differentiated lamellocytes express Filamin-240. Thus the analysis of the rescue experiments shows that introduction of the full-length cheerio cDNA into the *cher*[1] homozygotes results in the expression of Filamin-240 in lamellocytes as well in suppression of lamellocyte counts. We conclude therefore that in the rescue-strain, *P[hs-FLN1-20];cher*[1], filamin undergoes a normal post-transcriptional modificaton in lamellocytes as it does in the ovaries, and functions in blood cells of *Drosophila* as a suppressor of lamellocyte development.

Among the four existing isoforms of filamin arisen by post-transcriptonal modification, we have detected Filamin-240 and Filamin-90 using a monoclonal antibody, 4B8. Biological activity so far has been assigned to Filamin-240, containing the acting binding domain; the lack of this isoform results in female sterility. Here we show that this isoform is expressed in a subset of blood cells, the lamellocytes too. Lamellocytes undergo massive differentiation in response to infection caused by parasites and pathogens. Cell shape transformation requires dynamic reorganization of the actin cytoskeleton. It was found previously that rearrangement of microtubules accompanies the modification of lamellocyte shape from a disc to bipolar form during the cellular immune response. The terminal differentiation of lamellocytes, flattening must involve dynamic reorganization of the actin cytoskeleton and Filamin-240 is involved in this process. So far, it was found that Drosophila filamin interacts with Toll receptor and mutantions in Toll can induce lamellocyte differentiation. By attaching the cytoskeleton to the Toll signaling pathway it is possible that Filamin-240 enables the

dynamic rearrangements of the actin cytoskeleton required for lamellocyte differentiation. Further, it would be interesting to investigate how the actin cytoskeleton functions in the Toll pathway and how the interaction of Filamin-240 with Toll receptor may be regulated.

Summary

In this work I used a molecular marker with specific expression in *Drosophila* lamellocytes to understand the differentiation and function of this cell type. By the aid of this marker we have cloned and characterized a novel *Drosophila* blood cell antigen. The protein corresponds to *Drosophila* Filamin-240, an actin-network specific protein, detected so far in the ring canals of the ovaries. Our studies indicate that Filamin-240 is expressed in lamellocytes also and is involved in the regulation of lamellocyte development. In the *cher*^{1} homozygous larvae, which lack Filamin-240 protein, a vigorous lamellocyte differentiation occurs which is further enhanced upon *in vivo* immune challenge by a parasitic wasp, *Leptopilina boulardi*. By introducing a full-length transgene encoding the *Drosophila* Filamin-240 protein into the *cher*^{1} filamin deficient homozygous mutant, the mutant blood cell phenotype was rescued. These data demonstrate that the expression of Filamin-240 is strictly lamellocyte-specific in *Drosophila* blood cells and that the protein is a suppressor of lamellocyte development. Transformation of shape of lamellocytes, flattening requires dynamic reorganization of the actin cytoskeleton. Our studies suggest that Filamin-240 might play a role in the rearrangements of the actin network during the terminal stage of lamellocyte development.

Acknowledgements

First of all I wish to thank my supervisor Dr. István Andó for the possibility to join his group as well as for the support and advice in the progress of this study. I am very grateful to Dr. Éva Kurucz for the continuous help and for the critical remarks to my work. Special thanks to all the colleagues: Brabara Laurinyecz, István Nagy, Róbert Márkus, Balázs Váczi, János Zsámboki for their strong support and help.

I am also grateful to Dr. Dan Hultmark for the cDNA expression library (Umeå University,Sweden). I thank Dr. Lynn Cooley (Yale University, US) for providing us with the *P[hs-FLN1-20]* transgene and anti-C-Fil antibody and also Dr. Mátyás Gorjanácz for the *cher1* allele and for Tamás Matusek (BRC, Szeged, Hungary) for the comments on the rescue experiments. I wish to thanks Szilvia Tápai, Ágnes Árva, Olga Kovalcsik for their technical assitance.I am thankful to Dr. Ilona Dusha and Dr. Miklós Erdélyi for tutorial supervising of my progress.

List of publications

Rus Florentina, Kurucz Éva, Márkus Róbert, Sinenko Sergey A., Laurinyecz Barbara, Pataki Csilla, Gausz János, Hegedűs Zoltán, Udvardy Andor, Hultmark Dan, Andó István, **Expression pattern of Filamin-240 in *Drosophila* blood cells**, *Gene Expression Patterns*, doi:10.1016/j.modgep.2006.03.005

Márkus Róbert, Kurucz Éva, **Rus Florentina**, Andó István, **Sterile wounding is a minimal and sufficient trigger for a cellular immune response in *Drosophila melanogaster***, *Immunology Letters* 101,2005, 108-111.

Andó István, Laurinyecz Barbara, Márkus Róbert, **Rus Florentina**, Váczi Balázs, Zsámboki János, Kurucz Éva**: Our ancient heritage: innate immunity; the celular immunity of the *Drosophila*,** *Magyar Tudomány 2004/10*

Andó István, Laurinyecz Barbara, Nagy István, Márkus Róbert, **<u>Rus Florentina,</u>** Váczi Balázs, Zsámboki János, Fehér Lászlo, Gateff Elisabeth, Hultmark Dan, Kurucz Éva, **Our archaic heritage: the innate immunity. The cellular immunity of *Drosophila*,** 2003, *Magy. Immunol.*2(4):39-45.

Presentations

<u>Rus Florentina</u>, Kurucz Éva, Gausz János, Hegedűs Zoltán., Udvardy Andor, Andó István, **Expression and a novel function of Filamin-240 in lamellocyte development in *Drosophila melanogaster*,** A Magyar Immunológiai Társaság XXXIII. Kongresszusa, Győr, 2003, October 15-16.

Laurinyecz Barbara, Kurucz Éva, Nagy István, **<u>Rus Florentina</u>**, Andó István, **Az antigén-indukálta sejtes immunválasz folyamata Drosophilában**, A Magyar Immunológiai Társaság XXXII. Kongresszusa, Kaposvár, 2002, October 30-02.

<u>Rus Florentina</u>, Kurucz Éva, Andó István, **Identification of hemocyte-antigens in *Drosophila melanogaster***, ITC 200/2001, Proceeding of the Closing Seminar, BRC, Hungarian Academy of Sciences, 99-110.