Haemolymph clotting in *Drosophila melanogaster* and *Galleria mellonella*

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The experimental work presented in this study has been conducted at the Institute for Molecular Biology and Functional Genomics at Stockholm University (Sweden) from January 2001 to August 2004. It was performed under the supervision of Prof. Dr. Tina Trenczek (Institute for General and Special Zoology, Justus-Liebig-University Gießen, Germany) and Docent Dr. Ulrich Theopold (Institute for Molecular Biology and Functional Genomics, Stockholm University, Sweden). The work was supported by a PhD fellowship (No. D/01/41725) of the German Academic Exchange Service (DAAD) from May 2001 to April 2004.
This study will be electronically published and publicly available for download at http://geb.uni-giessen.de/geb/.

Die praktischen Arbeiten der vorliegenden Studie wurden am Institut für Molekularbiologie und Funktionsgenomik an der Universität Stockholm (Schweden) von Januar 2001 bis August 2004 durchgeführt. Sie wurde von Prof. Dr. Tina Trenczek (Institut für Allgemeine und Spezielle Zoologie, Fachbereich 08 der Justus-Liebig-Universität Gießen, Deutschland) und Dozent Dr. Ulrich Theopold (Institut für Molekularbiologie und Funktionsgenomik, Universität Stockholm, Schweden) betreut. Die Arbeit wurde von Mai 2001 bis April 2004 durch ein Promotionsstipendium (Kennziffer: D/01/41725) des Deutschen Akademischen Austauschdienstes (DAAD) unterstützt.
Diese Studie wird elektronisch veröffentlicht werden und unter der Adresse http://geb.uni-giessen.de/geb/ zum Download zur Verfügung stehen.

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Statement

With this I state that the present work, to the best of my knowledge and belief, does not contain material previously published or written by another person, except where due reference has been made in the text.

Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig durchgeführt und verfasst und keine anderen als die angegebenen Hilfsmittel verwendet zu haben.

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Gießen, September 2004

The Blind Men and the Elephant

(John Godfrey Saxe, 1816-1887, based on an Indian fable)

It was six men of Indostan
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy his mind

The First approached the Elephant
And happening to fall
Against his broad and sturdy side,
At once began to bawl:
"God bless me! but the Elephant
Is very like a wall!"

The Second, feeling of the tusk,
Cried, "Ho! what have we here
So very round and smooth and sharp?
To me `tis mighty clear
This wonder of an Elephant
Is very like a spear!"

The Third approached the animal,
And happening to take
The squirming trunk within his hands,
Thus boldly up and spake:
"I see," quoth he, "the Elephant
Is very like a snake!"

The Fourth reached out an eager hand,
And felt about the knee.
"What most this wondrous beast is like
Is mighty plain," quoth he;
`Tis clear enough the Elephant
Is very like a tree!"

The Fifth, who chanced to touch the ear,
Said: "E`en the blindest man
Can tell what this resembles most;
Deny the fact who can
This marvel of an Elephant
Is very like a fan!"

The Sixth no sooner had begun
About the beast to grope,
Then, seizing on the swinging tail
That fell within his scope,
"I see," quoth he, "the Elephant
Is very like a rope!"

And so these men of Indostan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and strong,
Though each was partly right,
And all were in the wrong!

Moral:

So oft in these scientific wars,
The disputants, I ween,
Rail on in utter ignorance
Of what each other mean,
And prate about an Elephant
Not one of them has seen!

Publications

Original Papers:

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Insect Biochem. Molec. Biol. **32**, 919-928.

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Isolation and characterization of hemolymph clotting factors in *Drosophila melanogaster* by a novel pull-out assay. Current Biology **14**, 625-629.

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Proteomic analysis of the *Drosophila* larval hemolymph clot.
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Korayem, A. M., Fabbri, M., Takahashi, K., **Scherfer, C.**, Lindgren, M., Schmidt, O., Ueda, R., Dushay, M. S. and Theopold, U. (2004)
A *Drosophila* salivary gland mucin is also expressed in immune tissues: evidence for a function in coagulation and the entrapment of bacteria.
Submitted to Insect Biochemistry and Molecular Biology.

Review article:

Theopold, U., Li, D., Fabbri, M., **Scherfer, C.**, Schmidt, O. (2002)
The coagulation of insect hemolymph. Cell. Mol. Life Sci. **59**, 363-372.

Abbreviations

ALP	alkaline phosphatase
Bc	Black cells
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
cDNA	complementary deoxyribonucleic acid
DAB	3',3'-diaminobenzidine tetrachloride
dig UTP	digoxygenin-modified UTP
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dom	domino (*D. melanogaster*)
dsRNA	double-stranded RNA
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis (beta-aminoethylether)-N,N,N`,N`-tetraacetic acid
EST	expressed sequence tag
FACS	fluorescence-activated cell sorter
fbp1	fat body protein 1 (*D. melanogaster*)
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram
g	gravity [in italics]
Gal4	yeast transcription factor involved in expression of galactose-induced genes
GFP	green fluorescent protein
h	hour(s)
H_2O dd	double distilled water
hml	hemolectin (*D. melanogaster*)
hmu	hemomucin (*D. melanogaster*)
HPL	*Helix pomatia* lectin
HS	hybridisation solution
IGEPAL CA-630	octylphenyl-polyethylene glycol (detergent)
IPTG	isopropylthio-β-D-galactoside
kDa	kilo Dalton
KGD	lysine-glycine-aspartate
K_m	half-maximal reaction rate (enzymes)
l	litre(s)
LPS	lipopolysaccharide
LRE	leucine-arginine-glutamate
lsp1β	larval serum protein 1β (*D. melanogaster*)
M	molar
mA	milliampere

mbn-2	tumourous cell-line derived from the haematopoietic organ of the *Drosophila* mutant *malignant blood neoplasm-2* (*mbn-2*)
MDC	monodansylcadaverine
mg	milligram
MHC	major histocompatibility complex
min	minute(s)
ml	millilitre(s)
mM	millimolar
mSSL	mouse strictosidine synthase-like
µg	microgram
µl	microlitre(s)
µM	micromolar
NBT	Nitro blue tetrazolium chloride
NFκB	nuclear factor κB
ng	nanogram
Ni-NTA	nickel nitrotriacetic acid
nm	nanometer (wavelength)
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline including 0,1 % Tween-20
PCR	polymerase chain reaction
PGRP	peptidoglycan recognition protein
PI	propidium iodide
PNA	peanut agglutinin (*Arachis hypogaea* lectin)
ppm	parts per million
PTU	phenylthiourea
RGD	arginine-glycine-aspartate
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RP49	ribosomal protein 49 (*D. melanogaster*)
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
sec	second(s)
serpin	serine protease inhibitor
SL2	Schneider's Line S2 (*Drosophila* haemocyte-like cell line)
SSC	sodium chloride - sodium citrate buffer
SSL	strictosidine synthase-like
TBS	Tris-buffered saline
TBST	Tris-buffered saline including 0,1 % Tween-20
tig	tiggrin (*D. melanogaster*)
Tris	Tris-hydroxymethyl-aminomethane
tRNA	transfer RNA
Tween-20	polyoxyethylenesorbitan monolaurate
UAS	upstream activation sequence (from yeast promoter)
VWD	von Willebrand disease
vWF	von Willebrand factor

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1 Introduction

1.1 Innate and adaptive immunity

In vertebrates different levels of adaptive immune reactions including T-cell responses and antibody production have evolved, leading to a highly adaptive defence system against microorganisms. Mammals can acquire immunity to pathogens during their individual life span, for example by memory cells for specific antibodies. However, the initial induction of an adaptive response takes several days, which is generally not sufficient for the survival of the organism. In addition many pathogens have evolved mechanisms to evade or destroy the host defence response. Therefore components of the vertebrate innate immune system, such as antimicrobial peptides and phagocytosing macrophages, are crucial during the first contact with a pathogen. Even all invertebrates including the phylogenetic ancient sponges possess innate immune responses.

1.2 Model organisms used in this study

Insects and other invertebrates rely exclusively on innate immunity. Hence they are promising model organisms for the investigation of this subject. The recently increasing number of studies on immune functions of Toll-like receptors in vertebrates was initiated and stimulated by insights from the insect defence system. Insects are also of high economical and medical importance. Some species are beneficial (like the honey-bee *Apis mellifera*), others are noxious (for example the diamondback moth *Plutella xylostella*) for economically important plants. Insect vectors transmit animal and human diseases like malaria or yellow fever and spread plant pathogens. Since insecticides often have hazardous side effects for the environment and because insects can evolve resistance mechanisms fairly quick, it may be a promising insect pest management strategy to interfere with novel targets of the insect immune system.
In our research project we use two insect species, the dipteran *Drosophila melanogaster* and the lepidopteran *Galleria mellonella*. *D. melanogaster* has been successfully

investigated for a long time, partly because it is easy to cultivate. Well-established genetics and the fully sequenced genome provide a good basis for molecular research. In addition *Drosophila* has been in the focus of insect immunity during the last decade.
Nevertheless, there are limitations to using *Drosophila* as a model system. Evolutionary, *D. melanogaster* and other dipterans represent a rather derived insect order (Burmester et al., 1998). Newly found immune proteins could be specific for *Drosophila* and lacking in other insects. At the same time, it is precisely these species-specific traits that may provide new insights into the adaptation of an immune system to a particular environment. For example, larvae of *D. melanogaster* live in rotten fruit rich in bacteria, which might necessitate stronger immune responses than in other insects. Furthermore the observed variety between insect immune systems is probably partly due to size differences and variable haemolymph amounts. The latter might be an important, but so far not investigated issue regarding haemolymph clotting.
Therefore I decided to also study the lepidopteran *Galleria mellonella* (greater wax moth). Morphological changes during coagulation in *G. mellonella* have been examined for a long time. The hundred times larger haemolymph amount of this species (approximately 50 µl versus 0,5 µl per individual in *Drosophila*) also facilitates quick pilot experiments.

1.3 Haemocyte types in *G. mellonella* and *D. melanogaster*

The body fluid of insects and other animals with an open circulatory system is called “haemolymph” to stress the fact that there is no separation between blood and the lymphatic fluid as in vertebrates. Similarly the cells circulating in the haemolymph are termed “haemocytes”. In insects these cells facilitate cellular immune reactions such as phagocytosis, nodulation and encapsulation (Ratcliffe & Rowley, 1979, Lackie, 1988, Lavine & Strand, 2002), which will be summarised in the following chapter. Moreover in many insects specific tasks can be exclusively assigned to certain haemocyte types with distinct morphological appearance. Recognition of a defined haemocyte class by a lectin or antiserum can substantially support functional data obtained with this labelling agent. The haemocyte classes present in insects differ between orders and even between

rather related species. Therefore I present here the haemocyte types of the two species investigated in the present work.

In lepidopterans like ***G. mellonella*** the main haemocyte types found in haemolymph preparations are plasmatocytes and granular cells (Chain & Anderson, 1983). In addition prohaemocytes, oenocytoids and spherule cells have been described. Prohaemocytes belong to a homogenous group of small progenitor cells of the other haemocyte types. Plasmatocytes can form large pseudopodia and possess the ability to attach to and spread on surfaces. They can form multicellular capsules around larger pathogens, which cannot be phagocytosed. Both granular cells and plasmatocytes of *G. mellonella* show phagocytic activity (Tojo et al., 2000). The cytoplasm of granular cells is filled with large numbers of granular inclusions, whose contents can be released into the medium upon activation. In contrast to plasmatocytes granular cells do not spread extensively, but can also adhere to surfaces via microspikes and mucous fibres of degranulated intracellular material (Chain & Anderson, 1983). This degranulation process may be crucial for the initiation of coagulation, nodule formation and encapsulation (Rowley & Ratcliffe, 1976, 1978). Oenocytoids are sites of prophenoloxidase production. Earlier descriptions of prophenoloxidase localisation in granular cells (Ratcliffe, 1984) can be explained by melanin deposition around granular cells (Ashida & Yamazaki, 1992). The function of spherule cells is not yet clear. These cells contain large amounts of mucopolysaccharides and were therefore suggested to contribute to extracellular matrix (ECM) formation in other insect species (Ashhurst, 1982, Horohov & Dunn, 1982). The construction of a basal lamina is required for successful wound healing and thus may be connected to the coagulation process.

The haemocyte classes described for ***D. melanogaster*** differ from the ones of *G. mellonella* considerably. Three haemocyte types can be distinguished in *Drosophila*: plasmatocytes, crystal cells and lamellocytes (Lanot et al., 2001). The podocyte, a fourth set of cells described in earlier studies (Rizki, 1957), is probably derived from plasmatocytes. Plasmatocytes do not spread as extensively as their lepidopteran counterparts and are mostly phagocytic active. While no granular cells are present in *Drosophila*, a subgroup of plasmatocytes containing numerous granules might fulfil similar functions. Crystal cells contain regular-shaped inclusions, which dissolve quickly as soon as these haemocytes disintegrate upon activation (Rizki & Rizki, 1985).

Through this cell rupture the enzyme prophenoloxidase and its tyrosine substrates, which are spatially separated inside the crystal cells, come into close contact in the haemolymph, leading to a localised enzymatic activity. Haemocytes isolated from *Drosophila* larvae are largely derived from the embryonic head mesoderm. Only towards the end of the third larval instar larval haemocytes are released from the larval haematopoietic organ (Holz et al., 2003). Differentiation of haemocyte precursor cells into lamellocytes in the lobes of the hematopoietic organ is induced by parasitoid infections in *Drosophila* (Lanot et al., 2001). Lamellocyte numbers also increase in the last larval instar towards pupation (personal observation).

Since similar cellular immune reactions occur in all insects, it may be assumed that there are some poorly understood molecular similarities between haemocyte types of different species and orders of insects. For example, the already described involvement of *Galleria* granular cells in haemolymph coagulation probably has its counterpart in *Drosophila* plasmatocytes or in a plasmatocyte subclass yet to be identified.

1.4 The insect immune system

Innate immunity in insects is conventionally divided into cellular and humoral responses (Gillespie et al., 1997, Vilmos & Kurucz, 1998). Phagocytosis, nodulation and encapsulation are summarised as cellular immune reactions. Coagulation, melanisation and induction of antimicrobial peptides are regarded as humoral defence reactions. However, this separation may be considered artificial, since many haemolymph proteins are originally released from haemocytes and others are directly connected to cellular immunity. Processes like clotting and wound healing might involve both cellular and humoral components. Hence, I will present the main aspects of an immune response in a temporal order (Figure 1). Because of the rapidity of events, mutants defective in clotting might not survive wounding, even if other aspects of the immune system are not directly affected. Moreover, it should be noted that most defence responses require a recognition mechanism that might be provided by the coagulum and the entrapped microorganisms at the wound site. In this chapter I will highlight the reactions that are probably closely related to coagulation, namely melanisation, encapsulation and wound healing.

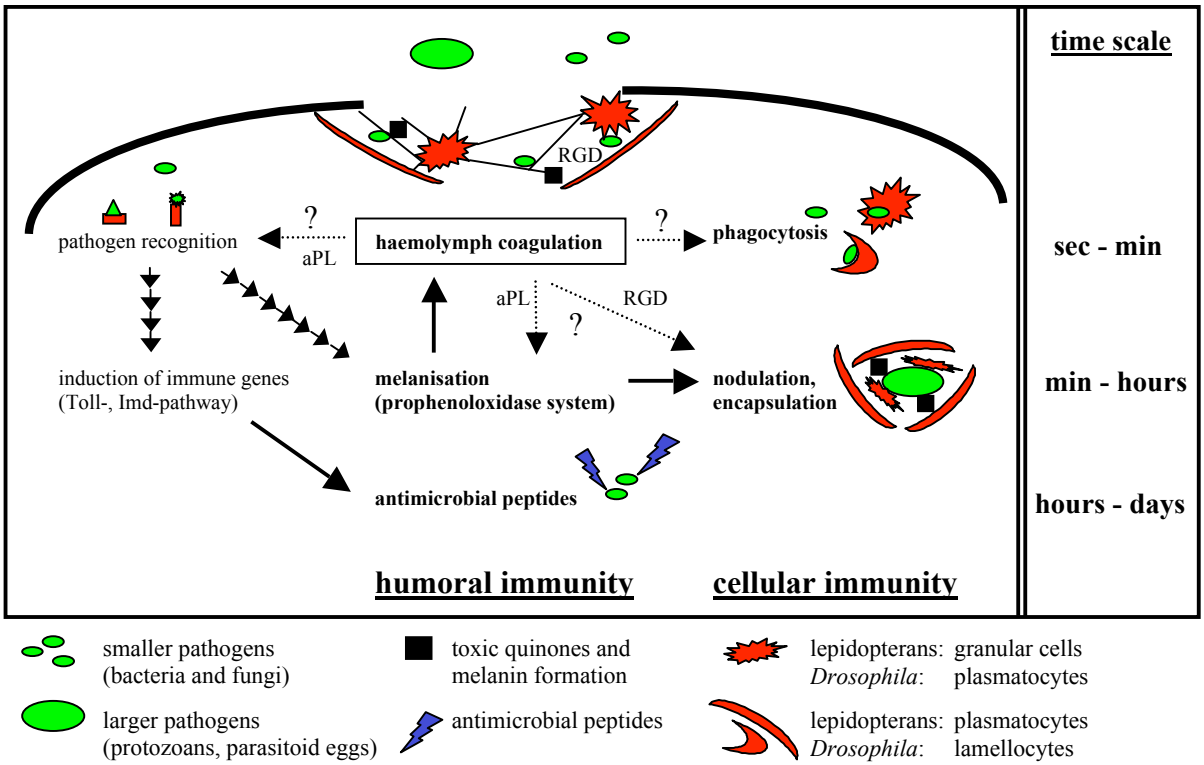

Figure 1: Insect immunity – an overview
This is a schematic summary of insect immune responses, emphasising the time element. Later wound healing events are not included in the picture. aPL: anionic phospholipids; RGD: arginine-glycine-asparagine

1.4.1 Cuticle and peritrophic membrane – the first line of defence

Microorganisms are ubiquitously present in large numbers in practically every environment and are potentially harmful to all organisms. In insects the hard chitinous exocuticle provides a first mechanical barrier for microorganisms, covering even the tracheal openings, fore- and hindgut. The midgut surface secretes a peritrophic membrane that mainly consists of glycoproteins and chitinous fibrils. One proposed function of peritrophic matrices is to prevent microorganisms in the ingested food from entering the haemocoele (Orihel, 1975). Digestive enzymes and the low pH in the gut create a hostile environment for many microbes. In addition, constitutively secreted antimicrobial proteins like lysozyme (Hultmark, 1996) may assist in quick killing of microorganisms. Some pathogens are able to penetrate the cuticle, especially at the thin synovial membranes. Wounds are even more vulnerable to intrusion by microorganisms.

1.4.2 Coagulation and phagocytosis – immediate immune reactions

Haemolymph coagulation fulfils at least two functions: it quickly seals wounds to avoid haemolymph loss and to prevent pathogens from entering the body cavity. Compared to vertebrate blood clotting pathogen, immobilisation might be a more critical issue in the open circulatory system of insects, because pathogens may easily spread to the inner organs and invade them. Simultaneously the danger of thrombosis is presumably lower in arthropods, allowing an extensive coagulation reaction without harmful consequences for the organism (Theopold et al., 2004). Morphological changes during clotting and biochemical characterisation of the involved proteins in vertebrates and invertebrates will be discussed in detail in chapter 1.5.

Two insect haemocyte subtypes, granular cells and plasmatocytes, phagocytose bacteria (Lackie, 1988). Interestingly, the presence of granular cells increases phagocytotic activity of plasmatocytes in *G. mellonella* (Anggraeni & Ratcliffe, 1991). It has been suggested that granular cells exocytose substances that facilitate phagocytosis, probably by opsonisation of the pathogens. Phagocytosis in the medfly *Ceratitis capitata* for example involves recognition of RGD-peptides by cell membrane integrins (Foukas et al., 1998). The RGD motif and other molecules of haemocyte origin were also implied earlier in stimulation of encapsulation (see chapter 1.4.4). Deposition of substances on pathogen surfaces by degranulating haemocytes during clotting might thus activate phagocytosis and other cellular immune responses.

Like vertebrate macrophages, insect haemocytes also produce reactive oxygen and nitrogen intermediates (ROI and RNI) that effectively kill microorganisms (Nappi & Ottaviani, 2000). This direct killing is possibly important for the clearance of pathogens that have become immobilised at the wound site by haemolymph clotting.

1.4.3 Melanisation – immobilisation and killing of pathogens

Melanisation fulfils multiple functions like sclerotisation, egg shell tanning and immunity. Melanin formation is catalysed by phenoloxidases, which belong to a protein family present in plants, fungi and animals including invertebrates and vertebrates. These enzymes are divided into three classes with overlapping substrate specificities: monophenoloxidases/tyrosinases, o-diphenoloxidases and p-diphenoloxidases/laccases (Sugumaran & Kanost, 1993). In horseshoe crabs and crustaceans the oxygen-transporting hemocyanins possess an intrinsic phenoloxidase activity (Decker et al., 2001, Nagai et al., 2001). Interestingly, hemocyanin activation is connected to initiation of the horseshoe crab clotting cascade (Nagai & Kawabata, 2000). Insects possibly evolved specialised phenoloxidases from an ancient hemocyanin precursor, primarily to sclerotise and harden the cuticle (Sugumaran, 1988). The cuticular prophenoloxidase of *Bombyx mori* is derived from the haemolymph enzyme by modification during its transport into the cuticle (Asano & Ashida, 2001a, 2001b). Haemolymph phenoloxidases are present as pro-forms (prophenoloxidases), which are activated by a protease cascade and regulated by serine protease inhibitors, serpins (Cerenius & Söderhäll, 2004). In addition to proteolytic induction after bacterial challenge (Ashida & Yamazaki, 1990) an alternative regulation pathway involving phospholipids from damaged cells (Sugumaran & Nellaiappan, 1991, Sugumaran & Kanost, 1993) has been described, which will be discussed in chapter 1.8. Activated phenoloxidases form quinones from phenolic substrates present in the haemolymph. Subsequently these products oligomerise, culminating in melanin deposition on surfaces. The hardening effect of quinones is commonly assigned to polymerisation, though some researchers regarded a dehydration effect of quinones as even more important (Vincent, 1990). Besides facilitating melanin formation, the intermediate quinone products are highly cytotoxic substances that might directly kill microorganisms immobilised in the coagulum. Several research groups in insect immunity suggested that phenoloxidases may represent the exclusive clotting- and wound healing-enzyme in insects. This idea is based on the observation that melanin is deposited in capsules around pathogens (Gupta, 1988) and at sites of injuries (Lai-Fook, 1966). Nevertheless, it was also reported that encapsulation even occurs in *Drosophila* phenoloxidase mutants like *Black cells* (Rizki

& Rizki, 1990) or after inhibition of the enzyme (Lavine & Strand, 2001). In a detailed study of wound healing in the mosquito *Armigeres subalbatus* it was shown that a gelatinous clot formed much earlier than melanisation became visible (Lai et al., 2002). This observation was recently confirmed for *Drosophila* wound healing (Galko & Krasnow, 2004). When I started the present work, it was therefore hypothesised that the initial clotting reaction might be independent of phenoloxidase activity.

1.4.4 Nodulation, encapsulation and wound healing – stabilising the scene

Large amounts of bacteria that cannot be phagocytosed, are enclosed in multicellular nodules. Bigger organisms like protozoans and eggs of parasitoid wasps are encapsulated. Both nodules and capsules are formed by haemocytes and have been predominantly studied in lepidopteran insects. Encapsulation occurs in a regulated manner in many lepidopterans (Ratcliffe & Gagen, 1977, Ratcliffe, 1986), though the location of haemocyte types in the capsule appears random in some species (Wiegand et al., 2000). According to the classical model suggested by Ratcliffe & Rowley (1979) haemocytes of the granular cell type, start to disintegrate on microorganism surfaces and exocytose molecules that form an extracellular aggregate. Among the released substances the "plasmatocyte spreading factor" may induce haemocyte chemotaxis (Clark et al., 1997) and thereby launch the second phase of capsule construction: Plasmatocytes are attracted, spread and flatten around the first layer formed by granular cells (Strand & Clark, 1999). RGD-containing proteins present in the degranulated material mediate plasmatocyte adhesion (Pech & Strand 1995, 1996). Though granular cells are missing in *D. melanogaster*, several plasmatocytes include numerous granules and thus may fulfil similar immune functions in the fly. However, to date only *Drosophila* lamellocytes are known to participate in capsule formation (Rizki & Rizki, 1986). It is interesting to note that in some dipterans with low haemocyte counts a so-called humoral encapsulation proceeds in the absence of cells (Götz, 1986). These "humoral capsules" might well correspond to what I call a haemolymph clot or coagulum throughout the present investigation. The first steps of nodulation and

encapsulation have repeatedly been characterised as morphologically similar to haemolymph coagulation (Bohn, 1986, Ratcliffe, 1986). Nodulation is even induced in the absence of microbial agents by saline injection in cockroaches and locusts (Gunnarsson & Lackie, 1985). This suggests an alternative pathogen-independent mechanism of haemocyte activation, which might be identical with clotting or induced by agents such as phospholipids released from or exposed on wounded cells (see chapter 1.8). Although capsules often become melanised, active phenoloxidase is not essentially required for encapsulation in *D. melanogaster* (Rizki & Rizki, 1990).

Haemolymph coagulation has long been recognised as the first phase of wound healing (Rowley & Ratcliffe, 1978). Primary plug formation is followed by melanisation of the scab and epidermal spreading reactions. Recently the mentioned steps in wound healing have been investigated in *Drosophila* (Galko & Krasnow, 2004). Analysing mutants of the phenoloxidase regulation, these authors concluded that melanisation is crucial for survival of the wounded animal by strengthening the wound plug. Yet the subsequent epidermal spreading reactions stimulated by the JNK pathway were stated to be independent of phenoloxidase activity. Epithelial cells orient towards the wound, fuse with each other and finally spread over the scab. These epithelial cell movements resemble dorsal closure during *Drosophila* embryogenesis (Wood et al., 2002), which is regulated by the Jun N-terminal kinase (JNK) signalling pathway as well (Rämet et al., 2002). The temporal order of the different phases of wound closure was studied in the mosquito *Armigeres subalbatus* (Lai et al., 2002). The authors of this study reported the establishment of a haemocyte plug already one minute after injury. Cells in this clump quickly released granular material that aggregated to an insoluble coagulum. Hardening of the primary clot by melanisation was observed after two hours. Flattening plasmatocytes or *Drosophila* lamellocytes might participate in the first cellular layer covering the wound as well, maybe adhering through RGD-dependent interactions, as proposed by Lackie (1988).

As the epidermal spreading and fusion reactions reach their end, a new basal membrane has to be synthesised around injuries or capsules to inhibit further haemocyte attachment. In *Pseudoplusia includens* apoptotic granular cells form a final layer at capsule peripheries (Pech & Strand, 1996, 2000). It remains to be investigated, if new basement membranes at wounds are produced by haemocytes or by epidermal cells.

1.4.5 Induction of antimicrobial peptides

Antimicrobial peptides are ubiquitously present in both prokaryotes and eukaryotes (Bulet et al., 2004). They are divided into several chemical classes and most of them act on microbial membranes through interaction with bacterial lipids (Bulet et al., 1999). The major amount of antimicrobial peptides in the haemolymph of insects is transcriptionally regulated after infection (Uttenweiler-Joseph et al., 1998, Engström, 1999) and they appear in the haemolymph after approximately two to four hours (Meister et al., 1997). Incorporation of these bioactive peptides in a haemolymph clot might promote killing of the entrapped microorganisms (not shown in Figure 1).

Antimicrobial peptide induction in *D. melanogaster* shows limited specificity for certain microorganism groups, namely fungi, Gram-positive and Gram-negative bacteria. Interestingly, the intracellular signalling pathways regulating *Drosophila* antimicrobial peptide genes share similarities with mammalian immune induction mechanisms (Hultmark, 2003). However, in difference to mammals *Drosophila* antimicrobial genes are regulated by two distinct cascades, the Toll and the Imd pathway (Hoffmann, 2003). Pattern recognition molecules on the pathogen surfaces are bound by peptidoglycan recognition proteins (PGRPs) which transmit the signal for peptide gene induction. In Gram-positive bacteria, Lys-type peptidoglycan is recognised by PGRP-SA in the haemolymph that activates the Toll pathway. The membrane-bound PGRP-LCx is specific for DAP-type peptidoglycan of Gram-negative bacteria and directly stimulates the Imd molecule, possibly as a heterodimer with PGRP-LCa (Steiner, 2004). Mammals widely rely on recognition of lipopolysaccharides (LPS). However, recent results suggest that the LPS molecule has no effect on the induction of antimicrobial peptide genes in *Drosophila* (Kaneko et al., 2004). Nevertheless, lipopolysaccharides are potent activator of the melanisation cascade in insects and may stimulate other immune reactions as well.

1.4.6 Towards an integrated view of coagulation in innate immunity

Haemolymph coagulation is one of the immediate reactions occurring after wounding. Since natural infections may occur through wounds, it is also likely that clotting is linked to immune processes. For example nodulation does not necessarily require the presence of bacteria (Gunnarsson & Lackie, 1985) and might thus be directly stimulated by primary wound reactions like coagulation. Furthermore it is possible that the immune response is directed by clotting, either towards a more cellular (nodulation, encapsulation) or towards a predominantly humoral reaction (melanisation, antimicrobial peptides). Regardless of potential regulatory functions, coagulation facilitates a localised immune response by immobilising and concentrating microorganisms at the site of injury. This may include local generation of cytotoxic phenoloxidase products by disintegrating cells in the coagulum and direction of antimicrobial activity to wound sites. Mutations with an affected nodulation, encapsulation or melanisation response may initially be caused by coagulation defects and should thus be re-investigated carefully. In summary, haemolymph clotting is clearly underrepresented in the current picture of insect immunity.

De novo induction of genes for clot proteins is probably too slow for efficient and quick wound sealing. These factors are rather expected to be constitutively present in the haemolymph or quickly released from activated haemocytes. Data available from *Drosophila* immune microarrays hence only provide limited information regarding possible clotting components, although replenishment of lowered haemolymph protein levels after coagulation might lead to a late gene induction pattern.

1.5 Comparison of coagulation in vertebrates and arthropods

1.5.1 Vertebrate blood clotting – primary and secondary haemostasis

Coagulation is "the change from a liquid to a thickened […] state (lat: *coagulatio*), not by evaporation, but by some kind of chemical reaction" (Webster`s dictionary, 1913). Clotting has historically long been regarded as a process separate from the immune response. Only recently it was suggested that coagulation and innate immunity evolved from a common ancestral immune system and continue to interact and cooperate with each other (Opal & Esmon, 2003). Localised coagulation not only enables wound closure, but also immobilises bacteria along with damaged cells and triggers inflammation. In vertebrates blood clotting is subdivided into primary and secondary reactions (Clemetson, 1999). During primary haemostasis aggregating platelets form a plug at the wound site (Figure 2). The scab is subsequently stabilised by fibrin polymerisation resulting from an activated clotting cascade (secondary haemostasis). Later the platelets retract to the edges of the lesion, followed by final spreading of endothelial cells over the injury. In the initial response von Willebrand factor (vWF) binds to collagen exposed on wounded subendothelium. A conformational change in vWF occurs after attachment, rendering it accessible to binding of the platelet membrane glycoprotein GPIb and thereby stimulates cell adhesion to the subendothelium (Sadler, 1998). Consequently, the platelet cytoskeleton is activated and the cells start to release further clot components through degranulation (Clemetson, 1999). In von Willebrand disease (VWD), characterised by mutated or absent vWF, primary haemostasis is impaired, which causes prolonged bleeding times (Sadler, 1998, Triplett, 2000). The von Willebrand factor also interacts with factor VIII, a component of the serine protease cascade (Jorieux et al., 2000).

This proteolytic cascade is regulated by two distinct pathways, an extravascular (extrinsic) and an intravascular (intrinsic) system. The extrinsic route is induced by the release of the intracellular tissue factor from ruptured cells at the wound site. In contrast the intrinsic system is stimulated by negatively charged surfaces, especially collagen exposed on damaged cell membranes of blood vessel walls. The two regulatory pathways combine to a common cascade initiated by clotting factor X. In addition to the

proteolytic activation, several factors require calcium and negatively charged phospholipids released from platelets as cofactors. The latter will be discussed in more detail later (see chapter 1.8). In the final step of the clotting cascade a transglutaminase, also known as factor XIII, cross-links lysine and glutamine residues of activated fibrin molecules with each other, thus hardening the clot. In addition vWF, fibronectin and other factors with high molecular weight are covalently linked to fibrin by factor XIII (Corbett et al., 1997). Many of the mentioned proteins like vWF, fibrinogen, fibronectin and endothelial collagen include the amino acid sequence RGD (Figure 2), which facilitates further integrin-mediated attachment of blood cells and links them together to stabilise the wound. As mentioned earlier, the RGD motif plays a crucial role in stimulation of cellular immune reactions in insects as well.

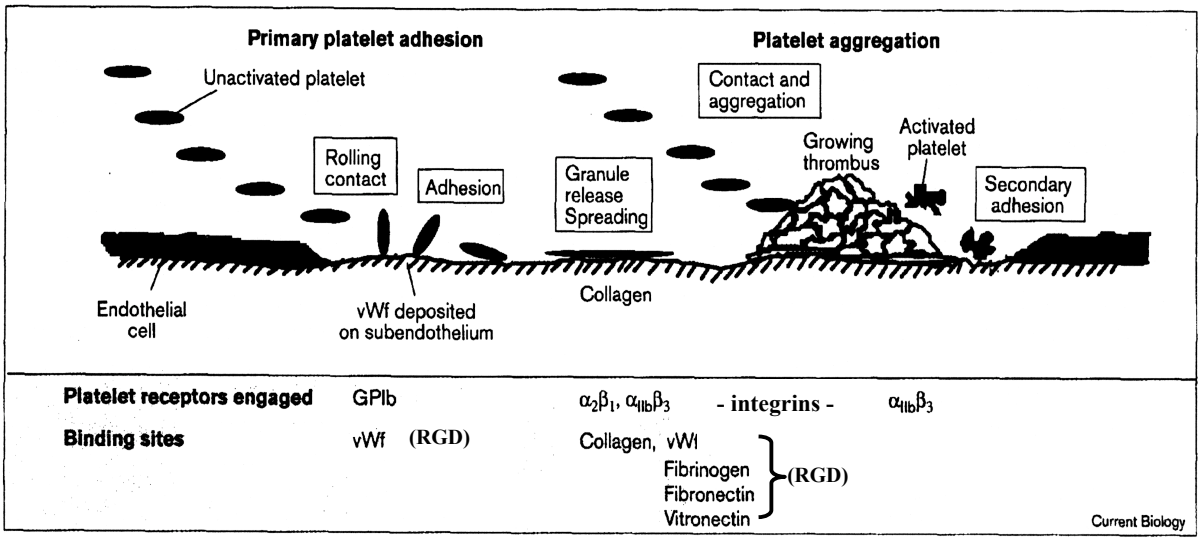

Figure 2: Vertebrate blood clotting: primary haemostasis
(modified after Clemetson, 1999)

1.5.2 The coagulation cascade of horseshoe crabs

Horseshoe crabs are the invertebrates, in which haemolymph coagulation has been studied most extensively. Clotting is activated by bacteria and more specifically by minute amounts of beta-glucan or lipopolysaccharide (Figure 3). This property of horseshoe crab haemolymph has been used since long as a quick clinical test for the

presence of LPS in patients (Nakamura et al., 1977). Activation of the clotting system by LPS was recently shown to be dependent on regulation by an NFkB motif (Wang et al., 2003), a promoter sequence that is also important for the regulation of several antibacterial peptide genes (Engström, 1999). Bacteria (Isakova & Armstrong, 2003) and immune proteins (Armstrong & Armstrong, 2003) are entrapped in the horseshoe crab coagulum, reflecting a protective function of the clot besides its haemostatic role. All elements of the proteolytic clotting cascade as well as regulating protease inhibitors are stored in haemocyte granules and released upon stimulation (Muta & Iwanaga, 1996, Iwanaga & Kawabata, 1998). Among the components of the horseshoe crab coagulation system there is only one orthologue to vertebrate clotting factors, namely transglutaminase, which is released from haemocytes into the plasma. An involvement of transglutaminase in coagulation was doubted for long, since another specific "clotting enzyme" proteolytically activates the haemolymph coagulogen (Figure 3). Recently it was reported, that primary coagulin aggregates are consequently cross-linked to residues on haemocyte membranes by transglutaminase (Osaki et al., 2002, Osaki & Kawabata, 2004).

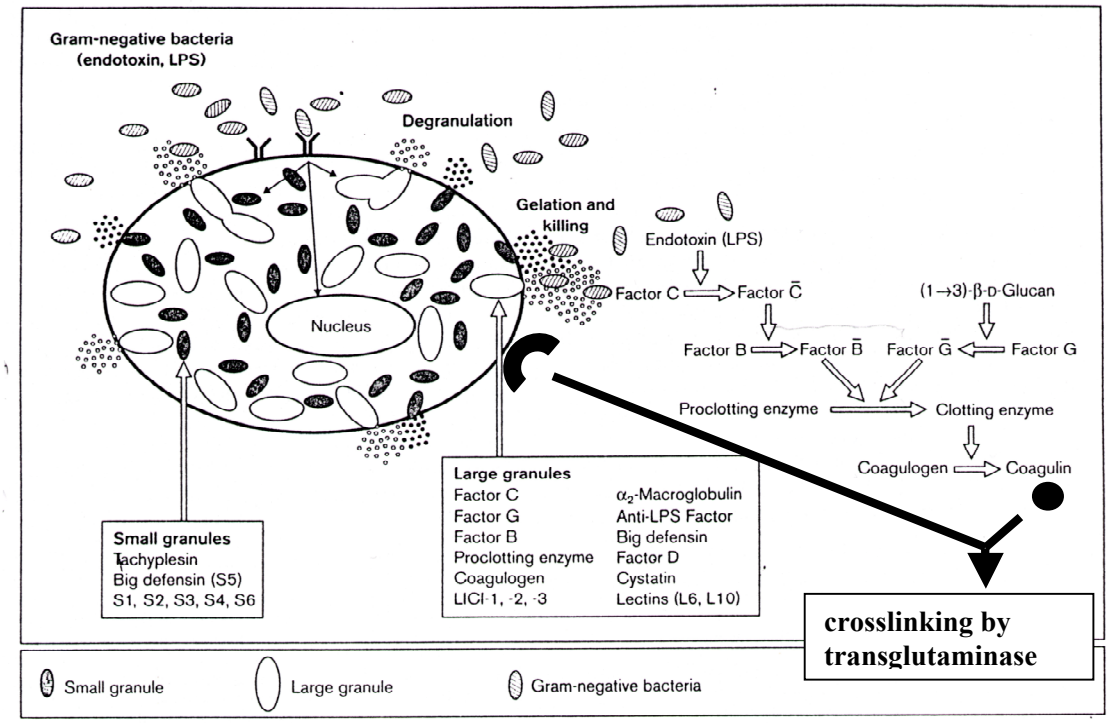

Figure 3: The haemolymph clotting system of the horseshoe crab
(changed after Muta & Iwanaga, 1996)

1.5.3 Haemolymph clotting in crustaceans

Like in other arthropods coagulation in crustaceans consists of two main steps, the degranulation of aggregating granular cells and subsequent formation of gelatinous material in the haemolymph (Ravindranath, 1980). The cross-linking enzyme transglutaminase is released from blood cells ("haemocyte coagulogen"), while the clottable protein is constitutively present in the haemolymph ("plasma coagulogen"). In contrast to horseshoe crabs and vertebrates, no proteolytic cascade seems to be involved in the activation process. However, coagulation essentially requires calcium as a transglutaminase cofactor and to initiate haemocyte degranulation (Hall et al., 1999).
The clotting protein in crustaceans was isolated from several species and characterised as a large lipoprotein related to insect vitellogenins (Fuller & Doolittle, 1971a, Komatsu & Andó, 1998, Yeh et al., 1999). In comparison to vitellogenins the clotting protein contains additional lysine- and glutamine-rich domains, which are targets for cross-linking by transglutaminase (Fuller & Doolittle, 1971b, Hall et al., 1999). It was proposed, that the crustacean prophenoloxidase system might act as a positive feedback mechanism by stimulating the release of transglutaminase and other clotting components from haemocytes (Hall et al., 1999). However, it may alternatively be the initial coagulation process that activates the prophenoloxidase cascade.

1.5.4 Insect haemolymph coagulation

Many morphological and cell-biological studies have been performed on insect haemolymph coagulation (Bohn, 1986). In contrast, only a limited amount of biochemical data is available on the molecular identity of factors involved in insect clotting (Theopold et al., 2002). Different requirements of vertebrate and invertebrate clotting systems due to certain characteristics of their physiology have been reviewed recently (Theopold et al., 2004).
Coagulation has to be activated quickly and regulated strictly. Constitutive presence of plasma factors might facilitate an almost immediate reaction, while proteins released

from haemocytes may help to avoid a premature activation of haemolymph clotting and to ensure a localised response. The initiation process probably involves oxygen originating from the air contact at the wound site. Under experimental conditions coagulation mostly occurs at the edges of cover slips covering haemolymph samples on slides and is slowed down by quick specimen preparation limiting oxygen access (Bohn, 1986 and personal observation). Possibly further components analogous to vertebrate tissue factor are released from damaged cells and induce the clotting process. Evidence for this assumption may be provided by the hypothetical, so far not purified protein hemokinin, which is released from damaged epidermal cells of *Hyalophora cecropia* and leads to formation of haemocyte clumps (Cherbas, 1973). Some researchers distinguished two general phases of insect haemolymph clotting, cellular aggregation and plasma gelation (Grégoire, 1974). After centrifugation of haemolymph samples coagulation was only observed in a small layer right above the sedimented haemocytes. In addition, clotting ability of the plasma could be restored by supplementation with haemocytes (Bohn, 1986). In conclusion it is likely that both haemocytes and cell-free plasma contribute to insect haemolymph clotting.

Haemocytes in the process of coagulation undergo a number of morphological changes: They increase in size, form vacuoles and cytoplasmatic blebs resembling apoptotic vesicles, before they finally degranulate and discharge intracellular material. Substances released from granules precipitate and form a dense meshwork (Bohn 1986). It is important to note that degranulation does not necessarily imply death of the involved haemocytes (Grégoire, 1974), although disintegration of cells participating in clotting frequently occurs (Rowley & Ratcliffe, 1976). Clot morphology and the contribution of haemocytes in its formation have been studied extensively since long (Yeager & Knight, 1933). Grégoire proposed three different patterns of haemolymph clotting depending on the presence of large veil-like structures and “coagulation islands”, the formation of pseudopodial meshworks or a combination of both (Grégoire, 1951). The possibility that the described cellular changes might simply represent a preparation artefact or necrotic cell death rather than a functional cellular process can be rejected, since haemocyte alterations are effectively inhibited by adding EDTA and other chemical agents to the medium (Bohn, 1986 and personal observations). Coagulation was investigated most extensively in lepidopterans, in which granular cells

are the subgroup of haemocytes involved in clotting (Bohn, 1986, Rowley & Ratcliffe, 1976). So-called coagulocytes, cystocytes or thrombocytoids were repeatedly described as a specialised haemocyte class with extremely large cell size, fragile membranes and numerous granules (Goffinet & Grégoire, 1975, Ravindranath, 1980). However, since these cells are not recognisable prior to clotting, it remains to be clarified if they "cause coagulation or are the result of coagulation", as noted by Gupta (1969).

The **haemolymph coagulogens** of the orthopteran insect *Locusta migratoria* (Brehélin, 1979, Gellissen, 1983, Duvic & Bréhélin, 1998) and the cockroach *Leucophaea maderae* (Barwig, 1985) were proposed to be identical with lipophorin. Intriguingly, locust lipophorin also aggregates spontaneously after wounding (Chino et al., 1987). In the lepidopteran *Manduca sexta* hemofibrin, a protein present in the cell-free plasma, forms a fibrous clot (Geng & Dunn, 1988). Other proteins in *M. sexta*, namely the serine protease-like scolexin (Finnerty et al., 1999) and the lectin M13 (Minnick et al., 1986), are possible activators of the coagulation reaction. Injection of a recombinantly produced part of the large protein hemocytin from *Bombyx mori* into larvae led to haemocyte aggregation (Kotani et al., 1995). A highly identical protein has been characterised on the nucleic acid level in *Manduca sexta* (Scholz, 2002). This protein is stored in the granules of granular cells in *M. sexta*. *D. melanogaster* has been the major model organism during the last decades in regard to immunity and other aspects of insect biology. However, until now no investigations have been exclusively devoted neither to clot morphology nor to the molecular identity of the implicated factors in this species. Recently hemolectin, a *Drosophila* protein with high similarity to *Bombyx mori* hemocytin, has been characterised (Goto et al., 2001) and a role in haemolymph coagulation on basis of a wound healing defect was proposed (Goto et al., 2003).

A gene for transglutaminase with high sequence similarity to the enzyme in crustaceans and horseshoe crabs is present in *Anopheles gambiae* and *D. melanogaster* and a function for this enzyme in insect haemolymph clotting is very likely (Korayem et al., unpublished data). Transglutaminases thus seem to represent the final cross-linking enzymes in all coagulation systems of vertebrates and arthropods investigated so far. Possible reasons for this conserved feature and the otherwise quite variable clotting factors in arthropods will be discussed in chapter 4.7.

1.6 Glycoproteins and lectins – interactions of haemocytes and pathogens

Many proteins involved in vertebrate immunity including most factors of the clotting cascade are glycosylated. The glycosylation state regulates assembly of the MHC- and T-cell complexes (Rudd et al., 2001). Cell-surface glycoproteins like CD43 might also contribute to the induction of apoptosis after association with lectins, carbohydrate-binding proteins (Tsuboi & Fukuda, 2001).

In general, gel-forming polymers often consist of glycoprotein monomers (Smith, 2002). Sugar modifications can be linked to a protein core by N- or O-glycosylation. **Mucins**, a group of multifunctional strongly O-glycosylated proteins, are mostly known for their presence in vertebrate mucus lubricant, but they can as well be integral cell membrane proteins (Hilkens et al., 1992). The mucus-type mucins are released from intracellular granules, oligomerise via disulfide bridges and form a gel by hydration (Hilkens et al., 1992). The function of mucins lining the peritrophic membrane of insects might be analogous to the ones present in vertebrate mucus, namely the inhibition of pathogen invasion into the midgut (Shen et al., 1999, Wilkins & Billingsley, 2001).

Cell agglutination is frequently catalysed by calcium-dependent multivalent **lectins** connecting cell surface glycoproteins (Sharon & Lis, 1989). In addition, lectins bind to microbial carbohydrates, thereby mediating host-pathogen interactions, which include both host invasion mechanisms and pathogen recognition systems (Mandrell et al., 1994). Lectins in insect haemolymph may thus establish contacts between haemocytes and pathogens and act as opsonins for phagocytosis (Pendland & Boucias, 1986, Renwrantz, 1986, Franc & White, 2000). A clustering effect of several glycoproteins on a small portion of the cell membrane might be important for activation (Vasta et al., 1996) and promote pathogen uptake. The bound state of insect membrane glycoproteins or lectins possibly also initiates the release of granular material, which includes large amounts of mucopolysaccharides (Gupta, 1991). As a result, the degranulated material might induce the prophenoloxidase cascade (Renwrantz, 1986), since carbohydrates and bacterial lipopolysaccharides trigger enzyme activation (Ashida & Yamazaki, 1990). On

the contrary, mucins on the surface of a cockroach parasite interfere with prophenoloxidase activation in the host by an unknown mechanism (Brennan & Cheng, 1975) and thereby block an effective immune response. *Manduca sexta* immulectin-1 stimulates prophenoloxidase activation (Yu et al., 1999). A lectin from the cockroach *Periplaneta americana* recognising galactose on pathogen surfaces is involved in encapsulation (Lackie & Vasta, 1988). Insect haemolymph lectins like M13 from *M. sexta* (Minnick et al., 1986) as well as galactose-binding lectins from the dipterans *Sarcophaga bullata* (Stynen et al., 1985), *Calliphora vomitoria* (McKenzie & Preston, 1992) and *D. melanogaster* (Haq et al., 1996) have repeatedly been implied in hemagglutination of sheep erythrocytes and insect haemolymph coagulation. Galactose modifications might therefore represent a general functional property of insect haemolymph clotting proteins. In agreement with this hypothesis galactose competitively inhibits adhesion of haemocytes to bacteria and especially interferes strongly with the attachment of granular cells (Dunphy & Chadwick, 1989). In summary, insect haemolymph lectins are involved in many immune reactions and seem to be of general importance for the stimulation of haemocyte aggregation.

In the present study I used lectins to visualise clot structures in *D. melanogaster*. In particular an N-acetyl-galactosamine-specific lectin from the snail *Helix pomatia* and a galactose-binding lectin from the peanut *Arachis hypogaea* ("peanut agglutinin") proved to be helpful tools. The snail lectin is a marker of vertebrate T-lymphocytes (Schadlich et al., 1984). In a *Drosophila* haemocyte cell line, *H. pomatia* lectin recognises hemomucin, a *Drosophila* haemocyte membrane glycoprotein (Theopold et al., 1996), which is additionally localised in microparticles released from haemocytes (Theopold & Schmidt, 1997). *A. hypogaea* lectin labels the *Drosophila* protein I71-7 (gp150), which is part of the *Drosophila* haemolymph coagulum (Korayem et al., unpublished data). The I71-7 gene is situated within a locus coding for various small salivary gland proteins of unknown function (Wright et al., 1996). In *M. sexta* peanut agglutinin labels the granules of granular cells, bacterial infections in addition lead to a significant labelling of spherule cells (Beetz, 2002).

1.7 Hemomucin, a glycoprotein with possible clot functions

The *Drosophila* haemocyte surface glycoprotein hemomucin (CG 3373) consists of a mucin-like segment and a domain with sequence similarity to plant strictosidine synthases, enzymes that catalyse a step in indole alkaloid biosynthesis (Theopold et al., 1996). Its mucin domain contains numerous potential O-glycosylation sites. Interestingly a strictosidine synthase-like domain is also present in several other animal genes, including two genes each in *Caenorhabditis elegans* and in *D. melanogaster* (CG 3373 and CG 11833) and one each in *Mus musculus* and *Homo sapiens* (Fabbri et al., 2000). These genes share the strictosidine synthase-like domain, but many of them lack the mucin-domain and hence cannot be considered as orthologues of hemomucin. Hence the term "strictosidine synthase-like" or "SSL" proteins will be used throughout the present study. There is very little information available about possible non immune-related functions of the mouse (Albrektsen et al., 2001) and the human member (Morita et al., 2000) of this protein family. It is appealing that the human protein is strongly expressed in hematopoietic tissues (Ulrich Theopold, unpublished data). Another interesting aspect is that both *Drosophila* hemomucin and the mouse protein are present in the central nervous system (Ulrich Theopold, unpublished data), which might implicate additional functions of these proteins.

In *Drosophila* larvae, hemomucin is found in salivary glands, imaginal discs, fat body, gut and ovaries (Theopold et al., 2001). In adult flies hemomucin is located in haemocytes, ovaries and in the peritrophic matrix (Theopold et al., 1996). From the adult ovaries hemomucin is also deposited on the surface of eggs. Using *H. pomatia* lectin to label hemomucin the protein was detected in granules of SL2 cells and particles released by the cells. Similarly, incubation with the lectin led to labelling of microparticle-like structures shed from *Galleria* haemocytes (Theopold & Schmidt, 1997), which may indicate the presence of a similar glycoprotein in this species that is released from haemocytes upon activation. *Drosophila* hemomucin also forms complexes with lipophorin from both *G. mellonella* and *D. melanogaster* (Theopold & Schmidt, 1997). From the data collected so far, hemomucin is likely to be a protein involved in clotting.

1.8 Phospholipids and eicosanoids – a link between apoptosis, clotting and immunity ?

In all eukaryotic cells, the lipid composition of the inner and outer cell membrane layer differs significantly, negatively charged phospholipids being confined to the inner leaflet. Three different energy-dependent pumps - translocases, floppases and scramblases – actively transport lipids to the opposite side of the bilayer to maintain the described lipid asymmetry (Bevers et al., 1999). Cell surface blebbing and the exposure of anionic phospholipids on the outer leaflet are features observed during early apoptotic, but not in necrotic processes (Bevers et al., 1999) and are evolutionarily conserved (van den Eijinde et al., 1998). The presence of normally intracellular components on vertebrate blood cell surfaces was suggested to act as an intrinsic "danger signal" to initiate an immune response that is pathogen-independent (Matzinger, 1998, Gallucci & Matzinger, 2001).

Loss of lipid asymmetry is also a significant characteristic of activated platelets (Zwaal et al., 1998) and regulates **vertebrate clotting** (Lentz, 2003). Increased levels of phospholipids, especially on platelet membranes, stimulate coagulation by reducing the K_m of clotting factors (Davie et al., 1991). Nevertheless, the well-established dogma that platelet surfaces are essentially required as crystallisation points for the reaction does no longer hold true, since membranes can be substituted by soluble phospholipids (Majumder et al., 2002). Conversely, anti-phospholipid autoantibodies in the blood of patients with systemic lupus erythematosus (SLE) block the normal regulation and cause coagulation defects (Casciola-Rosen et al., 1996). Suramin, an inhibitor of aminophospholipid translocase reduces activation of several human clotting factors (Horne et al., 1992).

Furthermore platelets form membraneous protrusions and release microparticles during clotting. Microparticles are small vesicles containing large amounts of anionic phospholipids and show both pro- and anticoagulant activity (Barry & FitzGerald, 1999). This regulative function of microparticles was suggested to be carried out mainly by supplying a phospholipid-rich surface as an assembly matrix for clotting factors, especially for the prothrombinase complex (Jy et al., 1995). Neutrophils are aggregated with each other in reaction to microparticle exposure (Jy et al., 1995). This

agglutination is achieved by the release of granular material from the neutrophils that forms fibrous networks, so-called NETs (neutrophil extracellular traps) binding to bacteria and killing them (Brinkmann et al., 2004). One bioactive compound in vertebrate microparticles is arachidonic acid, an eicosanoid precursor that is hydrolysed from membrane phospholipids by phospholipase A_2. It mediates inflammatory reactions of leukocytes (Forlow et al., 2000) and interactions with further platelets and endothelial cells (Barry et al., 1997). For example platelet-activating factor (PAF) is derived from phospholipids by phospholipase activity within minutes. The phospholipid-binding annexin V is a vertebrate anticoagulant by clustering on platelet surfaces (Andree et al., 1992). Moreover, annexin V also inhibits phospholipase A_2 activity, thus interfering with eicosanoid biosynthesis (Russo-Marie, 1999).

Intriguingly even in invertebrates, the **horseshoe crab clotting** factor C is not dependent on the presence of microbes, but can alternatively be achieved by anionic phospholipids (Nakamura et al., 1988).

Coagulating insect haemocytes form surface blebs and shed vesicles similar to vertebrate microparticles (Grégoire, 1951). Annexin V labels phospholipids on these microparticles in haemocyte lines (Theopold & Schmidt, 1997). It can therefore be assumed that there is a connection between apoptotic processes and haemolymph clotting in insects as well. Suramin, the mentioned inhibitor of vertebrate aminophospholipid translocase, effectively blocks coagulation in different insect groups (Grégoire, 1953), suggesting the requirement of negatively charged phospholipids for clotting. Besides the well-known pathogen-dependent prophenoloxidase activation cascade, enzyme stimulation was proposed to be alternatively elicited by phospholipids (Sugumaran & Nellaiappan, 1991, Sugumaran & Kanost, 1993). Prophenoloxidase activation by denatured lipophorin (Ashida et al., 1983, Leonard et al., 1985) or denaturing agents like SDS (Decker et al., 2001) could be explained along similar lines, since the bulk of insect lipophorins consists of about 40 to 50 % lipids (Blacklock & Ryan, 1994), which are exposed during denaturation. Conversely, native lipophorin may bind and block phospholipids or remove them from the haemolymph. While lipophorin-depleted haemolymph elevates haemolymph phenoloxidase activity by about 25 %,

native lipophorin inhibits stimulation of the enzyme (Duvic & Bréhélin, 1998). Haemocytes from parasitised immune-compromised *Pieris rapae* caterpillars do not render their membrane lipid distribution (Asgari et al., 1997), a finding that strengthens the possible significance of phospholipid exposure as a recognition signal in insect immunity.

Even in insects the eicosanoid precursor arachidonic acid is hydrolysed from phospholipids by phospholipase A_2 (Howard & Stanley, 1999). Eicosanoids stimulate insect phagocytosis and activate the prophenoloxidase system (Mandato et al., 1997). Furthermore they mediate microaggregation reactions in response to LPS (Bedick et al., 2000), nodulation around injected bacteria (Miller et al., 1994) and fungi (Lord et al., 2002). Conversely, nodulation is inhibited by the bacterium *Xenorhabdus nematophilus* that blocks eicosanoid biosynthesis (Park et al., 2003). The prevention of eicosanoid production similarly reduces the encapsulation frequency of parasitoid eggs in *Drosophila* (Carton et al., 2002). Intriguingly, a regulatory role for eicosanoids in the Imd pathway leading to antimicrobial peptide production (see chapter 1.4.5) was reported recently as well (Yajima et al., 2003). *Drosophila* annexin IX might have immune-modulating functions similar to vertebrate annexin V by interfering with eicosanoid synthesis. It is tempting to speculate that annexin IX and lipophorin may act as anticoagulants in insect haemolymph. Apoptotic features of coagulating cells possibly also stimulate phagocytosis of haemocytes from the clot by the *Drosophila* scavenger receptor encoded by the gene *croquemort* (Franc et al., 1996, 1999). Since most antimicrobial peptides act on negatively charged membranes (Boman, 1995), one might further speculate on an additional role for some peptides in the clearance of apoptotic cells. It remains to be clarified, if insect haemolymph clotting is triggered, accompanied or followed by apoptotic events.

In summary, phospholipids on haemocytes and their eicosanoid derivates likely provide a link to different aspects of insect immunity. This regulation appears quite similar to the situation in vertebrates and might represent an evolutionary conserved feature.

1.9 Aim of the projects

As mentioned, only very little information on *Drosophila* coagulation was available at the beginning of the present research program. The aim of my work was to fill this gap and identify and characterise putative insect clotting factors using different approaches. Since no other candidate clotting proteins of *D. melanogaster* were described at the time, I initially chose to study hemomucin, a glycoprotein that was known to be present in the clot and in insect microparticles. A methodical advantage with hemomucin was the possibility to use an already existing specific antiserum and *Helix pomatia* lectin for labelling and visualisation of the protein. A knock-down of hemomucin by RNA interference should assist in clarifying the function of the protein in immunity and development. A mouse protein with high sequence similarity to hemomucin (mSSL) should be analysed by production of a specific antiserum. It was intended to use this antiserum to clarify a possible role for the mSSL protein in mouse immune tissues, which was suggested by data from the human SSL homologue.

While it became increasingly clear that probably many more factors are involved in insect haemolymph clotting, it remained technically complicated to separate a coagulum from haemocytes and cellular material to characterise its components. Furthermore cross-linking of the proteins was supposed to hinder molecular identification. Especially isolation of the *Drosophila* clot turned out to be difficult for the mentioned reasons. Hence, I chose two different methods that both were designed to circumvent these problems. First I produced an antibody against a haemolymph coagulum isolated from *G. mellonella* cell-free plasma. For *Drosophila* I decided to use a direct proteomics approach in search for new haemostatic proteins. In *D. melanogaster* some putative coagulation factors were then further analysed using mutants and RNA interference.

2 Material and Methods

2.1 Maintenance of *Drosophila* and *Galleria* cultures

2.1.1 Rearing conditions

The *Drosophila* cultures were maintained in a culture room with a temperature of either 18°C or 25°C and a light/dark-cycle of 12 hours each. All crosses for RNA interference experiments were carried out at 25°C to ensure activity of the yeast UAS-promoter. For crosses of different *Drosophila* stocks virgin female flies were isolated from one strain and crossed with three to five males each from the other fly line. Experiments described in this study were carried out with late third instar wandering stage larvae that were picked from the walls of the culture vials with a brush. In some mutant stocks such as *domino* with increased lethality during late larval stages (Braun et al., 1998) the retrieved numbers of larvae were not sufficient, so that big feeding stage larvae were chosen from rinsed food in addition. In these instances the larvae used for control samples also included feeding stage larvae.
Galleria larvae were kept in hard plastic boxes and reared under the same temperature conditions as described above for *Drosophila*. Last larval instar wandering larvae were used in the experiments described in the present study. These were easily recognisable by the emptied hindgut visible through the integument. The larval development of *Galleria* initially takes a long time, but during the last larval instar the larvae grow rapidly. The cultures were reduced to some individuals only at this stage.

2.1.2 *Drosophila* food medium

For the preparation of *Drosophila* food 2,2 l of water were brought to the boil. Then the following constituents were added under vigorous mixing:

3	tablespoons	dry yeast
100	ml	syrup
80	g	mashed potato powder
20	g	agar

The blend was boiled up again for 15 min and then cooled down to 60-70°C under vigorous mixing to avoid clump formation. Afterwards 17 ml of 10 % nipagin (Sigma; 4-hydroxy-benzoate-methylester) dissolved in 95 % ethanol and 2,5 ml ascorbic acid were added. To avoid layering of the food, the mix was further cooled down under stirring to about 50°C (just above the agar solidification temperature) before portioning it into culture vials and flasks.

2.1.3 *Galleria* food medium

The following ingredients were mixed together:

100	g	baby cereals
200	g	wheat germs
1,5	g	dry yeast
0,15	g	nipagin (4-hydroxy-benzoate-methylester, SIGMA)
2		multi-vitamin capsules (TwinLab, "Daily One")

After reaching a homogenous blend, the remaining components were added slowly under vigorous stirring to avoid clumping of the food. The medium could be stored for several weeks at 4°C prior to use. New food was added to the top of the *Galleria* cultures in sufficient amounts.

2.2 *Drosophila* embryo fixation for *in situ* hybridisation

Freshly laid *Drosophila* embryos were collected from apple juice plates, dechorionated in 50 % chlorine solution for 2-3 min and washed several times with water. The embryos were transferred into an Eppendorf microtube and rinsed with a washing solution containing 0,04 % Triton X-100 and 0,7 % NaCl. Fixation was carried out in a ribofix-heptane mixture with the following ingredients and vortexed for 25 min:

1, 4	ml	16 % formaldehyde (electron microscopy grade, Polysciences)
0, 25	ml	10 x PBS
0, 25	ml	0,5 M EGTA, pH 8,0
0, 6	ml	H_2O dd
2, 5	ml	heptane

The solution was replaced with 0,5 ml heptane and 0,5 ml methanol and shaked vigorously for 1 min to devitellinise the embryos. After rinsing twice each with methanol and 99 % ethanol to remove the heptane, the embryos were stored in 99 % ethanol at -20°C until use.

2.3 DNA microinjection of *Drosophila* embryos

The vector containing the P-element excision sites and the hemomucin construct for *in vivo* production of dsRNA should be injected into *Drosophila* embryos to generate a transgenic fly stock. The transposase gene for the P-element transition was added to the solution on a helper plasmid ("Δ 2-3"; kindly provided by Ylva Engström) and brought into an "M cytotype" fly stock (w^{1118}) lacking the transposase gene and natural P-elements. Furthermore this helper plasmid construct was "wings-clipped", in which the IR-sites flanking the transposase gene were deleted. This ensured that the transposase would not become incorporated into the genome, and the enzyme would be exclusively active during the next germ cell development only. (Expression of the transposase in subsequent fly generations would have led to an unstable insertion.) Integration of the construct in the germ cells was possible, since the DNA was introduced at an early stage of embryogenesis, when no pole cells (germ cells) had formed yet in the syncytium lining the outer layer of the embryos. The presence of pole cells could easily be recognised under the microscope. Thus the construct could be stably integrated into the germ-line and transmitted to the next generation. Successful transformation of the plasmid into the genome was indicated by red-eyed flies in the G1-generation, since the integration plasmid included a copy of the *white* gene, for which the acceptor flies had a mutation. Transformed flies were then isolated and back-crossed with the parental stock for generation of strains with independent insertion sites. The following protocol for preparation of the embryos was adapted from Roberts (1998), the microinjection method was originally described by Rubin & Spradling (1982). Flies were allowed to deposit eggs for 1 h on apple juice agar plates with excess amounts of yeast to stimulate egg-laying activity. The early stage embryos were collected, dechorionated in 50 % chlorine solution for 2 to 3 min, washed several times

with water, followed by a brief rinse in PBS containing a weak detergent to remove embryos sticking to the walls of the collection sieve. The dechorionated embryos were mounted on a square of dark-coloured apple juice agar (including charcoal for better contrast of the white embryos) under the microscope with a fine needle. Later injection of the embryos at the pole cell-forming posterior end was facilitated by orienting them in this working step. The eggs were first adjusted with the anterior end (recognisable by the micropyle) to one side of the agar square and then transferred to a cover slip with a glue-covered sticky surface. The glue had been prepared from rubber tape dissolved in heptane over night and was applied to the cover slips some minutes before use to allow the heptane to evaporate. After 10 min of drying (time varied according to the temperature and humidity of the environment), the embryos were covered with oil (Voltalef grade “H10S”; elf atochem, France), which limited the loss of body fluid from the embryos into the medium in reaction to insertion of the microcapillary. These fine needles were produced with a needle pulling machine (Narishige, Japan) and filled with DNA solution (1 □g/□l in PBS) using a drawn-out glass pipette. The cover slip with the mounted embryos was adjusted close to the capillary under the injection microscope (Wilovert, Mikron AB) and manually moved into the needle. A defined DNA volume was injected from the capillary connected to a gas tube by shortly pressing a foot pedal**.** Embryos with already formed pole cells were killed by moving the needle right through them. Finally the cover slip with the injected embryos was placed into a humid chamber. The H10S-oil was replaced by “H3S” oil (elf atochem, France) with lower density to allow for sufficient air supply of the developing embryos. Emerging larvae, which had survived the injection procedure, were collected during the following days and transferred into food vials for further development.

2.4 Crossings for hemolectin RNA interference experiments

The UAS-*hml* (hemolectin) construct integrated into the genome of a wildtype fly codes for the expression of a single-stranded RNA that forms intramolecular base pairs and thereby is rendered into a double-stranded structure. The transformed flies were kindly provided by Dr. Akira Goto (Strasbourg, France).

To activate the yeast promoter UAS, the parental stock had to be crossed with a *Drosophila* Gal4-line. The Gal4-protein can be expressed at a certain developmental stage or in a single tissue by restricting its transcription with a heat-shock or tissue-specific promoter. In the present study I decided to utilise an Actin5C-Gal4 line to ensure high levels of ubiquitous Gal4 expression. In addition a haemocyte-specific *hml*-Gal4 line was used for other crosses (data not shown).
To guarantee induction of the temperature-sensitive UAS-promoter, the flies and their offspring were kept at 25°C.

2.5 Maintenance of mbn-2 cell cultures

The *Drosophila* cell line malignant blood neoplasm-2 (mbn-2) was established from hematopoietic organs of a haemocyte tumour mutant with the same name (Gateff et al., 1980). I used cells from this line in the *in vitro* experiments, since they show a haemocyte-like appearance and cell physiology. The culture bottles were maintained with 5 or 15 ml volume of medium. The *Drosophila* Schneider culture medium (GIBCO and PAN Biotech GmbH) was supplemented with 10 % fetal calf serum (FCS; Invitrogen) and 2 mM L-glutamate (GIBCO) before use.

2.6 Collection of haemolymph and haemocyte samples

Haemolymph from *G. mellonella* was collected by cutting a pro-leg with a pair of fine scissors, while *Drosophila* haemolymph was obtained by opening the larvae with a pair of forceps without disrupting the fat body or other inner organs. Under average conditions one drop of *Galleria* haemolymph or haemolymph from two *Drosophila* larvae per well were used. The amount of buffer added to *Drosophila* samples was minimised to about 5 µl to allow for maximal clot formation. The physiological buffers employed for haemocyte preparations on object slides were “insect Ringer” for *G. mellonella* (Theopold & Schmidt, 1997) and *Drosophila* Ringer (Goldstein & Fyrberg, 1994). The haemocytes were allowed to settle down and attach to the slide during a 10 min incubation time in a humid chamber before application of a cover slip.

Fixatives were not used, since clot structures were destroyed in the course of several tested fixation methods. Haemocyte preparations were alternatively labelled with lectins from *A. hypogaea* and *H. pomatia* (20 µg/ml; Sigma) during an incubation period of 15 min to 1 h at room temperature. The specimens were then carefully rinsed with PBS, mounted with a cover slip and analysed under a fluorescence microscope (Axioskop, Zeiss).

Haemolymph samples for gel electrophoresis (see chapter 2.13) were either bled directly into a microcentrifuge tube filled with *Drosophila* Ringer or collected on a concaveous depression object slide and transferred into a tube later. If coagulation should be inhibited, the buffers lacked $CaCl_2$ and instead contained 20 mM EDTA. To acquire a plasma sample (cell-free haemolymph) the haemocytes were centrifuged down at 200 *g* for 15 min. Preparation of the clot samples for the pull-out assay will be described in chapter 2.12.

2.7 Flow cytometry - Fluorescence-activated cell sorter (FACS)

Hemomucin expression on haemocyte-like mbn-2 cells was studied before and after addition of the calcium ionophore A23187. For each sample 5 ml of mbn-2 cells were incubated for 2 h with 2,5 µM, 5 µM, 10 µM and 20 µM calcium ionophore (A23187, Sigma; dissolved in DMSO) or 20 µM DMSO respectively in Ringer solution. An additional control sample did not include any chemical. Following incubation the cells were killed by addition of 0,05 % natrium azide to the medium to stop all cellular reactions and 0,5 ml were transferred to FACS tubes. *Helix pomatia* lectin (HPL, FITC-labelled; Sigma) was added in a final concentration of 20 µg/ml and the tubes incubated in the dark on ice for 10 min. The fluorescence signal for FITC-HPL was analysed in a FACScan flow cytometer (Becton Dickinson) and data collected with the CellQuest software. One sample was treated with propidium iodide (PI) (according to the manufacturers advice, Sigma) in addition to FITC-HPL in order to recognise the position of apoptotic cells and cell debris in the FACS output. These cells were excluded from the analysis. All remaining cells were considered as viable and their fluorescence levels were investigated at 495 nm.

2.8 *In situ* hybridisation of *Drosophila* embryos

The hemomucin DNA (provided by Dr. Ulrich Theopold) was available integrated in the pBluescript SK$^-$ vector (cloned into the Eco RI restriction site of the multi cloning site). To avoid cross-reaction of the RNA probe with other mucin RNAs in the embryo, the gene sequence exclusively contained the strictosidine synthase domain of hemomucin (internal laboratory plasmid name "Hmu 7"). In a restriction test with Bam HI and Hind III (method after Sambrook et al., 1989) it was confirmed that the hemomucin DNA was still present in the vector and no other DNA contamination was recognisable (gel not shown). To produce larger amounts of DNA, competent *E. coli* cells (strain JM 109) were transformed with the plasmid and the plasmid recovered from the cells according to a standard protocol (Sambrook et al., 1989). The templates for the production of a sense and antisense RNA probe were created by linearisation of the plasmid with two different restriction enzymes Bam HI and Hind III (Sambrook et al., 1989). The linearised DNA was cleaned in a phenol/chloroform extraction (after Sambrook et al., 1989) and subsequently used for an *in vitro* transcription including digoxigenin- modified UTP nucleotides to label the RNA probes for later detection.
Transcription with the T7 RNA polymerase led to production of the sense probe (control for *in situ* hybridisation), while the T3 RNA polymerase gave rise to the antisense RNA probe (see Figure 4).

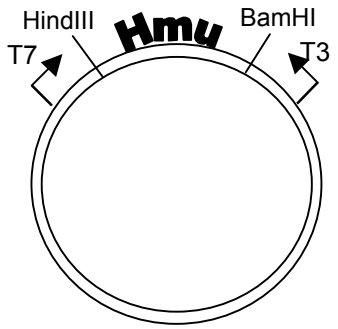

Figure 4: Schematic presentation of the plasmid used for production of the sense and antisense RNA probe of hemomucin

Protocols for *in vitro* transcription, dig UTP labelling and *in situ* hybridisation were adapted from Tautz & Pfeifle (1989). For the *in vitro* transcription the following mix was incubated at 37°C for 2h:

1 µl	10 x transcription buffer (see below)
1 µl	10 x dig U NTP mix (see below)
1 µl	50 mM DTT (Pierce)
1 µl	RNase inhibitor (stock 50 units/µl; Ambion)
2 µl	linearised DNA (approximately 1 µg)
1 µl	T7 or T3 RNA polymerase (Gibco)
3 µl	H_2O dd

After incubation 16 µl H_2O dd were added and 1 µl of the mixture saved to check the RNA product on a 1,2 % agarose gel together with a standard (gel not shown). Addition of 25 µl 2 x carbonate buffer (120 mM Na_2CO_3, 80 mM $NaHCO_3$; pH 10,2) and subsequent incubation at 65°C for 12 min ensured partial fragmentation of the RNA, so that it could penetrate the *Drosophila* embryos. To avoid complete degradation, the reaction was stopped by a pH change caused by addition of 0,2 M Na CH_3COOH (adjusted to pH 6,0 with acetic acid). Further addition of 10 µl 4 M LiCl, 5 µl phenol/chloroform-extracted tRNA (20 mg/ml) and 300 µl 99 % ethanol and freezing at -20°C for 15 min caused RNA precipitation. The RNA was then pelleted by sharp centrifugation in the cold, washed with 70 % ethanol, dried and dissolved in 150 µl of hybridisation solution (HS; composition see below). For hybridisation 20 µl of settled fixed embryos were washed five times for 5 min each in 0,1 % PBST (PBS with 0,1 % Tween 20) and afterwards once for 5 min in a 1:1 PBST/HS mixture. Afterwards the eggs were briefly washed three times with HS and pre-hybridised in HS at 55°C for 1 h. Incubation with 0,2 µl of the probe (two parallel samples for sense and antisense probe) in 50 µl HS was carried out over night at 55°C. The following day the embryos were rinsed four times 20 min each at 55°C in HS prior to detection with a preabsorbed alkaline phosphatase (ALP) labelled anti-dig antibody diluted 1:2000 in 0,5 ml PBST under shaking for 1 h. The eggs were washed four times with PBST for 15 min each and then three times in freshly prepared ALP buffer (see below) for 5 min each. The localisation of the anti-dig antibody was visualised by developing the samples in the dark with 0,5 ml ALP buffer including 1,75 µl X-phosphate and 2,25 µl NBT. The

embryos were checked under a dissecting microscope and after about 10 min the reaction was stopped by washing several times with PBST. Finally the embryos were embedded on object slides for long-time storage at 4°C.

10 x transcription buffer:

0,4 M	1 M Tris pH 7,5
0,06 M	1 M $MgCl_2$
0,1 M	1 M NaCl
0,02 M	1 M spermidine-HCl

10 x dig U NTP mix:

10 mM	ATP
10 mM	GTP
10 mM	CTP
6 mM	UTP
4 mM	Dig-labelled UTP (all Böhringer Mannheim)

hybridisation solution (HS):

50 %	formamide
5 x	SSC
100 µg/ml	heparin
0, 1 %	Tween-20
100 µg/ml	sonicated boiled salmon sperm DNA

ALP buffer (for alkaline phosphatase reaction):

100 mM	NaCl
50 mM	$MgCl_2$
100 mM	Tris-HCl, pH 9,5
0,1 %	Tween-20

2.9 *In vitro* transcription of hemomucin RNA for RNAi

The “Hmu 7” vector was used for RNA interference experiments. Since it did not contain the mucin domain of hemomucin, unspecific interference with other mucin-like sequences in the cells was avoided. The intron-exon boundaries of hemomucin were determined with help of the Berkeley *Drosophila* Genome Project (BDGP) web page (http://www.fruitfly.org). The DNA template of about 660 bp for the *in vitro* transcription was chosen from a region at the start of the fourth exon. It was amplified from the plasmid in a “nested PCR” consisting of two successive reactions (kit from Roche, Applied Biosystems) with different primers (Interactiva) to ensure a specific DNA product (Figure 5). The primers for the second PCR reaction included the sequence of the T7 promoter (GGA CGA ACT GGC TTT CG) utilised in the following *in vitro* transcription. In parallel a GFP dsRNA was amplified from another plasmid as a control. After the PCR reaction the samples were cleaned up using a PCR purification kit (QIAGEN) and used as DNA templates using the MEGASCRIPT T7 transcription kit (Ambion). The RNA products were ethanol-precipitated (method after Sambrook et al., 1989), resuspended in RNA storage buffer (Ambion) and annealed to dsRNA by incubation at 65°C for 30 min. RNA interference experiments were carried out according to the protocol by Clemens et al. (2000) with 15 µg dsRNA (coding for hemomucin or GFP) added to mbn-2 cell cultures in a concentration of 10^6 cells per ml.

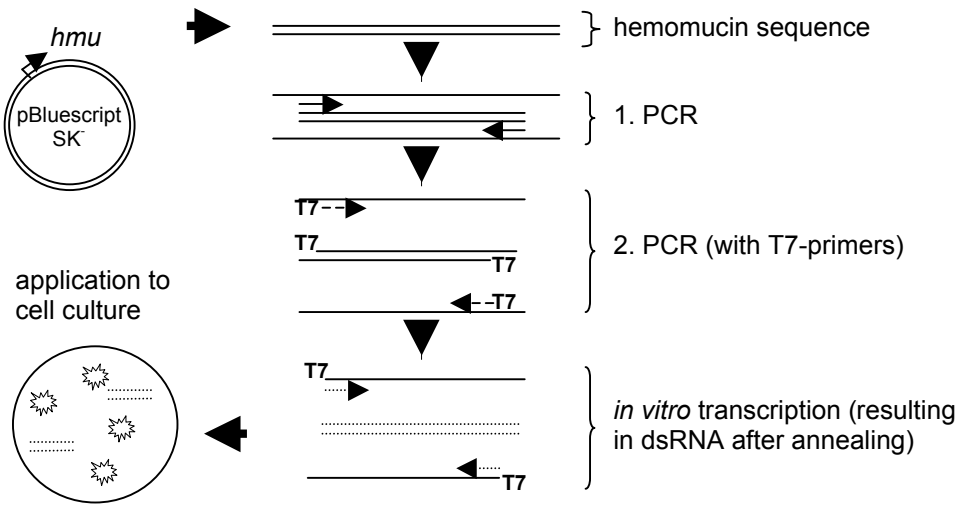

Figure 5: Production of a dsRNA for hemomucin RNA interference in cell culture

2.10 Synthesis of a DNA coding for a dsRNA hairpin construct

In addition to a cell culture knockdown of hemomucin, a construct for *in vivo* down-regulation of the protein in transgenic flies was produced. The most advanced available synthesis technique at the time was adapted from a study by Kennerdell & Carthew (2000) summarised in Figure 6. Briefly, the DNA for the hemomucin gene was multiplied by two parallel PCR reactions using primers with different restriction sites added to their ends. As a template the "Hmu 7" construct lacking the mucin domain was used. After cleaving the PCR products with the endonuclease Sfi I "sticky ends" were created, which were subsequently ligated to each other. The resulting product consisted of two copies of the gene sequence, but oriented in opposite directions. Endogenous transcription from this transgene would therefore lead to the formation of an RNA with two complementary strands hybridising to a double-stranded structure. Importantly, the restriction enzyme Sfi I has no specificity for the five central base pairs of the recognition site GGCC NNNNN GGCC. Using two different primers "Sfi Ia" and "Sfi Ib" (Figure 6, magnifying glass), the RNA transcripts would later on not be complementary in this small central region and form a "hairpin loop". The product from the first ligation and the cloning vector were subsequently restricted with another pair of enzymes, Eco RI and Xho I, and ligated into the pUAST vector, which had been linearised with the same restriction enzymes. After multiplication of the plasmid in transformed bacteria and recovery with help of "mini prep" plasmid DNA preparations this construct was ready for microinjection into *Drosophila* embryos (see chapter 2.3) for production of transgenic fly lines. To facilitate an easy screening method for successful integration of the transgene into the genome, the transposable element also included the *white* gene (w^+) for red eye colour. The red-eyed flies from the G2-offspring indicated successful integration of the construct into the fly genome. An ambiguous result after crosses with the transformants led me to send the construct for sequencing (KISeq unit at Karolinska Institute, Stockholm). Since the insert was not the expected hemomucin gene, I did not continue to analyse the transgenic flies. In theory, after mapping the insertion to the chromosome level, it would have been balanced to maintain the construct. The hemolectin RNAi-stocks used in the present study (kindly provided by Dr. Akira Goto) were produced by a similar approach (Goto et al., 2003).

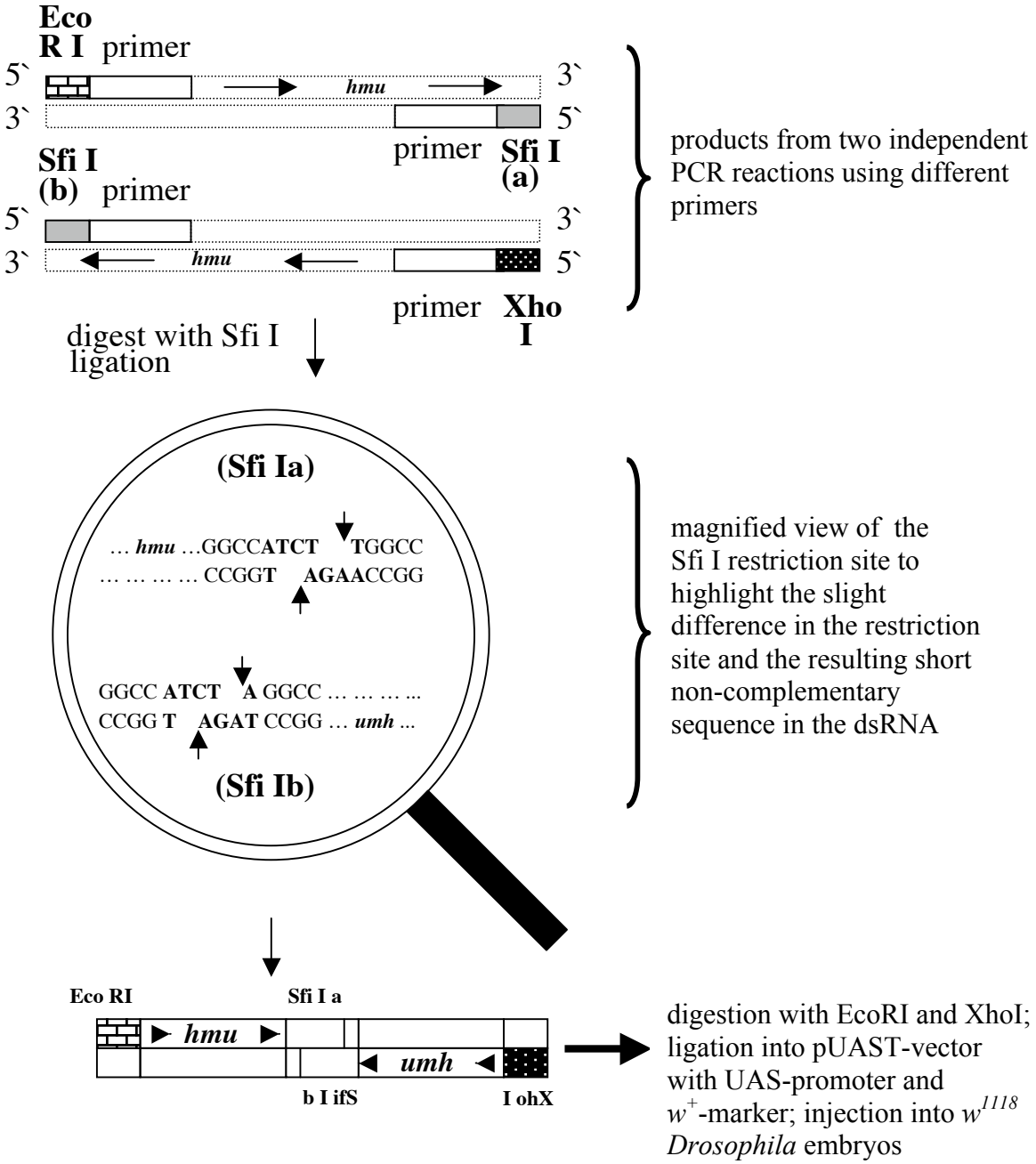

Figure 6: Schematic presentation of DNA synthesis for *in vivo* dsRNA production from a hemomucin transgene
(method after Kennerdell & Carthew, 2000)

The two PCR reactions were carried out in a thermocycler (Robo Cycler Gradient 96, Stratagene) utilising a kit (GeneAmp XL PCR kit, Applied Biosystems) for the needed components. All amounts and incubation times for the reaction were adapted from the supplied booklet; the reaction was extended for 30 cycles. The PCR products were cleaned up with the QIAquick PCR purification kit (Qiagen) and the samples resolved in 60 µl each.

The used primers were synthesised by Thermo Hybaid (Interactiva, Germany) and had the following sequences. (Restriction sites are presented underlined, the central parts of the variable Sfi I – recognition site with dotted lines.)

PCR 1: "Eco RI–*hmu*": 5`–G GAA TTC CGC TCG AAT GTT AGC–3`
"*hmu*–Sfi Ia": 5`–GGC CAT CTT GGC CCT TGG TAG TGG GTT TCG–3`

PCR 2: "Xho I–*hmu*": 5`–G CTC GAG CGC TCG AAG AAT GTT AGC–3`
"*hmu*–Sfi Ib": 5`-GGC CTA GAT GGC CCT TGG TAG TGG GTT TCG–3`

A 1 µl aliquot of the purified PCR product was used on a 1 % agarose gel (Sambrook et al., 1989) in order to confirm a specific PCR reaction. The subsequent restriction of both PCR products with Sfi I was carried out with the following components:

10 µl DNA (PCR product)
7 µl H_2O dd
1 µl Sfi I (Sigma)
2 µl restriction buffer (supplied with the enzyme)

The incubation time was initially adjusted to 1 h according to standard conditions, but later on prolonged to 2 h to ensure total restriction. Additionally the amount of enzyme used in the reaction was increased to 5 µl (and 3 µl H_2O dd only) in some repeated trials. After restriction the samples were cleaned from the small cleaved-off nucleotides from the ends of the construct using the PCR purification kit (QIAquick, Qiagen).

After Sfi I restriction the two PCR products were incubated prior to ligation at 45°C for 10 min to melt unspecifically hybridised DNA-fragments. Afterwards the following components were mixed together:

product from PCR 1:	5 µl
product from PCR 2:	5 µl
10 x ligase buffer:	2 µl (composition see below)
DNA ligase (Gibco):	1 µl
milliQ-deionised water:	7 µl

10 x ligase buffer:	0, 1 M MgCl2
	0, 5 M Tris-HCl (pH 7,5)
	10 mM ATP
	50 mM DTT (Pierce)
	(dissolved in milliQ-deionised water and stored at -80°C)

Initially the reaction was performed at room temperature for 2 h (according to the standard protocol for ligation of sticky end fragments). Since the ligation seemed to be quite ineffective under these conditions, it was later on extended to 24 h and even to 36 h at 4°C. The product of the ligation reaction was purified with the QIAquick PCR purification kit (Qiagen) and stored at -20°C. An aliquot of the sample was removed for a control 1 % agarose gel.

In the next working step the sample had to be restricted again with 0,5 µl each of the enzymes Eco RI and Xho I (Gibco) together with the supplied reaction buffers. In parallel the pUAST-vector used for the subsequent second ligation reaction was cleaved with the same enzymes to create sticky ends for the insert. After nucleotide purification the construct should be ligated into the pUAST-vector. The amounts used for this ligation were calculated roughly from the band intensities of vector and insert on an agarose gel (not shown), using the insert at approximately five times excess compared to the vector. The components for the second ligation reaction were therefore:

vector (pUAST):	2 µl
insert (ligated PCR products):	10 µl
10 x ligase buffer:	2 µl
DNA ligase:	1 µl
milliQ-deionised water:	5 µl

A control was prepared in a reaction mix with the linearised vector without the insert in the same total volume as for the other samples (15 µl H_2O dd added). Ligation of the control reaction was not expected, since the Eco RI- and Xho I-restricted ends should not be able to hybridise to each other. In a subsequent "PCR purification" step the samples were cleaned from the ligase and salts. Plasmid transformation was performed according to Sambrook et al. (1989) using 1 µl of each ligation reaction with and without the insert (control). Since the competent cells (*E. coli*, strain JM109, Promega) were ampicillin-sensitive, successful uptake of the plasmid with a resistance gene marker was indicated by growing colonies on LA bacteria plates (Sambrook et al., 1989) including 100 µg/ml of the antibiotic. The plasmid was then recovered from the bacteria (using the QIAprep SPIN miniprep kit, Qiagen), checked together with linearised plasmid DNA on a 1 % agarose gel after Eco RI / Xho I restriction and was ready for injection into *Drosophila* embryos.

2.11 Recombinant expression of the mouse SSL protein in *E. coli* for production of an antiserum

The EST clone "IMAGE:1512404" coding for the mouse SSL protein was ordered through the NCBI web page (http://www.ncbi.nlm.nih.gov/dbEST). The cells were cultured and the EST recovered from the vector (using a plasmid MIDI kit, Qiagen). A PCR reaction was performed (GeneAmp XL PCR kit, Applied Biosystems) under standard conditions assuggested by the manufacturer using 1 µl plasmid DNA as a template. The PCR primers included restriction sites for Bam HI and Hind III at their ends (underlined bases), facilitating cloning of the product into the pQE-32 vector with the same recognition sequences in its multi-cloning site. Cleaning of the PCR product was performed with the QIAquick PCR purification kit (Qiagen). An aliquot of 0,5 µl was applied to a 1 % agarose gel (not shown) together with linearised plasmid DNA to check for the right size of the oligonucleotide product and to estimate the volumes for the following ligation reaction.

Forward primer “M1” (including Bam HI-site):
5` – ATT AGG ATC CAG ATG TCC TTT GTG CTG – 3`

Reverse primer “M2” (including Hind III-site):
5` – TTA TAA GCT TTC ATT CTC TTA ATA CAG – 3`

After restriction of both PCR product and vector DNA with the enzymes Bam HI and Hind III, another purification step was performed. Then the ligation was carried out at 4°C for 24 h using 10 µl of the insert and 2,5 µl of the vector in a total volume of 20 µl (for all other components see protocol for hemomucin hairpin DNA construct in the last chapter). A control ligation using restricted vector only was also performed. The ligation products were cleaned from ligase and small nucleotide contaminants and transformed into competent *E. coli* JM 109 cells (Promega) on LA-plates containing 100 µg/ml ampicillin (Sambrook et al., 1989). Plasmid DNA was isolated from growing clones (QIAprep SPIN miniprep kit, Qiagen) and 5 µl DNA each as well as a sample of the restricted pQE-32 vector were applied to a 1 % agarose gel (Sambrook et al., 1989).
Transcription of the protein from the plasmid in the successfully transformed bacteria was stimulated in mid-log phase cultures by induction of the lac-promoter of the pQE-32 vector with 1 mM isopropylthio-β-D-galactoside (IPTG) for 1 and 2h at 37°C. Both *E. coli* clones including and missing the insert (judging from the agarose gel after transformation) were treated this way. Protein samples were taken from the cultures at both time points, separated on a 15 % SDS polyacrylamide gel and blotted to a nitrocellulose membrane for 1 h at 200 mA constant voltage (see chapter 2.13). The membrane was subsequently incubated with an antibody against a recombinantly produced part of hemomucin (see chapter 2.13, table 1; kindly provided by Dr. Ulrich Theopold) in a concentration of 1:5000 in TBST.
Since the used pQE-32 vector included a His-tag epitope, the induced protein with a molecular weight of about 20 kDa from the cells could be purified on a Ni-NTA column (Qiagen). The described working protocol was adapted from the Qiagen handbook “The QIAexpressionist” (chapter 19). Cells were harvested from 1 ml bacterial culture by centrifugation at 15 000 *g* for 1 min and lysed by vortexing the resuspended pellet in 200 µl lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8,0 with NaOH). Afterwards the cell debris was removed in a 10 min centrifugation step at 15 000 *g*.

The supernatant containing the His-tagged recombinant protein was mixed with 50 µl of a 50 % Ni-NTA resin (Qiagen) and incubated on a shaker for 30 min at room temperature. The resin with bound proteins was pelleted for 30 sec at 15 000 *g* and washed twice with 250 µl of a lysis buffer adjusted to pH 6,3 with HCl. Finally eluates were collected from three subsequent incubation steps with 25 µl of this buffer adjusted to pH 4,5. Aliquots of the samples were tested on a 15 % SDS polyacrylamide gel (not shown). Since the protein recovery from the column was limited, harsher elution conditions were tried using 10 M urea. The collected protein amount was still too small, thus it was finally decided to incubate the Ni-NTA beads in sample loading buffer at 90°C for 2 min. The sample was separated on a 15 % SDS gel (see chapter 2.13), which was destained using solutions lacking acetic acid, which would have interfered with following working steps. An estimated protein amount of 1280 mg was derived from 1 litre of bacterial culture, which was sufficient for antibody production in two rabbits (500 µg for each animal had been suggested by AgriSera AB). The 20 kDa protein band was cut from the gel and sent for antibody production in rabbits (AgriSera AB, Vännäs, Sweden). The collected antiserum received from the company was tested for reactivity and specificity on Western blots with different amounts of the recombinant protein from *E. coli* cells and with the endogenous mouse protein in different tissues including bone marrow and platelets. A pre-immune serum from the same rabbits was tested additionally in all cases to exclude positive signals due to endogenous rabbit antibodies already present in the serum prior to immunisation (not shown). All mouse tissues were applied to the gel in samples with a volume of 20 µl in a 1:10 dilution except bone marrow and platelets samples, which were tested undiluted. The mouse tissues were prepared under the guidance of Matthias W. Hornef (Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden). It was tried to improve the affinity of the antiserum for the 20 kDa protein by elution of the immobilised antibodies from the nitrocellulose membrane using a protocol by Smith & Fisher (1984).

2.12 Clot isolation with paramagnetic beads in the pull-out assay

A general problem connected to the isolation of a haemolymph clot is the fact that haemocytes become integrated in the coagulum during the clotting process. Therefore normal “pull-down” centrifugation methods fail to separate the cells from the protein fibres. I used paramagnetic beads (tosylactivated Dynabeads M-280, Dynal Oslo/Norway) to be able to isolate the detergent-resistant clot from the lysed haemocytes (Figure 7). The beads became passively integrated into the coagulum during the ongoing reaction. Two kinds of possible unspecific binding reactions to the beads, physical and chemical, were reported by the manufacturer. Physical binding occurs in the time range of minutes, but can be reversed through treatment with weak detergents, as used in the present study. Chemical interactions with the tosyl-activated groups take place rather in the time scale of several hours and are thus unlikely for the protocol described here. However, to minimise the risk for unspecifically bound proteins, the activated groups were blocked with 0,2 M Tris (pH 8,5) as suggested by the manufacturer. In some cases the beads were coated with PNA over night to enhance binding of clotting factors (Figure 24). The same proteins, however, were retrieved using untreated beads.

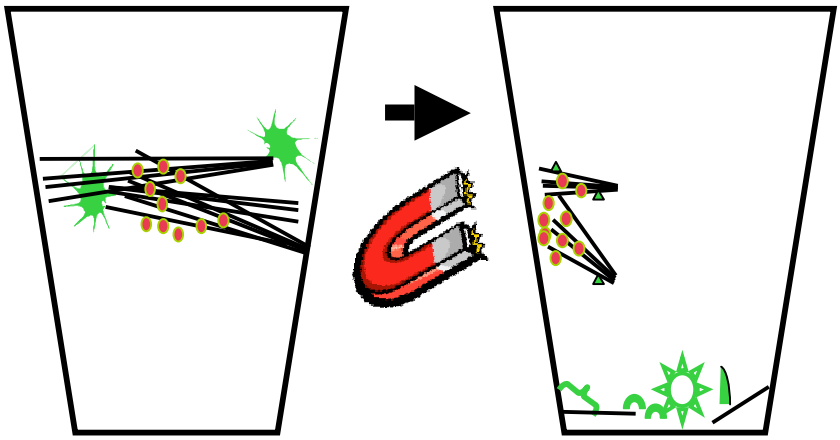

Figure 7: Principle of the pull-out assay
Paramagnetic beads become passively incorporated into the haemolymph clot (left). They can be separated from the cells together with the detergent-resistant clot after lysis of the haemocytes (right). Green symbols: haemocytes and cell fragments; red circles: beads; black lines: clot fibres.

50 µl of *Drosophila* Ringer including PTU and 50 µl of washed paramagnetic beads in *Drosophila* Ringer solution were transferred to a concaveous object slide. Haemolymph from twenty washed larvae was added by opening them with antimagnetic forceps submersed in the solution and swirling the sample to maximise contact of the clot with the beads. Bead aggregation was followed under the dissection microscope during preparation and photos were taken at several time points with a camera attached to the microscope. Twenty larvae were used, since the aggregation did not increase visibly in reaction to further added haemolymph to the sample. In some mutants like *domino* homozygous larvae have an increased lethality and are considerably smaller (Braun et al., 1998). In these cases thirty individuals were used to adjust the haemolymph volume to a comparable amount.

The beads were transferred together with the buffer into a microcentrifuge tube. The preparation slide was rinsed with additional 100 µl of *Drosophila* Ringer to collect remaining beads, which were added to the sample. The tube was placed into a magnet holder (Dynal Oslo/Norway) so that the coloured paramagnetic beads were collected at the tube wall. Subsequently the cleared solution was removed with a pipette, leaving behind only the beads together with the bound haemolymph clot. Three washing steps were performed with 100 µl of *Drosophila* Ringer, three additional rinses with Ringer including 0,5 % IGEPAL CA-630 (Sigma) and protease inhibitor mix (Complete, Roche) and finally three washing steps with Ringer. The protease inhibitor was added to avoid a possible destruction of the clot proteins by intracellular proteases released from the lysed haemocytes. The beads were incubated with the washing solution for 1 min each, the first detergent washing step was allowed to proceed for 2-3 min. The washed beads were resuspended in 20 µl 1x sample loading buffer (Laemmli, 1970).

2.13 Polyacrylamide SDS gel electrophoresis and Western blots

For polyacrylamide SDS gel electrophoresis and Western blots a minigel system (MiniProtean, BioRad) was used. The standard method described by Laemmli (1970) was followed; all buffers and solutions were prepared according to Sambrook et al. (1989). All samples were mixed with sample loading buffer to a final 1x buffer concentration and denatured prior to freezing and prior to gel electrophoresis,

respectively, by incubation at 80°C for 2 min. The proteins and a standard (Cat. No. 161-0373, BioRad) were separated by electrophoresis in 10 % and 5 % resolving gels. The electrophoresis was performed at constant voltage of 25 mA for the stacking gel and 30 mA for the resolving gel. The protein bands were visualised in the gel by either staining with Coomassie (Coomassie Brilliant Blue G, Sigma) or a silver staining kit (Amersham Biosciences). Destaining of the gel was carried out as suggested by the suppliers, but acetic acid was excluded from all buffers, if a protein sample from the gel should be sent for sequencing.

For labelling with antibodies and lectins, the proteins were blotted to a nitrocellulose membrane (BioRad) at 200 mA for 2 h. The membrane was blocked in TBST (TBS including 0,05 % Tween 20) for 1 h and incubated with the lectin or antiserum (see table 1). Hemomucin was detected by one of two antibodies (kindly provided by Dr. Ulrich Theopold) or the *H. pomatia* lectin (HPL; peroxidase conjugated; 1:10 000 v/v; Sigma). Similarly the lectin peanut-agglutinin (PNA; peroxidase-labelled; working solution 1:1000 v/v; Sigma) was used to visualise clot components on Western blots applying the same incubation times and washing steps as described before. The blots were washed four times 15 min each in TBST under vigorous shaking. A secondary antibody (goat-anti rabbit; alkaline phosphatase (ALP) conjugated; 1:20 000 v/v; Sigma) was added for 1 h and the membranes were washed again four times, 15 min each with TBST. Development of the ALP-reaction was carried out with BCIP and NBT (Sambrook et al., 1989). Membranes labelled with HPL were developed with 1:1000 v/v H_2O_2 (40%) and 6 mg/10 ml 3'3'-diaminobenzidine tetrachloride (DAB) in 10 mM Tris pH 7,5 was started directly after lectin treatment and four TBST washing steps (Sambrook et al., 1989).

Table 1: Primary antisera used in the present study

antiserum	specificity	origin	raised against	reference
anti-hemomucin	polyclonal	rabbit	protein from *Dr. mel.*	Theopold et al., 1996
anti-hemomucin recombinant	polyclonal	rabbit	rec. protein in *E. coli* (part of SSL-domain)	Fabbri et al., 2000
anti-hemolectin	polyclonal	rabbit	rec. protein in *E. coli*	Goto et al., 2001
"anti-clot "	polyclonal	rabbit	*Galleria in vitro* clot	Li et al., 2002
anti-mouse SSL (anti-mSSL)	polyclonal	rabbit	rec. protein in *E. coli* (from EST-clone)	present work (chapter 2.11)

2.14 Mass spectrometry of proteins from the pull-out assay

The most prominent protein bands from a pull-out sample were cut out with sterile stainless steel blades from a Coomassie-stained gel for mass spectrometry characterisation, transferred into siliconised tubes ("Maxymum Recovery", Axygen) and sliced into small gel bits. The sliced gel pieces were washed in three or more successive incubation steps by vortexing in 100 µl of 25 mM NH_4HCO_3 in 50 % CH_3CN until they were completely destained. Next the gel was dehydrated by vortexing in 100 µl CH_3CN for 5 min and then dried in a Speed Vac. The proteins were digested in 20 µl of 12.5 ng/µl sequencing grade trypsin (Promega V511A) in 50 mM NH_4HCO_3. After vortexing the gel pieces in this solution for 10 min, the tubes were covered with parafilm and incubated at 37°C for 16-20 h (over night). The peptides were solubilised by vortexing for 1 h in 50 µl of 50 % CH_3CN/5 % trifluoroacetic acid. Afterwards the gel pieces were pelleted and the supernatant transferred to a new siliconised tube. This extraction step was repeated twice. Finally the combined supernatants were centrifuged under vacuum until dry (approximately 2 to 4 h). Prior to mass spectrometry analysis the pellet of recovered peptides was resuspended in 2 to 5 µl of 50 % CH_3CN/0.1 % trifluoroacetic acid, depending on the estimated amount of protein. Mass spectra were recorded in positive reflection mode by using a mass spectrometer equipped with a delayed ion extraction technology (MALDI-TOF Voyager-DE STR, Applied Biosystems). As a matrix α-Cyano-4-hydroxy-cinnamic acid (CHCA) was chosen. External calibration was performed using the Sequazyme Peptide Mass Standard kit (Angiotensin I and ACTH, clips 1-17, 18-39, 7-38) and auto digestion peaks of bovine trypsin were used for internal calibration. The peptide profiles produced by mass spectrometry were analysed using the programs Mascot (http://www.matrixsciences.com/), ProFound (http://prowl.rockefeller.edu/), MS-Fit (http://prospector.ucsf.edu/) and PeptIdent (http://www.expasy.org/tools/peptident.html). The monoisotopic (MH^+) peptide masses were compared with the theoretical masses derived from the NCBI database entries for *Drosophila melanogaster*. Known masses of trypsin autodigest products were excluded from the searches. Search parameters included allowed mass accuracy of 50 ppm, more than four peptide mass hits required for a

protein match, consideration of maximal one missed enzymatic cleavage, a pI range of 0.0-14.0, and a molecular mass range of 1-3000 kDa. Carbamido-methylation of cysteine residues was set as a complete modification for the retrieved peptide sequences and partial oxidisation of methionine was allowed. The protein matches were only accepted as meaningful, if analysis with two of the mentioned programs independently led to significant matches for the same protein. In ProFound e-values above 1,645 were considered significant (synonymous with a significance level of 95 %), while the inbuilt significance threshold of 95 % in Mascot was adapted. Peptide mass data collection was kindly performed by Christine Karlsson and Olga Loseva (Department of Molecular Biology & Functional Genomics, Stockholm University).

2.15 Preparation of total RNA from *Drosophila* larvae for RT-PCR

I investigated a possible induction of hemolectin in larvae and adults from different time points after poking with a fine tungsten needle (Fine Science Tools). Larvae were pricked at the posterior end close to the spiracles, in adults the needle was applied at the weaker cuticle at the wing attachment sites. Poked larvae and adults were transferred to a vial with fly food to ensure sufficient food availability and air humidity. In parallel non-poked larvae and adults were transferred to new tubes and used as control samples to check for possible developmental effects on the RNA level (1 h and 24 h). The pricked individuals were collected after 1, 6, 12 and 24 h, washed with water, transferred into microtubes and stored in liquid nitrogen to immediately stop all transcriptional activity. Larvae that had died from too harsh pricking were excluded from the experiment. (All adult flies survived the procedure.) RNA preparation was performed using a kit (RNeasy, Qiagen) and following the supplied protocol. Five larvae in 700 µl RLT-buffer were used for each sample and homogenised using polypropylene pellet pestles (Sigma). The RNA was extracted in the final elution step with 50 µl RNase-free water per sample and RNA-concentration was measured at 260 nm in a photometer (BioRad). A calculated RNA equivalent of 0,5 µg each was further tested on a 1,2 % agarose gel including formaldehyde (Sambrook et al., 1989; gel not shown). The calculated RNA-concentrations proved to fit quite well with the actual band strengths.

2.16 Test for wound-induced induction of hemolectin using RT-PCR

A possible induction of hemolectin transcription by pricking should be checked using reverse transcription polymerase chain reaction (RT-PCR). Since the RNA concentrations for the reactions were adjusted approximately, the RT-PCR may be characterised as "semi-quantitative". Two different pairs of forward and reverse complementary primers were used in two parallel test reactions, one pair was adapted from a previous study on hemolectin (242 bp fragment complementary to amino acids 6819-7061; Goto et al., 2001), the other pair was chosen from a list of suggested primers employed for microarrays (http://www.affymetrix.com). Since both primer pairs proved to lead to a comparable band intensity for hemolectin in test gels (not shown), only the primers from Goto et al. (2001) were used for the RT-PCR reactions shown here. Primers for the ribosomal protein RP49 (constitutive expression) were employed in control samples for each time point (480 bp product; primer sequences adapted from Goto et al., 2001). The optimal hybridisation temperature for the RP49-primers was determined as 52°C, while the hemolectin-primers annealed best at 59°C. In test reactions 21 reaction cycles were found to lead to a weak but visible signal for hemolectin (not shown), thus ensuring the recognition of an induction elevating the low constitutive background transcription level. Each RNA-sample was used for two independent PCR reactions using primers for RP49 or hemolectin. The RT-PCR was performed in a thermocycler (Robo Cycler Gradient 96, Stratagene). A thermostable DNA polymerase from *Thermus thermophilus* with intrinsic reverse transcriptase activity (Gene Amp EZ rTth RNA PCR Kit; Applied Biosystems) allowed the performance of both reactions (reverse transcription and DNA amplification) in a single sample mix. The used RNA amounts, components of the PCR mix and all cycling conditions were adapted from the manual supplied by the manufacturer. Briefly, an initial reverse transcription reaction at 60°C for 30 min was followed by a standard PCR protocol for 21 cycles with each cycle consisting of a denaturation step at 94°C, a combined primer annealing and extension step at 52 or 59°C respectively and a final elongation step at 72°C. The different optimal annealing temperatures for each primer pair were applied by using the gradient heating block of the thermocycler.

3 Results

3.1 Studies on localisation and functions of hemomucin

3.1.1 Detection with *Helix pomatia* lectin and an antiserum

One of the first candidate proteins for a haemolymph clotting factor in *Drosophila melanogaster* was hemomucin, since it was known to be localised in haemolymph clots. Detection of hemomucin can be achieved by labelling with the lectin from *Helix pomatia* (HPL) in both the clot preparation and on Western blots. Alternatively a hemomucin-specific antiserum can be used which recognises antigens of the denatured protein only (Theopold et al., 1996), while labelling of the native protein is not possible. The expression of hemomucin in other larval and adult tissues than haemocytes (Theopold et al., 1996, 2001) suggested a broader biological role for this protein. Hemomucin is poorly detected with the lectin on membranes with *Drosophila* haemocyte samples (Figure 8 and Dr. Ulrich Theopold, personal communication). In cell samples from mbn-2 cultures the lectin binds hemomucin more efficiently (Figure 13, first lane). The antiserum clearly labels hemomucin in the haemolymph (Figure 8, arrow) and weakly binds to the protein present in haemocytes (not visible in Figure 8). Hence the antiserum was utilised on Western blots, while haemocyte monolayer preparations were labelled with HPL.

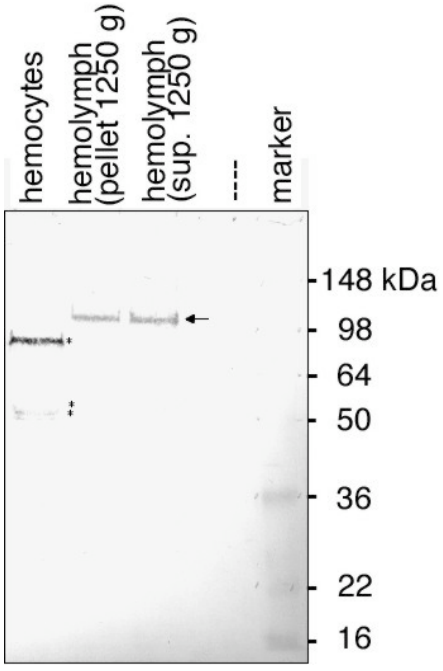

Figure 8: Simultaneous double detection with an antiserum against hemomucin and HPL-reactive proteins in a clotted haemolymph sample of *Drosophila*

arrow: specific detection of hemomucin with an antiserum directed against the full-length protein (see chapter 2.13, table 1; secondary antibody labelled with alkaline phosphatase); weak hemomucin signal in haemocytes not visible here

stars: several cellular HPL-reactive haemocyte proteins (HPL labelled with peroxidase)

3.1.2 *In situ* hybridisation of *Drosophila* embryos

Based on expression data from larval (Theopold et al., 2001) and adult stages (Theopold et al., 1996) of *D. melanogaster* it was hypothesised that hemomucin might be involved in hardening processes in general, which might include embryonic development as well. Furthermore it had not yet been investigated, if hemomucin is also produced by embryonic hemocytes. Since no data was available at the time on the presence of hemomucin in *Drosophila* embryos, its expression was studied by *in situ* hybridisation (see chapter 2.8). The probe was synthesised from a part of the strictosidine synthase-like domain to avoid unspecific cross-reactions with the mucin domain of other proteins. Hemomucin transcripts were detected in the distal part of all blastoderm cells after cellularisation in stage 5 embryos (Figure 9). During later time points of embryonic development, at about stage 14, hemomucin RNA was expressed in fore- and hindgut, salivary glands and tracheae. Examination of the preparations at higher magnifications revealed that the probe was located in the epithelia of trachea and gut and not in their lumen (not shown), speaking against a possible unspecific staining reaction. The control hemomucin sense RNA probe did not give rise to any signal (not shown).

In addition to *in situ* hybridisations with hemomucin probes, labelling was alternatively performed with an RNA probe against the second strictosidine synthase-like protein in *D. melanogaster*, CG 11833 (Figure 10). RNA coding for this protein was present in the trachea as shown for hemomucin. In addition a paired structure in the pseudo-cephalic region (ps) and the pharynx (p) were labelled.

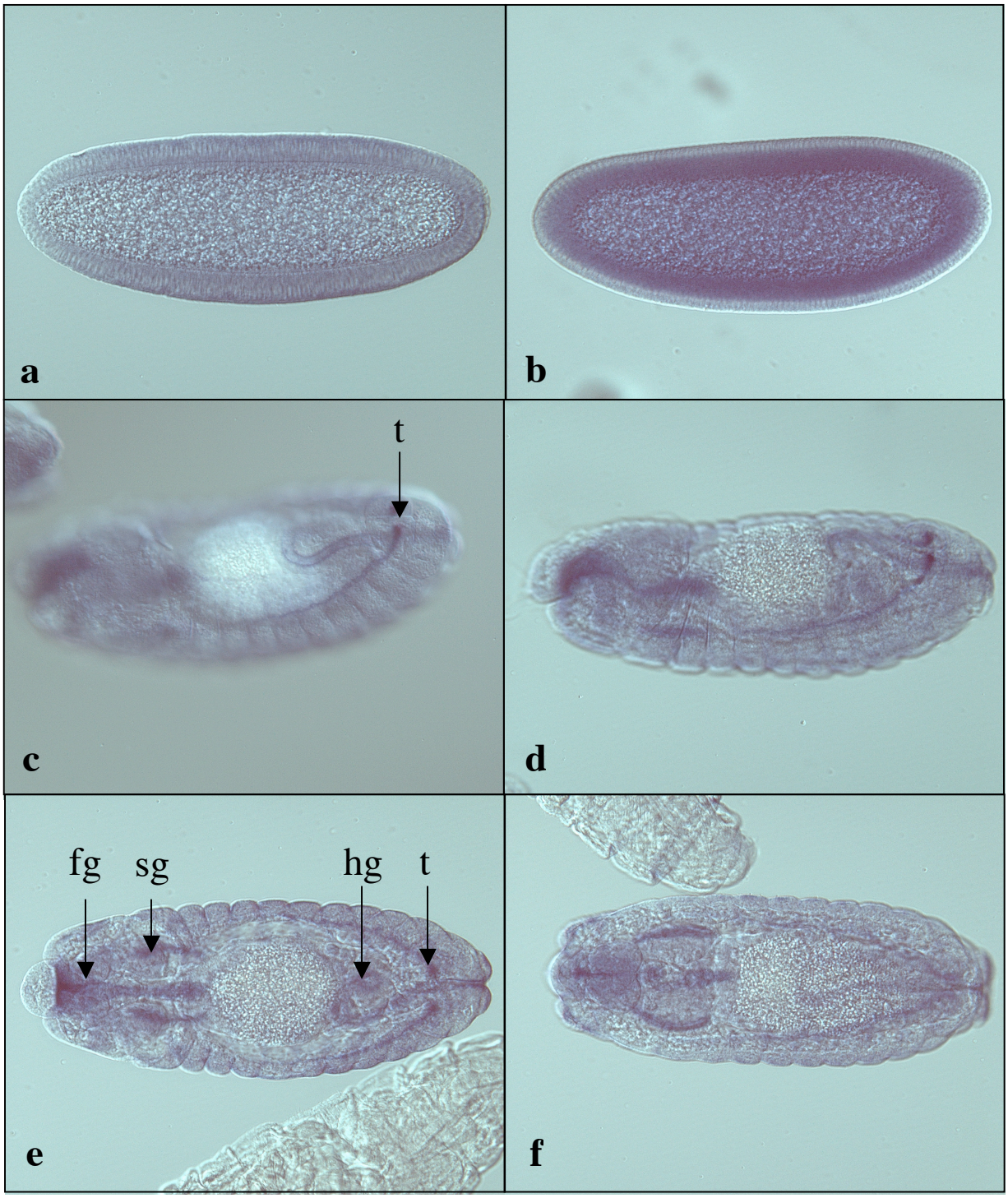

Figure 9: *in situ* hybridisation of *Drosophila* embryos with a hemomucin RNA probe

a: stage 5: weak expression in the whole embryo (?)
b: stage 5/6: strong expression in the basal parts of the syncytium
c-f: stage14: expression of hemomucin in fore- (fg) and hindgut (hg), salivary glands (sg) and trachea (t)

(Figures c/d: lateral view, Figures e/f: dorsal view; in all pictures the anterior end is oriented to the left side, the posterior end to the right)

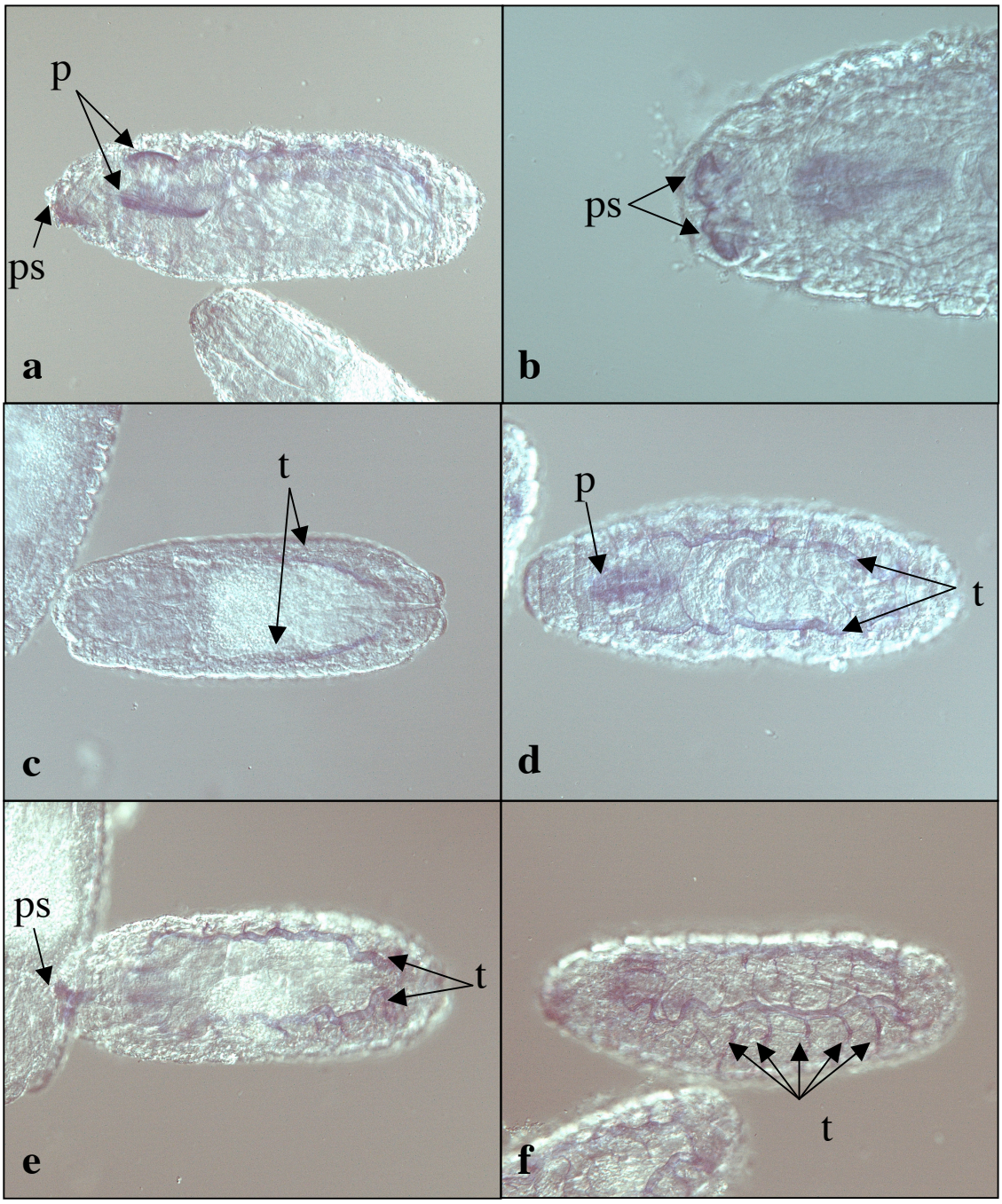

Figure 10 a-f: *in situ* hybridisations with an RNA probe for the second strictosidine synthase-like *Drosophila* protein (CG 11833)
(stage16/17), pharynx (p),"pseudo-cephalic region" (ps), trachea (t)

3.1.3 Studies on microparticle formation in a haemocyte-like cell line

Since hemomucin is localised on microparticles released from insect haemocytes (Theopold & Schmidt, 1997), it was hypothesised that it may be shed from the haemocytes into these particles, leading to a depletion of the protein from the cells. The calcium ionophore A23187 is used in vertebrate studies to induce platelet microparticle formation *in vitro* and stimulates comparable cell changes in *Drosophila* mbn-2 cell cultures (not shown). HPL was used as a tool to detect hemomucin in a FACS analysis of cells from the haemocyte-derived *Drosophila* cell line mbn-2 (see chapter 2.7), since antibody labelling was not feasible for the native protein. The working hypothesis was that the lectin signal intensity of ionophore-treated cells would be lower than the one of untreated cells. In fact mbn-2 cells showed a weaker HPL-labelling after incubation with the ionophore A23187. This effect could already be observed after treatment with comparably low ionophore concentrations of 2,5 µM and was even stronger at higher concentrations (Figure 11). Since the ionophore had to be diluted in DMSO, a sample treated with this chemical alone in a high concentration was used as a control, which did not cause any reduction of HPL-labelling. A differential reaction of the cells to the ionophore could be observed, the cell population II (Figure 12C; “Pop II”) did not show any change in HPL-labelling in contrast to cell population I (Figure 12B, “Pop I”).

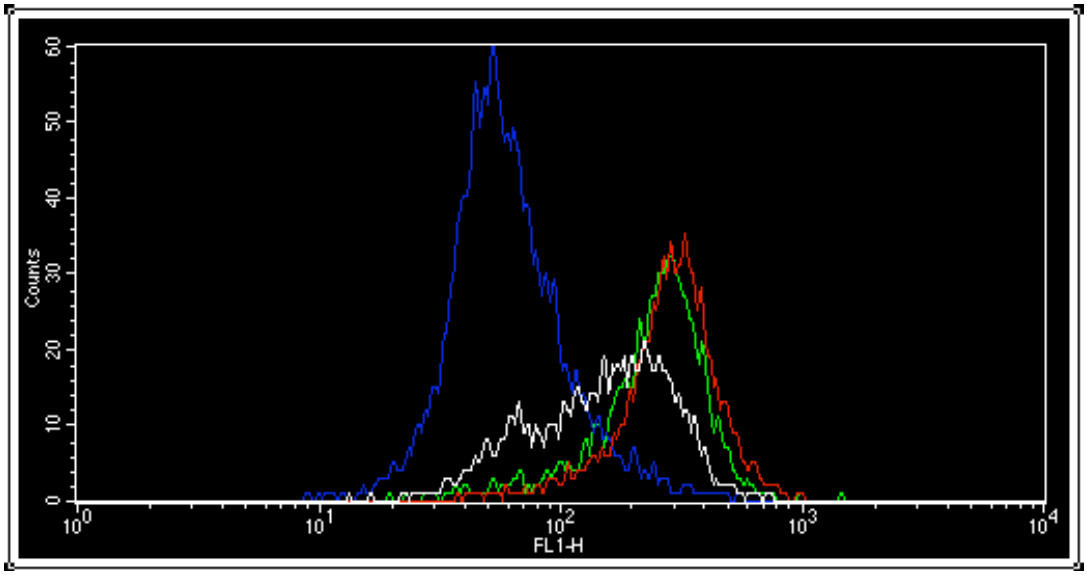

Figure 11: FACS analysis of hemomucin levels in mbn-2 cells treated with the calcium ionophore A23187, detected with FITC-labelled HPL
green: untreated cells; red: 20 µM DMSO (control);
white: 2,5 µM ionophore A23187; blue: 20 µM ionophore A23187

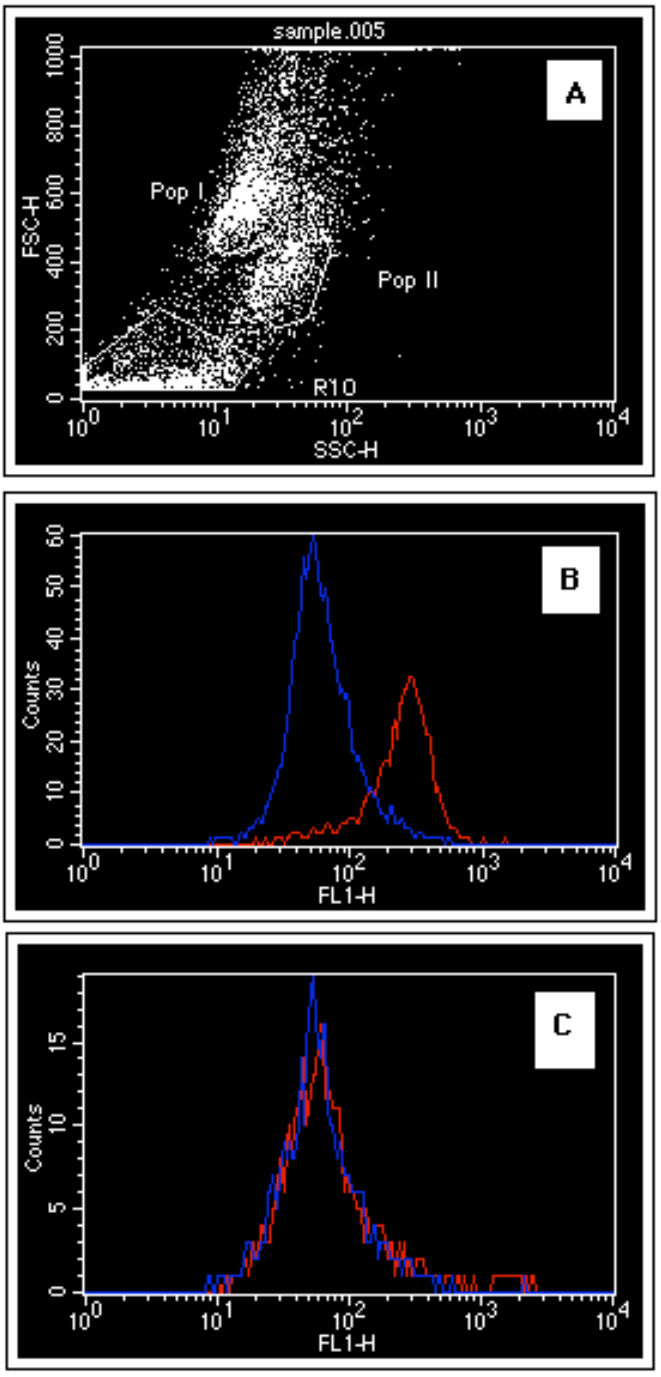

Figure 12: Differential reaction of two cell populations to ionophore treatment
A: fluorescence signal of the total cell sample; the lectin labelling was different for two cell populations “Pop I” and “Pop II”
B: cell population I, C: cell population II
blue: 20 µM ionophore A23187
red: control sample (no ionophore added)

3.1.4 *In vitro* transcription and hemomucin RNAi in cell cultures

No *Drosophila* mutant for hemomucin was available from the common stock centres, hence I decided to down-regulate the protein by RNA interference (RNAi) in haemocyte-like cell cultures (see chapter 2.9). Hemomucin dsRNA as well as a control dsRNA of the GFP gene were applied directly to an mbn-2 cell culture. After three days of incubation (the usual initiation time for RNAi) the cells were sedimented by centrifugation and labelling with *H. pomatia* lectin was performed on a Western blot. A substantial down-regulation of hemomucin could be observed in the treated cells (Figure 13). Nevertheless no obvious changes in cell morphology had occurred after three days and even after fourteen days and repeated dsRNA application. The membrane of RNAi-treated mbn-2 cells could also still labelled with HPL (not shown).

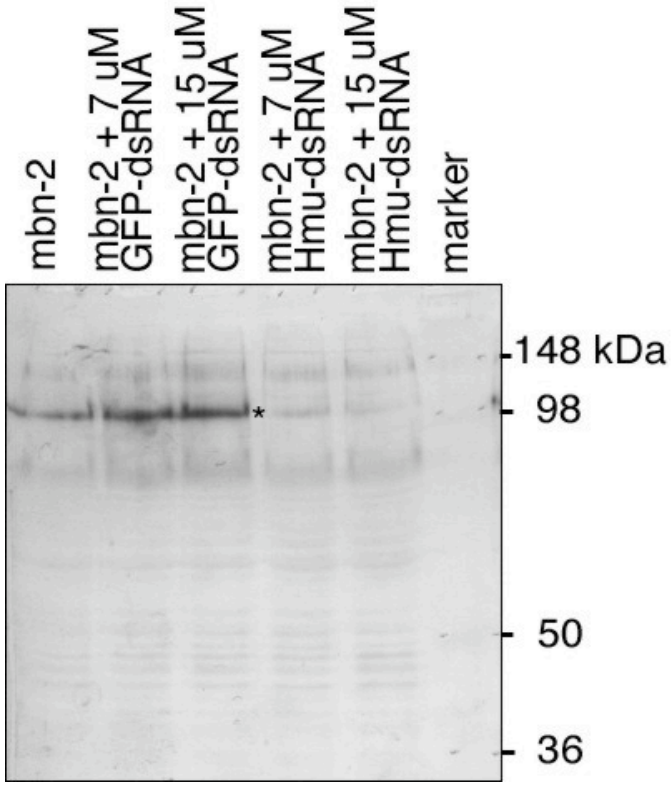

Figure 13: Decrease of hemomucin in mbn-2 cell cultures after treatment with hemomucin dsRNA and a control GFP dsRNA
(10 % SDS-polyacrylamide gel; detection with HPL, labelled with alkaline phosphatase; star indicates position of hemomucin)

3.1.5 Synthesis of a DNA construct for hemomucin RNAi *in vivo*

Since the RNAi technique worked well for hemomucin in cell culture (Figure 13), I decided to produce a DNA hairpin construct for endogenous expression of a hemomucin dsRNA in a transgenic fly stock (see chapter 2.10). All steps of the working protocol were controlled on agarose gels. Both PCR products were found to consist of a single oligonucleotide with the expected size of about 650 bp each (Figure 14). The Sfi I restriction and the subsequent ligation were repeated several times and modifications to the working protocol were adapted after discussions with colleagues. However, these experiments led to only few successfully ligated products. The band in the 1350 bp range was always very weak (Figure 15, lane "Lig"), but reproducibly disappeared after restricting the product again with Sfi I, indicating successful ligation (Figure 15, lane "R"), indicating that the construct sequence was right.

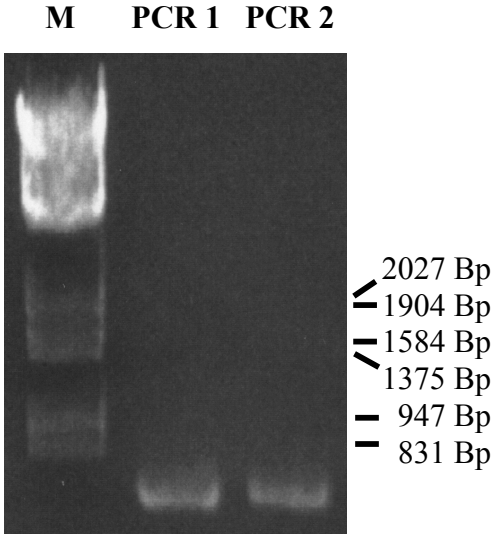

Figure 14: Control for the two PCR products for *in vivo* down-regulation of hemomucin
M: molecular weight standard;
PCR1, 2: two independent PCR reactions

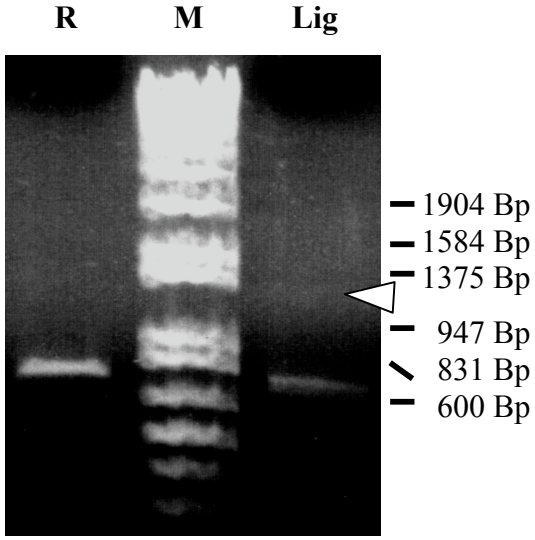

Figure 15: Test gel to check efficacy of Sfi I-restriction and the first ligation

M: molecular weight standard;
R: ligation product restricted with Sfi I;
Lig: ligation product (light arrowhead)
(lower band: non-ligated DNA)

Even the final ligation of the insert into the vector was controlled on a gel after Eco RI / Xho I restriction. Only few bacterial colonies seemed to have integrated an insert. DNA from these clones was analysed on the gel presented in Figure 16. The size of the fragments integrated in the plasmid from preparations 1, 3 and 6 was in the 1500 bp range expected for the *hmu-umh* inverted-repeat insert.

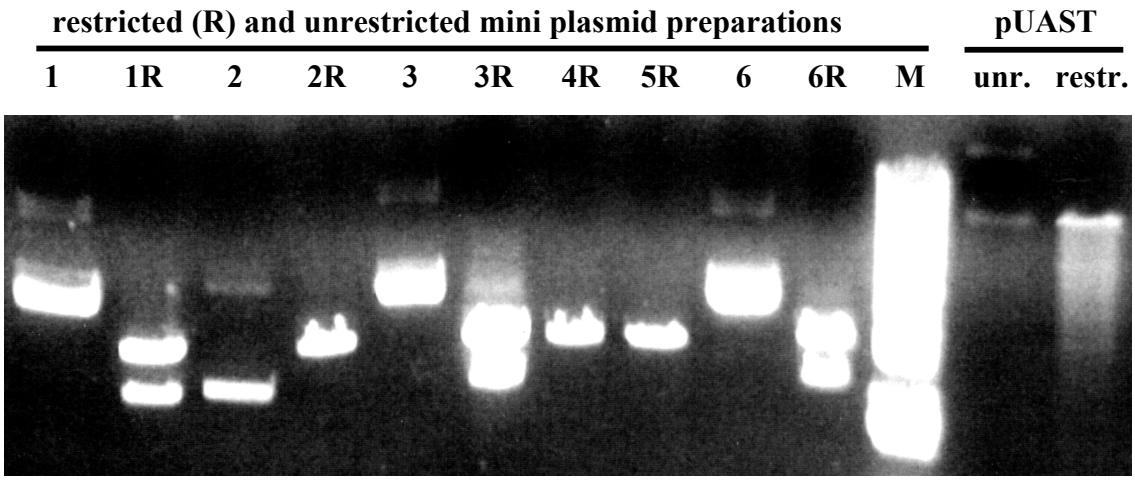

Figure 16: Control gel with plasmid DNA preparations after the second ligation
(1-6: different unrestricted plasmid preparations, 1R-6R: samples restricted with Eco RI and Xho I prior to gel electrophoresis to recognise insert sizes)

Since the cloning was considered successful, the plasmid was amplified and injected into *Drosophila* embryos. However, when the insert was sequenced N-terminally at a later stage (done by the KISeq unit at Karolinska Institute, Stockholm), it turned out that it was in fact a gene coding for an *E. coli* transposon. In summary, the attempt to synthesise a DNA construct for inheritable *in vivo* RNA interference of hemomucin was not successful despite numerous attempts.

3.2 Recombinant expression of the mouse SSL protein in *E. coli* for production of an antiserum

The genomes of several organisms include one or more copies of genes with similarity to the strictosidine synthase-like domain of hemomucin and are therefore termed "SSL proteins" (see chapter 1.7). Interestingly the human SSL protein is strongly expressed in hematopoietic tissues (Ulrich Theopold, unpublished results). Since the presence of the mouse member of the SSL-family (mSSL) in immune tissues had not yet been investigated, I tried to produce an antiserum against a recombinant mouse SSL protein (see chapter 2.11). A publicly available EST clone for mSSL was obtained and restriction sites were added to its sequence in a PCR reaction. After ligation into the pQE32-vector successful integration of the mouse SSL DNA into the plasmid was tested on an agarose gel (Figure 17).

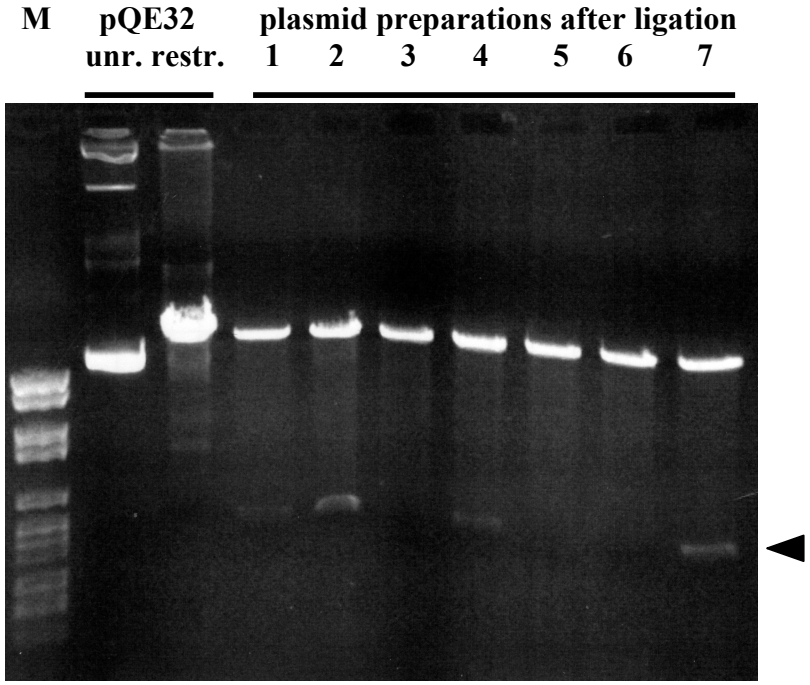

Figure 17: Control of the ligation of the EST-derived sequence for mouse SSL into the pQE-32 vector after Bam H I / Eco RI restriction
(M: marker; unrestricted and restricted plasmid;
1-7: different plasmid preparations of bacterial colonies after ligation;
black arrowhead indicates the position of the insert)

Four of the eight tested ligation reactions (1, 2, 4 and 7) had led to integration of the DNA coding for the mouse protein. Total protein samples from the IPTG-induced *E. coli* cells were analysed on a nitrocellulose membrane with the antiserum against a recombinant part of the *Drosophila* hemomucin SSL-domain (Figure 18). The major band recognised by the antiserum was in the 20 kDa range, indicating a good affinity of the hemomucin antiserum for the recombinant mSSL protein from *E. coli*.

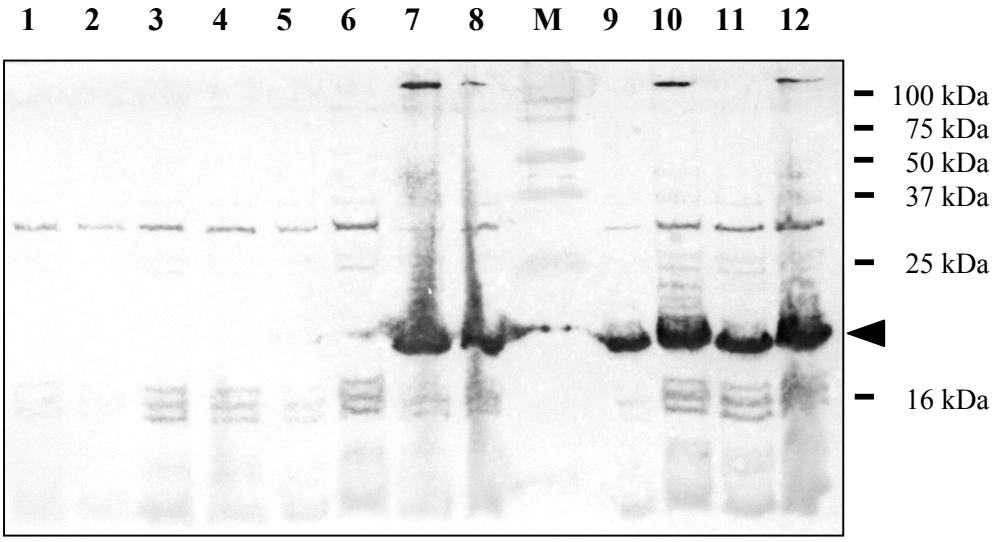

Figure 18: Expression of recombinant mSSL protein detected with an antiserum against hemomucin (15 % SDS-polyacrylamide gel;
1-6: clones without integrated mSSL construct; mSSL-expression after 2 h (7, 9, 10) and 1h (8, 11, 12) IPTG-induction indicated by arrowhead)

It was attempted to purify the recombinant mouse SSL protein over a Ni-NTA column utilising the His-tag of the pQE-32 vector. It turned out that the eluate of the proteins bound to the bead matrix included not only mSSL, but numerous additional proteins (not shown). Elution efficiency of the mSSL protein increased after prolonged incubation, suggesting a strong binding affinity of the protein to the beads. After testing harsher elution conditions, finally the beads were cooked to bring all proteins into solution. A huge amount of mSSL protein gained choosing this approach was isolated from a 15 % gel and used for antibody production (see chapter 2.11).
The antiserum was initially tested on the recombinant produced mSSL-protein from *E. coli* cells to confirm the specificity of the antibody (Figure 19).

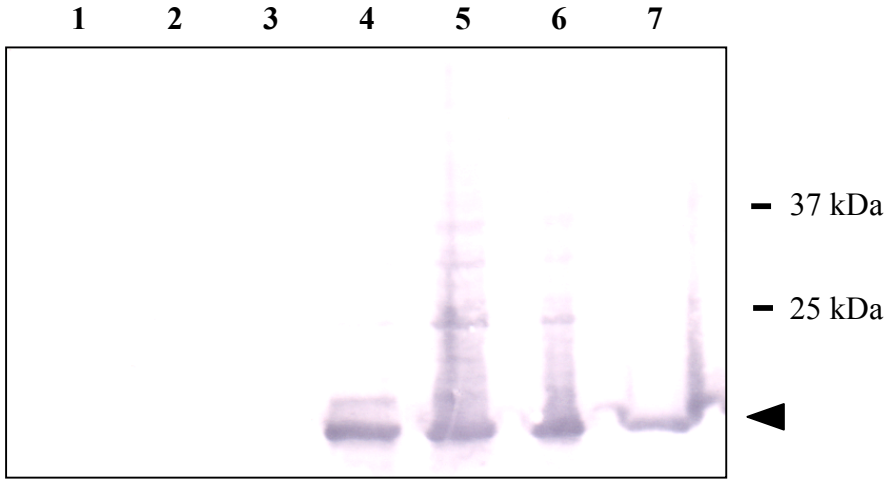

Figure 19: Incubation of total protein samples from *E. coli* with the antiserum against recombinant mSSL on a Western Blot (antibody diluted 1:1000; 1: *E. coli*, 2/3: *E. coli* + 1 mM IPTG, 4: *E. coli* + mSSL, 5-7: *E. coli* + mSSL + 1 mM IPTG, marker not shown; black arrowhead: ~ 20 kDa band represents recombinant mSSL protein)

Among proteins from the transformed bacteria the antiserum showed strongest activity against the 20 kDa band representing mSSL (Figure 19, arrowhead), even though additional proteins were recognised. Only weak unspecific cross-reactions of the antiserum with endogenous *E. coli* proteins were observed (Figure 19, gel lanes 1-3, not visible in figure). The detection signal of the 20 kDa protein could even be slightly improved by using a higher dilution of 1:5000 v/v of the antiserum (not shown). The mSSL protein was already expressed by transformed bacteria prior to induction of the *lac* promoter with IPTG (Figure 19, gel lane 4). No signals were visible on a membrane incubated with preimmune serum of the same rabbit (not shown).

The mSSL protein, initially named APMAP, had been reported to have a molecular weight of approximately 47 kDa (Albrektsen et al., 2001). The reactivity of the antiserum was repeated for the tissues used in this study. In addition bone marrow as an immune organ, platelets and a stomach sample were tested (Figure 20).

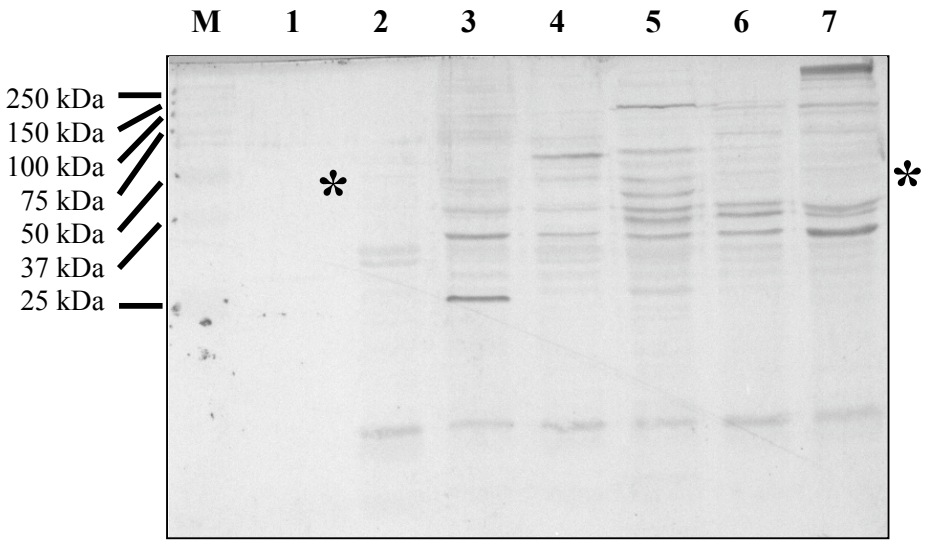

Figure 20: Antiserum against recombinant mSSL tested on different mouse tissues:
1: platelets
2: bone marrow
} undiluted samples
3: brain
4: stomach
5: liver
6: kidney
7: heart
} samples diluted 1:10
(labelled ~ 50 kDa protein indicated by stars; antiserum diluted 1:5000)

The antibody reacted against a protein of about 47 kDa in all tested mouse tissues, which resembles the reported size of the mouse SSL protein (Albrektsen et al., 2001). However, a large number of additional proteins were recognised as well, in many instances with an even stronger intensity. I tried to improve the signal/noise ratio by using higher dilutions of the tissue samples and the antiserum. Nevertheless the detection of proteins did not change qualitatively. An affinity purification approach, in which the 47 kDa-reactive subset of the antiserum was recovered from a nitrocellulose membrane for incubation of a second Western blot, did not improve the results considerably either (not shown).

3.3 Use of a clot-specific antiserum in *Galleria mellonella*

It is difficult to isolate clot components from insects, mostly because the coagulation process itself is quick and involves covalent cross-linking of proteins, which prevents molecular characterisation. One way around this problem is to detect the constituents of the coagulum rather indirectly using an antiserum. This was done using an antiserum against a washed clot of *G. mellonella* produced by Dongmei Li (Li et al., 2002). Briefly, a plasma clot from *G. mellonella* was formed *in vitro* around rabbit red blood cells. This cell type was chosen for two reasons: They were not immunogenic for the rabbit they were derived from, assuring specificity of the antiserum. In addition, erythrocytes are covered with large numbers of glycoproteins, which provide a good reaction matrix for a clot. The coagulum isolated this way was washed extensively prior to injection to remove haemocytes and unspecifically bound haemolymph proteins.
I used this antiserum against the *in vitro* clot to characterise the *Galleria* haemolymph coagulum. In a haemocyte preparation of *Galleria* the coagulum was visualised adjacent to granular cells, underlining clot specificity of the antiserum. The only haemocytes labelled by the anti-clot antibody were identified as granular cells (Figure 21).

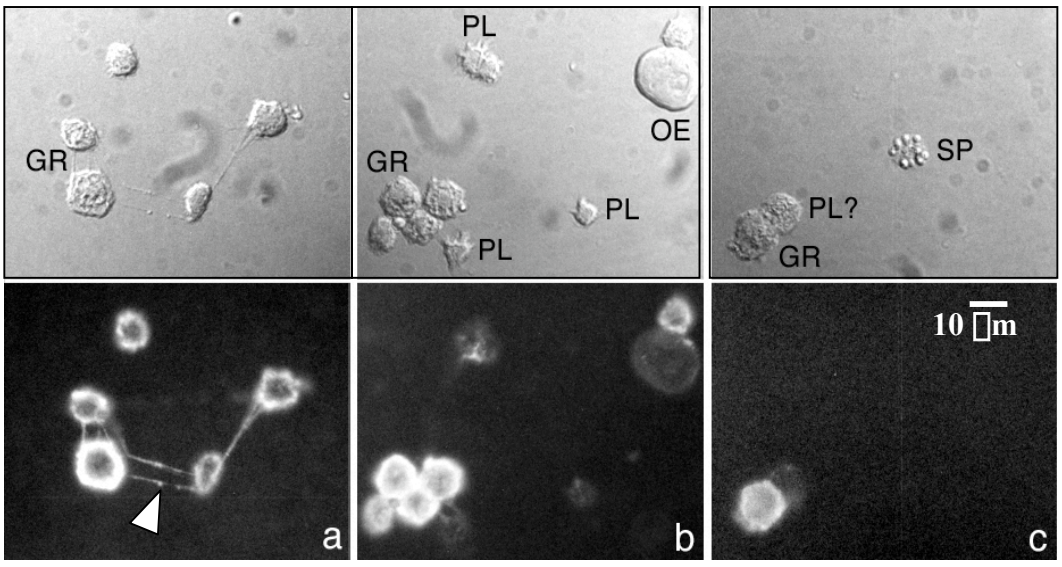

Figure 21: Labelling *Galleria mellonella* haemocytes using a clot-specific antiserum
(top: phase contrast pictures, bottom: fluorescence microscope pictures)
GR: granular cells, PL: plasmatocytes, OE: oenocytoids, SP: spherule cells;
white arrowhead indicates clot labelled with the antiserum (Li et al., 2002)

3.4 An assay for isolation of clot components in *Drosophila*

It would probably have been extremely time-consuming to isolate sufficient amounts of haemolymph clots from *Drosophila* for the production of an antiserum as described for *G. mellonella*. As an attempt to characterise new candidate clotting factors in *D. melanogaster*, I therefore chose a different approach. Since the complete sequence of the *Drosophila* genome was available, it was more promising to directly identify the isolated proteins using mass spectrometry analysis instead of screening DNA-libraries indirectly with an antibody. The pull-out method (see chapter 2.12) was expected to represent a more specific way to identify functional clot components than by detection with a polyclonal antiserum and allows additional controls. The general idea behind the pull-out assay was to use paramagnetic beads for isolation of a detergent-resistant haemocyte- and nuclei-free coagulum (Figure 7). The name "pull-out" was chosen to stress the difference to conventional "pull-down" centrifugation methods. Proteins in the retrieved sample were subsequently characterised molecularly by mass spectrometry. The results presented here were published together with additional data in Scherfer et al. (2004).

The haemolymph clotting ability of wild type *Drosophila* was assessed by two means, the aggregation strength of the beads during sample preparation and the abundance of certain proteins isolated from them. The bead aggregation strength was proportional to the added haemolymph amount (Figure 22, upper row). Clot fibres bound to the beads could be visualised using PNA. When larval haemolymph from *domino* mutants lacking haemocytes (Braun et al., 1998) was added to the beads, bead aggregation was almost absent (Figure 22, lower row).

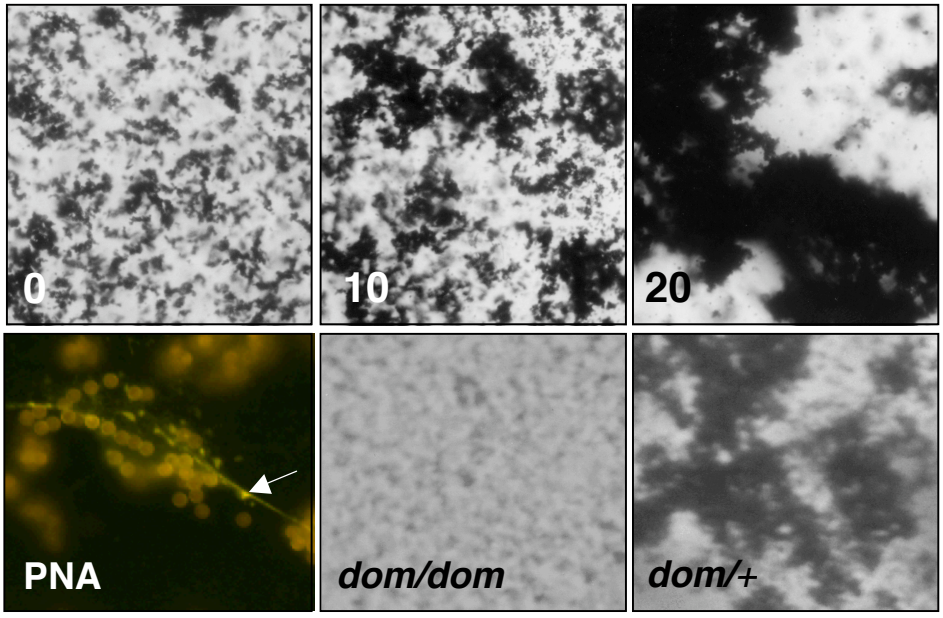

Figure 22: Haemolymph clotting followed by bead aggregation
upper row: addition of haemolymph from 0, 10 and 20 larvae to the beads
lower row: PNA: peanut agglutinin labelling of fibres between beads
(beads: orange-brown, labelled clot fibres yellow-green;
white arrow indicates PNA-labelled clot structures)
dom/dom: *domino* homozygous mutant larvae
dom/+: haemolymph from *domino* heterozygous larvae (control)

In order to test the specificity of protein binding to the beads, the supernatant including unbound proteins was separated from the clumped beads and added to a new aliquot of beads ("depletion assay"). This procedure was repeated several times. Proteins were retrieved from the beads in each step and applied to a polyacrylamide gel (Figure 23) together with the supernatant from the last binding reaction ("S"). The protein subset isolated by this method was different from the total haemolymph composition with many of the main *Drosophila* plasma proteins missing and some factors being strongly enriched on the beads. Most of the bead-associated proteins could be depleted completely from the haemolymph sample during the first binding reaction (Figure 23, lane 1 compared to following lanes). The presence of hemolectin and tiggrin in these samples correlated strongly with the intensity of bead aggregation. In contrast to other isolated proteins, the apolipophorin subunits I and II continued to associate with the paramagnetic beads after several reactions (Figure 23, open arrowheads).

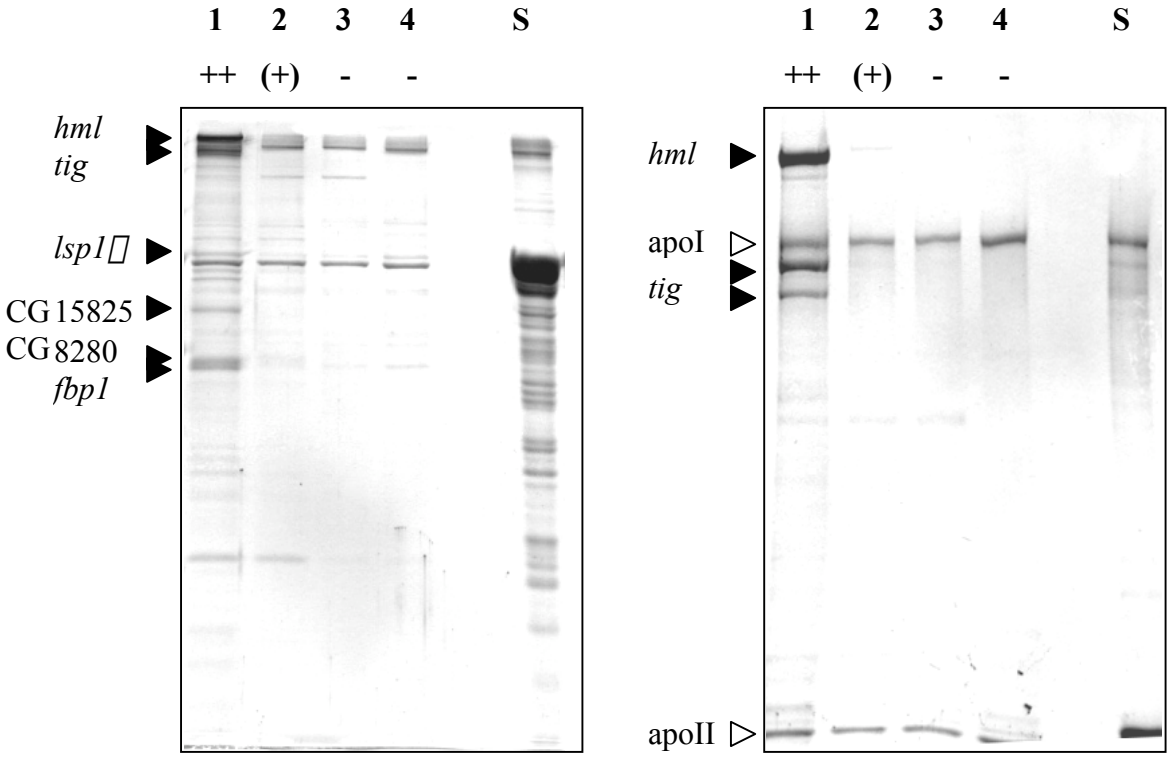

Figure 23: "Depletion assay" for proteins binding to the beads *in vitro*
(left picture: 10 % gel; right picture: 5 % gel)
1-4: successive binding reactions, S: supernatant after last binding reaction;
++ strong (+) very weak and – no bead aggregation;
filled arrowheads: proteins that are depleted from the sample;
open arrowheads: proteins that cannot be depleted (apolipophorin I and II);
hml: hemolectin, *tig*: tiggrin, *lsp1*: larval serum protein 1,
fbp1: fat body protein1, apo I and II: apolipophorin subunits I and II

The protein constituents of the clot bound to the beads were separated by gel electrophoresis, major bands were cut out from a gel and characterised by mass spectrometry (Figure 24; for method see chapter 2.14). It was not possible to redissolve all shown proteins for mass spectrometry analysis. The molecular identity of other proteins could not be determined by *in silico* analysis due to insignificant matches with the database entries. Since transglutaminase is an important component of the clotting system in several invertebrate and vertebrate groups, the transglutaminase inhibiting substance monodansylcadaverine (MDC) was included in a sample (Figure 24). No major inhibition of the protein binding to the beads was observed.

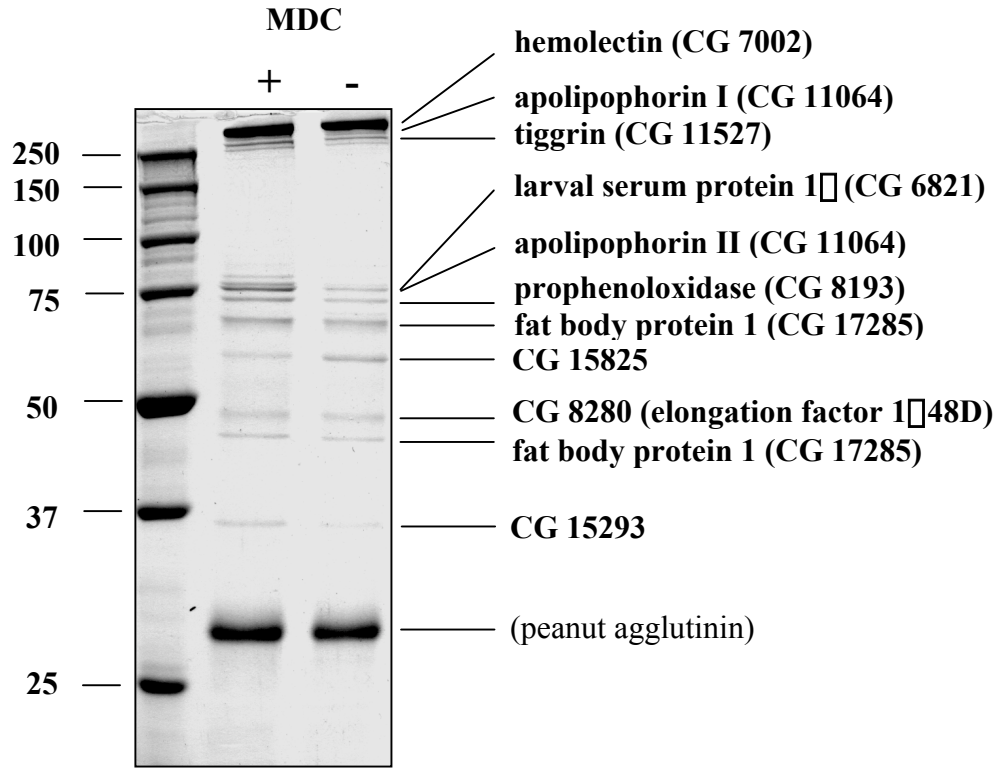

Figure 24: Putative clotting proteins isolated with the pull-out assay
Peanut agglutinin was coupled to the beads in this experiment to improve clot protein recovery (gel performed by Christine Karlsson). Nevertheless, the same proteins bound even in the absence of PNA.

One of the characterised proteins was hemolectin, which previously had been implied in coagulation and wound healing processes (Goto et al., 2001, 2003). The same working group also produced an antiserum against hemolectin, which I used in immunofluorescence experiments to show that hemolectin is a major compound of the clot fibre structures (not shown). The clot-labelling lectin PNA (Figure 22) exclusively detected hemolectin in the protein mix from the pull-out on a Western blot (Figure 25).

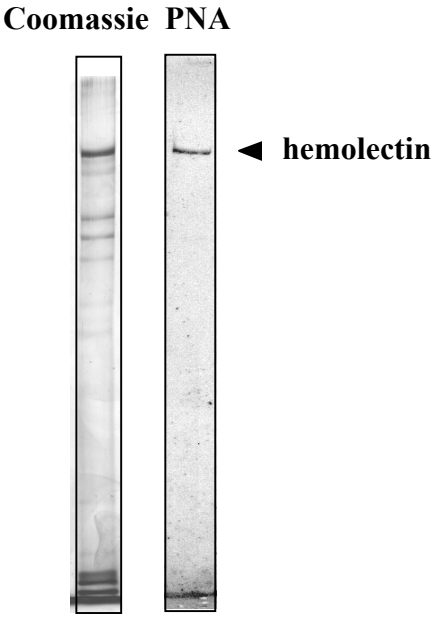

Figure 25: Peanut agglutinin exclusively recognises hemolectin from proteins isolated with the pull-out assay (5 % SDS-polyacrylamide gel)
left: Coomassie-stained gel, right: PNA-labelling on blot;
black arrowhead indicates position of hemolectin

Tiggrin, an extracellular matrix protein, was found in the preparation in two forms with slightly different molecular masses. These two forms probably reflect two novel isoforms or glycosylation states of the protein. No other extracellular matrix proteins were identified amongst the proteins isolated with the pull-out assay.

The apolipophorin subunits I and II of *Drosophila* "high-density lipophorin" (HDLp, originally described as "retinoid- and fatty-acid binding glycoprotein"; Kutty et al., 1996) continued to associate with the beads in the depletion test (Figure 23). Therefore it is possible that they do not represent specifically bound clotting proteins. An alternative explanation will be discussed.

The retrieved peptides from the characterised prophenoloxidase match best with the prophenoloxidase CG 8193 (judging from the matching profile in the Mascot database).

To date there are no known homologues to the uncharacterised product of gene CG 15825 in any other species including insects. A decreased amount of this protein was retrieved from the beads after transglutaminase inhibition with MDC (Figure 24), but was not followed up later.

To functionally confirm an involvement of hemolectin in clotting, I tested the effects of RNA interference (RNAi)-mediated down-regulation of hemolectin on the aggregation

ability in the assay. Crossing a UAS-*hml* RNAi line to an Actin5C-Gal4 strain, dsRNA production was expected to be induced strongly and ubiquitously in all cells starting from early development. The RNAi crosses were viable and did not show any morphologically visible defects. The addition of haemolymph from these larvae to beads did not cause a normal aggregation reaction. Haemolymph from the RNAi crosses contained haemocytes, in which the regulation of clotting seemed to be impaired, since an extracellular clot meshwork was missing (Figure 26). Furthermore the haemocytes included numerous oversized granules. This altered cell morphology was similar to haemocytes collected in anti-coagulation buffer lacking calcium ions and including EDTA (personal observation). It should be highlighted that coagulation did occur even after hemolectin RNAi knockdown, albeit with much delay and less extent.

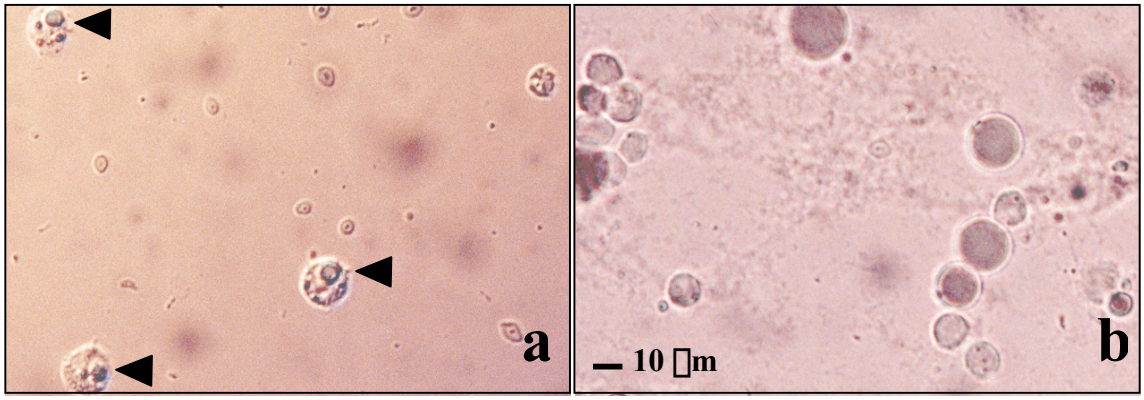

Figure 26: Clot formation and haemocyte morphology after hemolectin RNAi (a) and in wildtype larvae (b); arrowheads: large granular inclusions.

The RNAi-knockdown of hemolectin was confirmed by a SDS-gel of the proteins derived from a pull-out assay with knockdown larvae. A substantial decrease of hemolectin could be demonstrated, while the amounts of the other characterised proteins did not seem to be changed (data not shown). Bead aggregation was strongly impaired in haemolymph from hemolectin RNAi larvae (Figure 27, upper row). Haemolymph prepared as a control from the offspring of the same parental lines, but lacking the actin promoter-driven Gal4 gene (instead bearing a balancer chromosome), aggregated the beads comparable to control haemolymph (Figure 27, lower row).

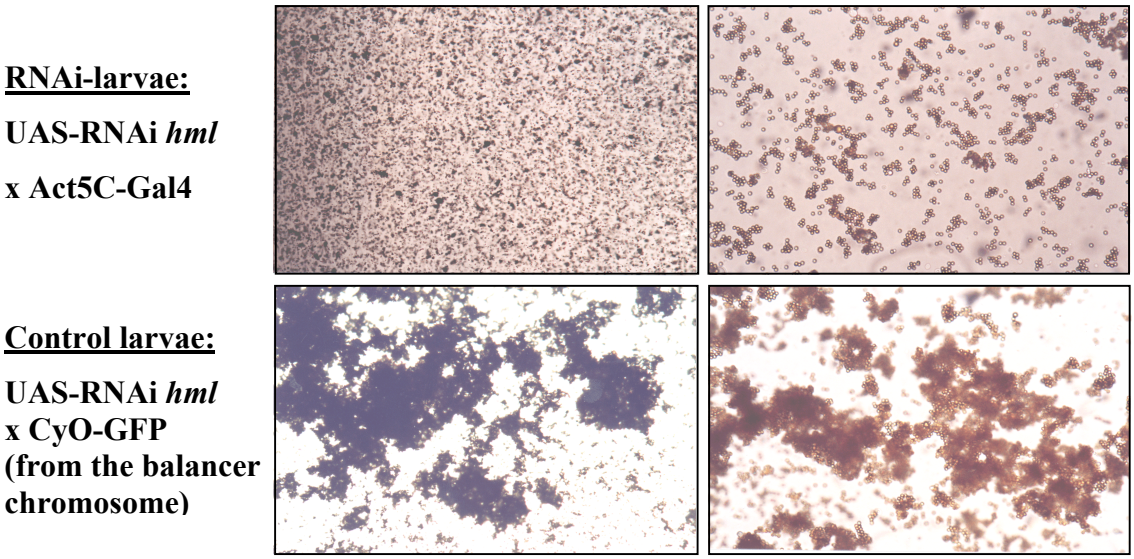

Figure 27: Bead aggregation is lacking in hemolectin RNAi larvae
(light microscopy pictures, magnification: left 10 x, right 40 x)

3.5 Regulation of haemolymph coagulation - preliminary results

Putative clotting factors were characterised by the pull-out assay as well as by 2D-gel comparisons of haemolymph samples before and after clotting (Karlsson et al., unpublished results). The functional significance of most of these proteins is still to be elucidated. Furthermore it is still widely unclear, how the different factors interact with each other. Even if it is too early to construct a detailed reaction scheme of the process, data from some pilot experiments might give hints to certain features of the system.

In order to confirm a reported induction of hemolectin transcription during development and after pricking (Goto et al., 2001), a RT-PCR reaction was performed with RNA samples from pricked and non-pricked animals (see chapter 2.16). I aimed to re-investigate the induction kinetics of hemolectin more carefully by adding additional time points. Furthermore the infection studies in the mentioned report had only been performed on adult flies. The use of larvae for clotting studies in our laboratory prompted me to additionally check the effect of poking in this developmental stage. Contrary to the previously published RT-PCR experiment, hemolectin was neither up-regulated during development nor in response to needle-pricking in the present study (Figure 28).

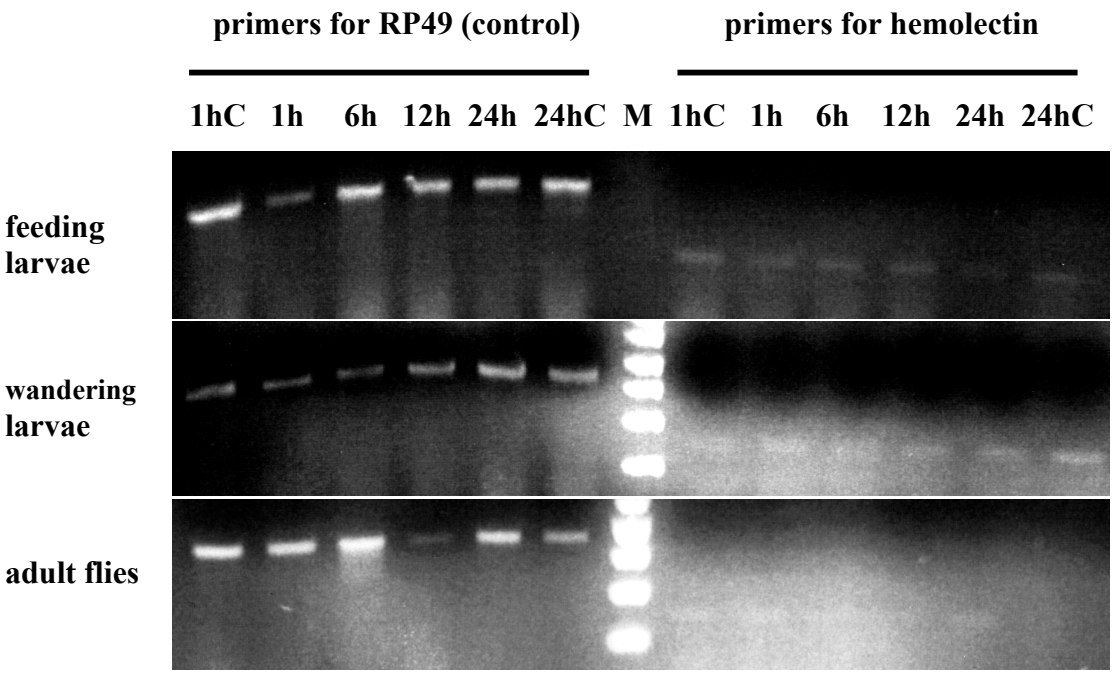

Figure 28: RT-PCR with primers for hemolectin (right) before and after poking different developmental stages of *Drosophila* with a fine tungsten needle
C: non-poked animals kept under the same conditions as a control;
1h, 6h, 12h, 24h: time points after poking (induction time)
left side of gel pictures: control primers for ribosomal protein 49 (RP49)

The influence of several putative coagulation inhibitors was tested for a possible impact on the composition of the protein subset isolated with the pull-out assay (Figure 29). The differences in the protein amounts in response to chemicals and even intra-sample variations turned out to be high. In general, a decrease of lower molecular weight bands like CG 15825 and tiggrin (Figure 29, arrows) correlated with the appearance of higher molecular weight complexes and a larger amount of hemolectin retrieved. The combinatory effect of all putative effectors led to a strong inhibition of this cross-linking (Figure 29, lane 7), although this effect was not always as intense as shown here. Nevertheless I decided to use Ringer solution with 20 mM EDTA and lacking calcium for samples simulating clotting inhibition to keep the reaction buffer as unmodified as possible.

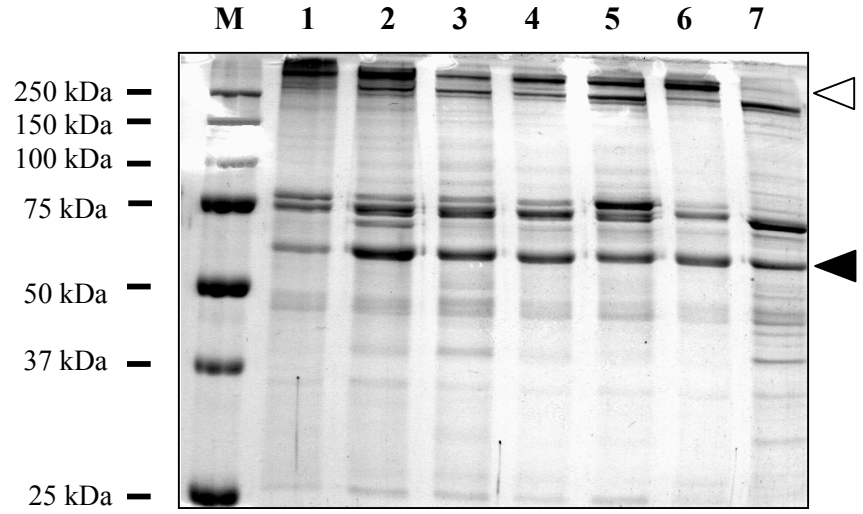

Figure 29: Addition of putative anticoagulants to the pull-out reaction
1: *Drosophila* Ringer – PTU; 2: Ringer + PTU;
3: Ringer – calcium / + 20 mM EDTA; 4: Ringer + 1 mM ascorbic acid;
5: Ringer + 5 mM MDC; 6: Ringer + 0,1 % natrium azide;
7: Ringer – calcium; + EDTA/PTU/ascorbate/MDC/azide
(haemolymph from 20 larvae each; white arrowhead indicates tiggrin;
black arrowhead shows position of CG 15825)

Since a mutant for *serpin-27A* (a serine protease regulating the phenoloxidase cascade) had become available, we tested larvae of this stock for a possible haemolymph coagulation defect. In addition a stock with a double mutation for *serpin-27A* and *Black cells* (kindly provided by Dr. Bruno Lemaitre) was used to check for the *in vitro* clotting ability. While haemolymph from both single mutants for *Black cells* and *serpin-27A* retained its ability to aggregate beads, the combined effect of both mutations caused a clear impact (not shown, comparable to Figure 22, *domino*). More intriguingly, this observation was reflected by the amount of hemolectin retrieved from the beads compared to the single mutants or the wildtype larvae (Figure 30).

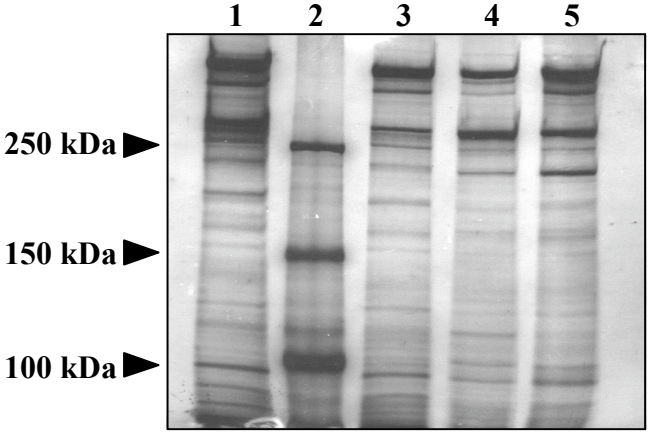

Figure 30: Comparison of pull-out retrieved proteins from mutants (5 % SDS-gel)
1 wildtype
2 molecular weight standard
3 *Black cells*
4 *Black cells*/*serpin-27A*
5 *serpin-27A*
Haemolymph from 30 instead of 20 larvae was used for the double mutant (4) to compensate for a smaller amount of haemolymph.

4 Discussion

4.1 Studies on localisation and functions of hemomucin

The hybridisation binding pattern of hemomucin probes during embryonic development (Figure 9) together with already published expression data from larvae and adult flies (Theopold et al., 1996, 2001) suggests broad biological functions for hemomucin. These possibly include hardening and sclerotisation or an involvement in immune reactions by pathogen immobilisation. Trachea, fore- and hindgut of the embryos, the peritrophic matrix of adults and eggshells are all sites of hardening processes. The also labelled larval salivary glands secrete sticky proteins prior to pupation that aid to attach the larvae to surfaces by forming a solidifying glue. Hemomucin may contribute to all these processes by aggregating to protein oligomers, since it tends to dimerise (Theopold et al., 1996). The galactose and N-acetyl-galactosamine glycodeterminants of hemomucin might cross-link with each other, possibly by endogenous lectin activity. Interestingly the pattern of hemomucin expression is similar to the one obtained by labelling of the embryo with wheat germ agglutinin (Callaerts et al., 1995).

Alternate to a sclerotising function, hemomucin might bind and immobilise bacteria on organ surfaces to prevent infections from spreading further into the haemocoele. Cross-linking was proposed to occur between hemomucin and sugar determinants on bacterial cell surfaces catalysed by haemolymph lectin activity (Theopold et al., 1996). It is intriguing, that most hemomucin expression sites as embryonic trachea, fore- and hindgut, adult peritrophic matrix and egg surfaces are potentially exposed to microorganisms. Both hypotheses point towards a dual function of hemomucin in immunity and development.

Transcripts of the second strictosidine synthase-like gene CG 11833 are present in the trachea, while tissue labelling is different from the one with the hemomucin probe (Figure 10). In contrast to hemomucin the CG 11833 protein does not include a mucin domain but consists only of the strictosidine synthase-like sequence. The similar binding of the probe to the trachea may therefore reflect a function of the SSL-domain in this specific organ.

Using the hemomucin antiserum for detection on a Western blot (Figure 8), it could be shown that the membrane protein hemomucin is released from the haemocytes during clotting, probably mostly on microparticle surfaces. Detection of hemomucin with HPL on membranes does not lead to a sufficient signal on *Drosophila* haemocytes. On this membrane the release of the protein from the cells into the haemolymph can be seen clearly (Figure 8). HPL does not recognise hemomucin in the cell fraction either, probably due to the fact that the membrane was already incubated before with the antiserum, which might have blocked all hemomucin binding sites. However, the lectin shows a quite good labelling for hemomucin in mbn-2 cell samples on Western blots (Figure 13 and Theopold et al., 1996). The strong HPL signal on haemocyte monolayer preparations in contrast to the weak recognition on Western blots might be explained by HPL binding to other hemocyte membrane molecules such as N-acetyl-galactosamine determinants of glycolipids, which are not recognisable on the Western blots (Dr. Ulrich Theopold, personal communication).

Microparticle formation in the *Drosophila* cell culture in reaction to ionophore treatment (not shown) correlated well with the depletion of hemomucin from haemocyte membranes (Figure 11). Microparticles are often found attached to haemolymph clots and may thus induce clotting analogous to vertebrate microparticles. It seems likely that hemomucin is actively shed into microparticles. The protein might therefore be a functional component of the particle surface and leads to stimulation of clotting. It is interesting to note that not all mbn-2 cells were sensitive to ionophore treatment and that those who were not showed a lowered lectin labelling already prior to addition of the ionophore (Figure 12). This could be interpreted in different ways: It is possible that these cells released particles together with hemomucin prior to the start of the experiment and could thus be characterised as non-reactive altered cells. However, it cannot be excluded that there might in fact be separate subpopulations in the cell culture that differ in their hemomucin content. It is tempting to speculate that this reflects the differential reaction of two haemocyte classes *in vivo*. Finally it is possible that this non-reactive cell population represents cells right after cell division with a thus lowered capacity for protein synthesis. Unfortunately it might be hard to morphologically characterise a difference between these two haemocyte groups in *D. melanogaster*, since this species predominantly contains plasmatocytes.

Knock-down of hemomucin in mbn-2 cells by RNAi proved to be successful (Figure 13). No obvious changes in cell morphology and behaviour could be observed and HPL membrane labelling was comparable to untreated cells. This observation might be explained by a slow turnover rate for hemomucin in the membrane, while intracellular hemomucin production would already be impaired, leading to a lower protein level in the electrophoresis sample. Hence it would be interesting to investigate the immune effect of hemomucin RNAi *in vivo* in a transgenic *Drosophila* fly line with an inducible promoter for a dsRNA hairpin construct. Furthermore the study of putative functions of the protein during development would be feasible in such flies. I aimed to generate a transgenic fly stock for hemomucin RNAi *in vivo*. However, this approach was finally not successful. As shown in Figure 15, the efficacy of Sfi I restriction and/or the first ligation was quite low. It has long been recognised that some restriction enzymes do not cut optimally at the ends of a PCR fragment (Kaufman & Evans, 1990). Nevertheless the study of Kennerdell & Carthew (2000) demonstrates that the technique used here (see chapter 2.10) is in general feasible with the chosen restriction enzymes. It is possible that the experimental conditions used did not favour a successful ligation. A number of additional difficulties connected with the method by Kennerdell & Carthew (2000) were pointed out later. Importantly transgenes with an inverted repeat on a plasmid exhibit a comparably weak stability in *E. coli* cells (Lee & Carthew, 2003). This finding is in agreement with the low occurrence of successful transformations obtained in the present study. Lee & Carthew (2003) therefore proposed to stabilise the plasmid by insertion of a longer intron-based spacer sequence between the inverted repeated sequences. After *in vivo* transcription this spacer is spliced out leading to a "loop-less" double-stranded RNA molecule. This technique led to considerably better results and is commonly used today, but was only reported towards the end phase of the present work and thus not used. A transgenic fly line for hemomucin RNA interference is now available in our group (kindly provided by Dr. Bruno Lemaitre) and will help to elucidate the probably various functions of hemomucin *in vivo*. Another possibility to address the putative roles of hemomucin in immunity and development would have been the injection of the *in vitro* transcribed dsRNA into *Drosophila* embryos. Due to time limitations of the project this approach was not followed up either. A general problem is that injections include the generation of a wound and thus analysis of

clotting defects in such animals is ambiguous. In summary, a specific function has yet to be assigned to hemomucin. It is likely that its ability to bind sugars on pathogen surfaces and to form oligomers is crucial for its biological role. A pro-inflammatory reaction might be regulated by association of hemomucin with lipophorin (Theopold & Schmidt, 1997). In the same study it was also suggested that putative lipid-binding properties of hemomucin (due to a large amount of hydrophobic amino acid residues in its sequence) may regulate association of hemomucin with lipophorin. Interestingly, the latter property is probably exploited by a parasitoid wasp to block an immune response by recruiting host lipophorin to wasp hemomucin deposited on eggs (Kinuthia et al., 1999).

According to this model, hemomucin may be a functional component of the clot matrix accessible to both cross-linking by lectins and regulative blocking by lipophorin (see also chapter 4.6). Hemomucin homologues are present in two lepidopteran species (Kotani et al., 1995, Scholz, 2002) and in the mosquito *A. gambiae* even SSL-proteins with similarity to both *Drosophila* SSL-proteins (with and without the mucin domain) are conserved (gene accession numbers "XP_309617" and "XP_321354", http://www.ncbi.nhlm.nih.gov). The fact that hemomucin is induced during *Plasmodium* infections of *Anopheles* mosquitoes (George Dimopoulos, personal communication) strengthens the suggestion that hemomucin is a factor involved in insect immune reactions.

4.2 Recombinant expression of the mouse SSL protein in *E. coli* for production of an antiserum

An antiserum against a recombinantly expressed mouse SSL protein (mSSL) was raised. This mSSL-protein produced in *E. coli* was even recognised by the antiserum against the SSL-domain of hemomucin (Figure 18), confirming that the EST was coding for the right sequence. The anti-mSSL antiserum had a considerably good specificity for the recombinantly expressed protein from *E. coli* cells (Figure 19). The mouse SSL protein of about 47 kDa was initially described as APMAP, "adipocyte plasma membrane-associated protein" (Albrektsen et al., 2001). In this study expression of the

protein in heart, brain, liver and kidneys of mice has been demonstrated. Nevertheless the polyclonal antiserum raised in both rabbits during the present work predominantly recognised a large number of additional mouse proteins (Figure 20) and thus appears to cross-react non-specifically. An affinity purification of the anti-mSSL antiserum from the nitrocellulose membrane did not improve the results considerably. Therefore the signals retrieved from the mouse tissues have to be discussed with caution. Since the mSSL protein includes no mucin domain like hemomucin (Albrektsen et al., 2001), a cross-reaction with other serine- and threonine-rich sequences can be excluded. It is possible that the mSSL protein is strongly N-glycosylated *in vivo*, as suggested by Albrektsen et al. (2001). The antibodies are likely to be directed against the unmodified amino acid backbone of the recombinantly produced protein which is buried under bulky carbohydrate groups in the native mouse protein. Other post-translational modifications might be lacking in the *E. coli* cells as well. This may explain, why labelling of the recombinantly produced mSSL protein with the antiserum is quite unsatisfying. Unfortunately the weak binding specificity of the antibody produced in the present study did not allow any further experiments. We also obtained an mSSL antiserum produced by another group (Albrektsen et al., 2001), which did not show sufficient binding in Western blot analysis either. This may indicate a general problem to create an antiserum against the mSSL protein.

Interestingly a strong expression of the human SSL protein has been observed in human brain and immature immune organs (Ulrich Theopold, unpublished results). It remains therefore tempting to investigate the expression of mSSL in bone marrow and platelets of mice. Plant strictosidine synthases are important for alkaloid biosynthesis and vertebrate brains are target organs for alkaloids interfering with dopaminergic synapses. However, *in situ* hybridisations of mouse brains with an mSSL RNA probe did not lead to labelling of dopaminergic regions (Christine Karlsson, personal communication). In summary, the functions of SSL proteins in vertebrates remain enigmatic and will be an interesting research subject in the future. An additional fact points towards the importance of SSL proteins in immunity: Most sequenced bacterial genomes contain no SSL homologs. However, *Pseudomonas aeruginosa*, a facultative bacterial pathogen of plants, flies and man, seems to have acquired an SSL gene through horizontal gene transfer from one of the vertebrate genes, with which it shares the highest similarity

4.3 Use of a clot-specific antiserum in *Galleria mellonella*

Initially we aimed to molecularly characterise clot components in *G. mellonella*, since the morphology of haemolymph coagulation is well described for this insect species. As a part of this project I used an anti-clot antiserum (Li et al., 2002) in immune fluorescence microscopy investigations of *Galleria* haemocyte preparations. The antiserum bound strongly to extracellular clot structures as well as attached microparticles (Figure 21 a). From the five described haemocyte types of *G. mellonella* only granular cells were labelled (Figure 21 b and c). This is in agreement with earlier morphological studies showing that the granular cells of lepidopterans are the haemocyte type involved in primary clot formation by degranulation of fibrous material (Bohn, 1986, Rowley & Ratcliffe, 1976). Hence the antiserum can be considered an interesting tool to identify clotting factors of *G. mellonella*. Further results obtained with the same antiserum in a library screen (done by Dongmei Li, Li et al., 2002) provided insights into the identity of the constituents of the *Galleria* haemolymph coagulum. While recognition of a **mucin-like** sequence by the antiserum once more highlighted the importance of glycoproteins in coagulation, **prophenoloxidase**-specific antibodies highlighted a connection between these enzymes and the clotting process that had been proposed earlier. The detection of a **hexamerin** in the clot is interesting, since an arylphorin-like gallysin from *G. mellonella* was shown to lyse erythrocytes and kill Gram-negative bacteria (Beresford et al., 1997). An immune function for hexamerins was further demonstrated for other insect species, including inhibition of degranulation, encapsulation and nodule formation in *Pseudaletia separata* armyworm larvae (Hayakawa, 1994). Several **lipophorin** expression clones were found in the library screen. Lipophorin became incorporated into the *Galleria* clot quite late during the ongoing coagulation process compared to other proteins (Li et al., 2002). Lipophorin involvement in haemolymph coagulation was reported earlier for cockroaches (Barwig, 1985) and locusts (Duvic & Bréhélin, 1998) and will be discussed in detail in chapter 4.4. Finally a large number of antibodies recognised a so far uncharacterised protein with little similarity to the **-crystallin** family (MacRae, 2000). Interestingly crystallins are known to differ considerably in their sequence, but not in their hydrophobicity, they easily self-aggregate and are cross-linking targets for

transglutaminases (de Jong, 1993, Shridas et al., 2001). The β-crystallin-like protein might therefore be one of the transglutaminase substrates in *Galleria*, supported by the presence of numerous cross-linkable lysine residues in its amino acid sequence.
In general, the production of an antiserum against an *in vitro* formed clot proved to be a successful approach and may be used in further comparative studies in different insect orders. Nevertheless, there are limits to conclusions concerning the *in vivo* situation in the insect. It is likely that parts of the native clot are of cellular origin and could thus not be retrieved, since cell-free haemolymph was used for antiserum production. Furthermore it is possible that certain clotting factors already precipitated prior to contact with the erythrocyte membranes and are no longer available for the *in vitro* clot. Since no inhibitors of phenoloxidase activity like PTU were added to the *in vitro* clotting reaction used for antiserum production, it cannot be excluded that some of the antibodies actually recognise phenoloxidase substrates in *G. mellonella* and not only the initial clot components. Further restrictions of the anti-clot antiserum will be pointed out in chapter 4.5.

4.4 A novel assay for isolation of clot components in *Drosophila*

In search for a quick and inexpensive method to isolate a haemolymph clot from *D. melanogaster* I used paramagnetic beads, which were passively incorporated into the forming coagulum and facilitated separation of the clot from haemocytes and cell debris (Figure 7). In addition the strength of bead aggregation seemed to reflect *in vitro* clotting ability in a reproducible manner (Figure 22). The clot proteins characterised by mass spectrometry clearly showed a composition different from the total haemolymph and were depleted from the buffer quite quickly in subsequent binding reactions (Figure 23), strengthening their putative role in coagulation. A remarkable feature of many of the pull-out retrieved proteins (Figure 24) like e. g. hemolectin and tiggrin is a relatively high molecular weight. In is interesting to note that non-crosslinked protein gels form more likely from large protein monomers that tend to entangle already at low concentrations of only one percent or less (Doi & Edwards, 1988).

Hemolectin (*hml*), the protein isolated from the beads in the highest quantity, was furthermore labelled by peanut agglutinin in a Western blot reaction (Figure 25). This was in accordance with the detection of PNA-reactive proteins in the coagulum in immunofluorescence studies (Figure 22), showing that hemolectin is part of the fibrous clot. An antiserum against hemolectin (kindly provided by Dr. Akira Goto) confirmed this conclusion in immunohistochemical clot preparations (not shown), strongly labelling the fibres of the coagulum. There is at least one additional PNA-reactive protein "gp150" (gene name "I71-7") present in the total haemolymph, which presumably is involved in the clotting process as well (Korayem et al., unpublished results). This protein gp150 could not be retrieved with the pull-out assay, probably due to its rapid cross-linking in the free haemolymph prior to potential binding to the bead matrix. In the lepidopteran insect *Manduca sexta* the PNA-reactive proteins are present in granular cells throughout development (Nardi, 2004). Intriguingly PNA-labelling even increased in the granules of this haemocyte subtype in *M. sexta* pupae compared to larvae (Beetz, 2002). In agreement with this observation the hemolectin homologue in *M. sexta*, hemocytin, is expressed in granular cells (Scholz, 2002). These results underline a possible immune function for PNA-reactive proteins in insects and putative endogenous haemolymph galactose-specific lectin activities that may transmit the signal to other components of the immune system.

Interestingly, hemolectin displays high similarity to vertebrate von Willebrand factor and had recently been proposed to influence the wound healing ability of larvae (Goto et al., 2003). The impaired bead aggregation after hemolectin RNAi (Figure 27) possibly reflects a defect in a primary step of haemolymph coagulation in insects, possibly resembling primary haemostasis in vertebrates. Intriguingly the presented change in haemocyte morphology (Figure 26) is reminiscent of an impairment of platelet degranulation in patients with von Willebrand disease (VWD). With an incidence rate of about 1 % in the whole population, VWD represents the most common inherited bleeding disorder in men (Sadler, 1998, Triplett, 2000). In vertebrates vWF is constitutively present in the blood and endothelial cells, even in preformed high molecular weight polymers. Binding to wounded subendothelium and shear stress leads to a conformational change of the protein that further stimulates protein aggregation (Sadler, 1998). Similarly insect clotting may require a concentration threshold or

conformation of hemolectin that causes haemocytes to degranulate at wounds and release intracellular clotting factors. Even in hemolectin RNAi knockdown larvae coagulation finally occurred, though after prolonged time periods, similar to the situation in patients with vWF mutations.

Hemolectin is a large protein of 427 kDa with a number of conserved domains (Figure 31) that may facilitate interactions with haemocytes and the wound edges or with other clot proteins. Most intriguingly, in *Drosophila* hemolectin (Goto et al., 2001) as well as in hemocytin of *B. mori* (Kotani et al., 1995) and of *M. sexta* (Scholz, 2002) there are several domains with similarity to vertebrate von Willebrand factor (vWF). Five copies of a vWF D-like domain, which accounts for self-aggregation of vWF (Mayadas & Wagner, 1992) are present in hemolectin. In addition four short sequence stretches resemble the vWF C domain, which is also important for protein oligomerisation. Two hemolectin domains display high similarity to vertebrate coagulation factor 5/8 and are also known as discoidin domains. Members of the discoidin family were initially described to bind galactose, but are even known to recognise phosphatidylserine and collagen and promote cell aggregation and adhesion (Baumgartner et al., 1998). This broad spectrum of putative ligands raises the possibility that association of a binding partner to the discoidin domain of hemolectin may change its conformation and activate the clotting process. A low-density lipoprotein (LDL) receptor domain may bind lipophorin at later steps of clotting (see below). The C-terminal cystine knot-like domain probably catalyses the initial dimerisation of hemolectin prior to further multimer formation (Goto et al., 2001). Two N-terminal epidermal growth factor (EGF)-like domains and a central peritrophin A-like chitin binding domain might enable hemolectin to bind to the wound site and thereby ensure a localised clotting reaction. Even an N-terminal sequence with weak but significant similarity to laccases can be recognised. Laccases are cross-linking enzymes with phenoloxidase-like activity, which in this case may be specialised for the requirements of clotting. The presence of an autocatalytic cleavage site GDPH similar to human mucins (Lidell et al., 2003) in the amino acid sequence suggests a so far undiscovered activation mechanism for hemolectin.

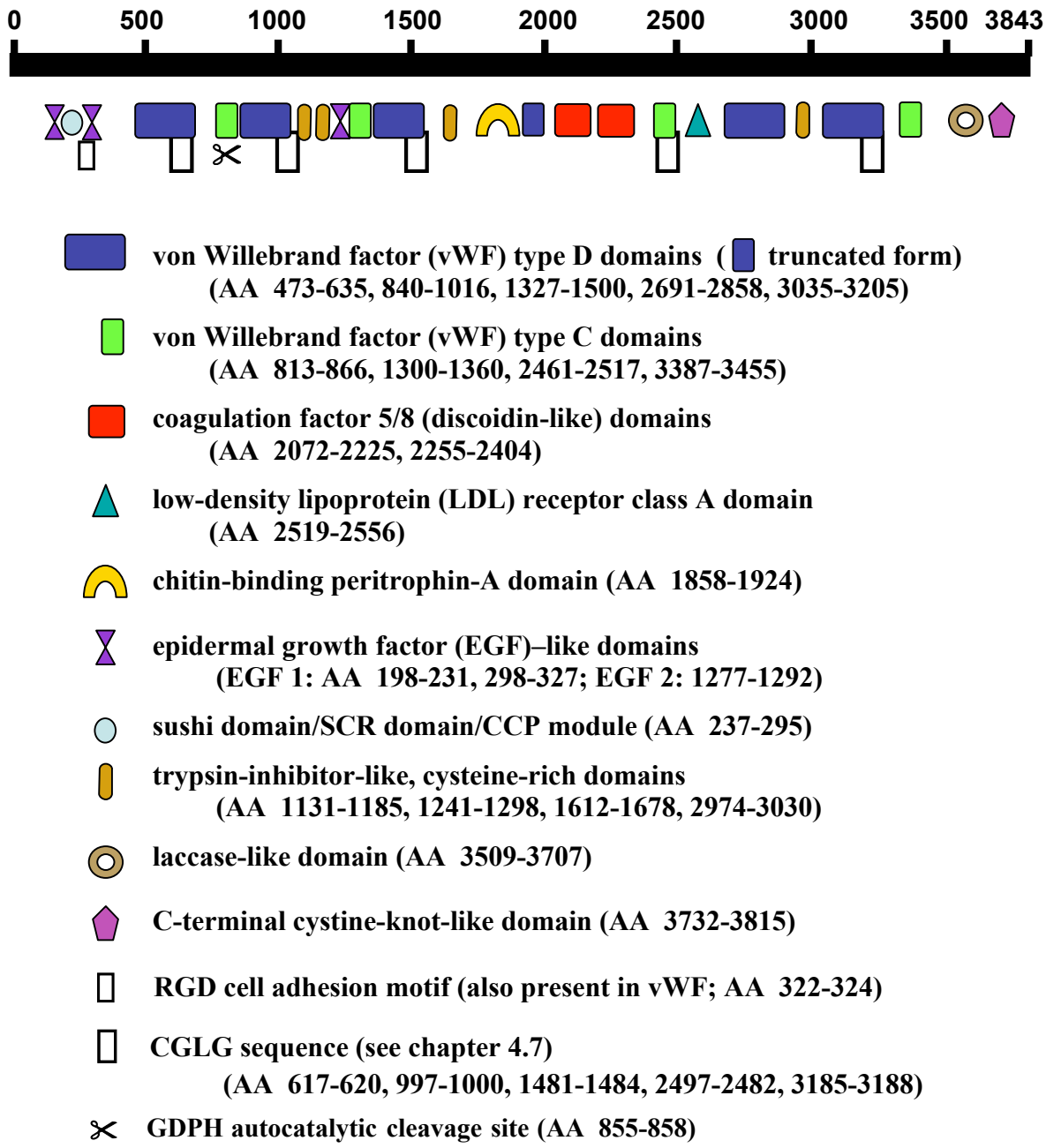

Figure 31: Domain structure of *Drosophila* hemolectin (CG 7002)
(including domains identified by Goto et al., 2001)
Additional domain similarities were identified with NCBI conserved domain search (http://www.ncbi.nhlm.nih.gov) and SRS Interpro Entry Search (http://srs.ebi.ac.uk). The small sequence patterns RGD, CGLG and GDPH were detected by manual search. The amino acid (AA) locations of The motifs are noted in brackets. In cases where different domain lengths were found by the used programs, the broadest motif borders are given here.

Tiggrin is an extracellular matrix protein expressed in haemocytes, fat body and other tissues. It is also present at muscle attachment sites of *Drosophila* (Fogerty et al., 1994), where it interestingly colocalises with lipophorin (Kutty et al., 1996). No orthologue of tiggrin is known from other insect species to date. The amino acid sequence of the protein includes an RGD peptide that mediates integrin contacts of muscle cells with the tendon matrix, thereby confering stability and elasticity to pre-existing muscle-epidermis contacts (Bunch et al., 1998). In vertebrates the fibrillar collagen receptor □1□1-integrin is involved in wound contraction through myofibroblasts (Racine-Samson et al., 1997). In insects there is no fibrillar collagen (Hynes & Zhao, 2000). It is thus intriguing to speculate that similarly tiggrin may contribute to clot strength by interaction with haemocyte surface integrin □PS2□PS, so that the scar can withstand body movements of the wounded larva. If this proves to be the case, tiggrin may even be considered a functional analogue of vertebrate fibronectin, since no sequence coding for a fibronectin is present in the *Drosophila* genome. A possible role in immunity is also supported by the observation that tiggrin is induced after injury (Irving et al., 2001). A general putative role for integrin binding sites in clotting proteins will be discussed later.

Other products of injury-induced genes isolated with the pull-out assay include the proteins encoded by the genes **CG 15825** and **CG 15293** (De Gregorio et al., 2001). Both gene products show no significant homology with any protein in the databases and are induced with slow kinetics. This expression pattern might be expected for genes involved in clotting and is similar to the one of mosquito genes induced by aseptic wounding (Dimopoulos et al., 2002). Additional support for a role of the CG 15825 protein in clotting is added by recent results which show that it is a substrate for transglutaminase (Karlsson et al., unpublished results). There is evidence for an involvement of the Toll pathway in the induction of both CG 15825 and CG 15293 (De Gregorio et al., 2002).

Additional proteins isolated with the pull-out assay are **larval serum protein 1 gamma (*lsp1□*)**, which is one of the major haemolymph proteins in third instar larvae of *Drosophila* (Kanost et al., 1990) and **fat body protein 1 (*fbp1*)**, which has been shown to bind *lsp1□* (Burmester et al., 1999). *Fbp1* was detected in two fragments of 69 and 49 kDa. Both *lsp1□* and *fbp1* belong to the hexamerin family, a group of haemolymph

storage proteins (Burmester et al., 1999). It is possible that hexamerin binding to the beads is a non-specific interaction resulting from the high concentration of these proteins in the haemolymph. However, both hexamerins and in particular lipophorin have been implied in clotting of *Galleria* haemolymph earlier (Li et al., 2002). Hence it seems plausible that haemolymph coagulation, which has to be fast and efficient, relies at least partly on the recruitment of some abundant haemolymph proteins.

The retrieved fragments of **prophenoloxidase CG 8193** include peptides from the N-terminal region and the protein sequence does not possess a predicted cleavage site. This indicates a non-proteolytic activation mechanism of the enzyme. Possibly binding of the enzyme to the clot or simply crossing a concentration threshold at the wound site leads to a conformational change that stimulates phenoloxidase activity. The prophenoloxidase CG 8193 may fulfil specialised haemostatic functions in *Drosophila* haemolymph, since its haemolymph levels also differ considerably between clotted and not clotted samples (Karlsson et al., unpublished results). Since coagulation occurs even in the absence of haemolymph phenoloxidase activity in *Black cell*s mutant larvae (Scherfer et al., 2004; Figure 2), it can be excluded that clotting is dependent on this cross-linking step. Even the composition of a *Black cells* haemolymph clot isolated with the pull-out assay or transglutaminase inhibition by MDC did not differ from the protein pattern in wildtype larvae. However, phenoloxidase and transglutaminase might very well harden the clot and apply stability to it, preventing its destruction through larval body movements. It remains an open question, if phenoloxidase and transglutaminase activities complement each other or if they represent subsequent reactions. It is interesting to note that synthetic melanin was recently reported to effectively inhibit vertebrate transglutaminases (Ikura et al., 2002).

CG 15825, tiggrin and *fbp1* contain a large number of **glutamine residues** (9, 12 and 14 % respectively). The average amino acid composition of proteins includes 4 % glutamines (McCaldon & Argos, 1988), thus an increased proportion may well reflect a functional feature of the mentioned proteins. Additional factors incorporated in the haemolymph clot like hemolectin, CG 8280, *lsp1* and lipophorin display elevated levels of lysine residues. Glutamine-rich proteins self-associate easily to form aggregates, a pathological feature observed in human prion diseases (Michelitsch &

Weissmann, 2000) and neurodegenerative disorders like Chorea Huntington (Masino & Pastore, 2002). Even transglutaminase activity is involved in the progress of these diseases (Violante et al., 2001). Since the glutamine residues occur through CAG nucleotide expansion in the mentioned cases, the non-clustered glutamines in putative insect clot proteins are probably not acquired through the same mechanism. However, glutamines together with lysines are also substrates for the terminal cross-linking of clot proteins by transglutaminase in vertebrates. In *Drosophila* CG 15825 and lipophorin have already been shown to be transglutaminase substrates (Karlsson et al., unpublished results). The transglutaminase inhibitor MDC caused a lowered level of CG 15825 in the clot. An "early stage encapsulation protein" from the beetle *Tenebrio molitor* (Cho et al., 1999) even includes 17 % glutamines and might also be a clot protein.

Besides tiggrin, hemolectin (see Figure 31), *fbp1* and transglutaminase of *D. melanogaster* also contain an **RGD motif**. A **KGD** integrin-binding site from crayfish protein peroxinectin (Johansson, 1999) stimulating encapsulation reactions *in vitro* (Kobayashi et al., 1990) is as well present in *Drosophila* annexin IX and lipophorin. Hemolectin, *fbp1*, *lsp1* and CG 15293 contain an additional cell-adhesion motif **LRE** (Yamada, 1991), tiggrin two copies. Interestingly clustered RGD-peptides are focal adhesion sites for stress fibre formation (Maheshwari et al., 2000). A point mutation of the RGD motif to AGD in a recombinant human vWF protein caused a total loss of binding to glycoprotein IIb/IIIa (Jumilly et al., 2001), underlining the importance of this peptide for the clotting process in vertebrates. Vertebrate integrin antagonists called disintegrins contain a RGD or KGD motif and are inhibitors of platelet aggregation (Andrews & Berndt, 2000). Similarly, the high abundance of the mentioned cell adhesion patterns RGD, KGD and LRE in factors implied in insect haemolymph coagulation probably reflects a secondary function of the clot, namely the attraction of flattening lamellocytes that express high levels of integrins (Irving et al., 2003) and form capsules around foreign objects and organisms. Moreover it was suggested earlier, that RGD-containing haemolymph proteins may promote attachment of insect haemocytes to wounds (Lackie, 1988).

Both subunits I and II of the *Drosophila* **lipophorin** ("retinoid- and fatty-acid binding glycoprotein"; Kutty et al., 1996) were present in the pull-out derived clot. Apolipophorins are multifunctional proteins with the ability to store and transport large amounts of lipids and represent a major constituent of insect haemolymph (Blacklock & Ryan, 1994). Earlier studies proposing apolipophorin as the clotting protein in several insect species may therefore be explained in two different ways: Either the sheer amount of protein in the plasma (Kanost et al., 1990) causes unspecific binding to the coagulum, as it might be the case for *lsp1* and *fbp1*. Alternatively this association is due to a functional binding, which may for example be achieved through the low density lipid protein receptor domain of hemolectin. Lipophorin is a transglutaminase substrate in *Drosophila* (Karlsson et al., unpublished results). In *Galleria* it binds to the coagulum quite late during its formation, as shown with the anti-clot antibody (Li et al., 2002). The protein is involved in several aspects of insect immunity: It inhibits haemocyte spreading *in vitro* (Coodin & Caveney, 1992, Mandato et al., 1996) and was successfully used to prevent cell aggregation in *H. cecropia* (Lanz-Mendoza et al., 1996). In addition lipophorin blocks proteolytic prophenoloxidase activation (Duvic & Bréhélin, 1998), induces reactive superoxide production (Arakawa et al., 1996) and assists in LPS detoxification from the haemolymph (Kato et al., 1994). Eggs of a parasitoid wasp block an effective immune response by binding host lipophorin (Kinuthia et al., 1999). If a protein with such numerous inhibitory functions is incorporated into a clot, this might lead to negative feedback regulation for several immune reactions at the wound. This hypothesis is also strengthened by the slow binding kinetics of lipophorin to the *G. mellonella* clot. The inhibitory mechanism may be achieved by competitive clustering on integrin binding sites through the KGD motif or blockage of immune-stimulating phospholipids.

Conversely, a lower molecular weight insect lipoprotein, apolipophorin III, stimulates phagocytosis and induces immune genes in response to infection (Wiesner et al., 1997), long after clotting has reached its end. Apolipophorin III was proposed to possess an inflammatory activity by associating with lipids and apolipophorin I and II (Dettloff et al., 2001). This complex, also called "low-density lipophorin" (LDLp) is not constitutively present in insect haemolymph (Soulages & Wells, 1994). Under normal conditions larval apolipophorin III does not contain any lipids (Cole & Wells, 1990).

The occurrence of LDLp in moulting larvae of *M. sexta* might be essential for an activated immune state during this life period due to a potentially higher susceptibility of the integument to infections (Engler et al., 1996). Hemagglutinating properties of apolipophorin III were ascribed to recognition of cellular glycolipids resembling bacterial LPS (Chung & Ourth, 2002), since interaction with glycoproteins could be excluded (Iimura et al., 1998). Moreover, this binding reaction seems to possess some specificity, since only certain LPS types added to the *in vitro* reaction interfered with cell aggregation (Iimura et al., 1998). Interestingly the immune-induced serine protease scolexin from *M. sexta* binds to LDLp (Dettloff et al., 2001). This observation is in good agreement with the reported glucose-specific hemagglutinating activity of this protein (Minnick et al., 1986), which could provide an additional mechanism to immobilise microorganisms at the wound site. On basis of the summarised data I propose a model for the involvement of lipophorin in the insect wound reaction: During late coagulation low-density lipophorin including apolipophorin III subunits possibly associates with lipids in the clot. In the immediate phase this may inhibit haemocyte spreading and block phenoloxidase activity, thereby preventing an over-activation of the immune system. Simultaneously the LDLp complex probably also promotes rapid LPS detoxification, production of reactive oxygen species and phagocytosis of bacteria and apoptotic cells in the coagulum. As a secondary result of LDLp formation, gene induction might also lead to expression of antimicrobial peptides at later time points.

It is fascinating to note that the presented model for possible immune functions of lipophorin is similar to the situation in vertebrates: **Mammalian lipoproteins** are important not only for lipid transport, but are also involved in the regulation of clotting: Low-density lipoprotein (LDL) activates platelets, while high-density lipoprotein (HDL) blocks platelet aggregation, even in this case by interaction with lipids (Rosenson & Lowe, 1998). In a pathological defect lipoprotein (a) is deposited on blood vessel walls by transglutaminase (Borth et al., 1991). In addition LDL enhances the phagocytotic activity of macrophages (Carvalho et al., 2002) and stimulates release of nitric oxide from platelets (Riddell et al., 1997). LDL uptake elevates cytokine production in vertebrate macrophages and T-cells (Tedgui & Bernard, 1994). The oxidation state of LDL and HDL can influence clotting intensity as well (Mertens & Holvoet, 2001).

The fact that haemolymph from *domino* mutants of *Drosophila* failed to aggregate the beads (Figure 22) suggests a major contribution of haemocytes to coagulation. Hemolectin and transglutaminase are originally cellular proteins and become localised at the wound sites by release from degranulating or rupturing haemocytes. Furthermore cells may act as effective "filling material" in the clot and add mechanical strength to it. In later steps of clotting the large flattened lamellocytes of *D. melanogaster* possibly play an essential role by attaching to RGD-containing clot proteins which might activate integrin-mediated immune responses.

The pull-out assay led to the first molecular description of proteins involved in *Drosophila* haemolymph coagulation and the protein pattern is clearly different from the total haemolymph proteins (Figure 23). A refined proteomics approach allowed the identification of additional putative clotting factors (Karlsson et al., unpublished results). It remains to be clarified if the less abundant proteins in the coagulum reflect a functional contribution to the clotting process or if they might be caught in the clot as "filling substance" without being crucial for haemolymph coagulation. A disadvantage with the pull-out assay is that it is impossible to conclude from the bound protein amount to the *in vivo* situation. The harsh washing conditions including detergents applied in the pull-out may remove weakly bound proteins, which thus escape detection. Clotting factors might aggregate so quickly in the haemolymph, that they do not get in contact with the beads. Alternatively proteins may be cross-linked so strongly that they cannot be recovered from the beads by denaturation in sample loading buffer. Similarly some putative clot proteins with low abundance in the insect may be missed by the assay, though they still might have a significant impact on the reaction. In addition the amount bound of a certain protein cannot be compared easily between different samples: A weaker band could be due to a lower haemolymph level, but might also be explained by stronger cross-linking in the haemolymph competing with the bead contact. A significant difference in the clot protein quantity of a mutant *Drosophila* stock or in changed buffer conditions might well represent an indirect effect on the physiology not directly related to clotting. In summary the pull-out assay is an efficient approach to isolate the haemolymph clot and even allows quick and inexpensive initial screening for clotting defects in *D. melanogaster*. However biological roles of putative coagulation factors have to be addressed using additional functional assays.

4.5 Comparison of clotting proteins in *Galleria* and *Drosophila*

Several of the identified proteins associated with a haemolymph clot in *G. mellonella* and *D. melanogaster* share similarities. Arylphorins were found in the coagulum of both *Galleria* (gallysin) and *Drosophila* (*lsp1* and *fbp1*). Similarly, lipophorin was present in clot preparations from both species. These protein belong to the most abundant haemolymph compounds and might thus be used for quick and efficient association at the wound site. In addition, lipophorin might interact with exposed phospholipids at the injury and aid in immune activation (see chapter 4.4). Also phenoloxidases were identified as integral constituents of haemolymph clots in both *Galleria* and *Drosophila*. It may be assumed that the processes clotting and melanisation are connected to each other by localised binding of phenoloxidases to the clot, which would prevent intrahaemocoelic enzymatic activity.

Nevertheless, it may seem surprising that most of the clot-associated proteins differ in both species. This may be explained in several ways: It is likely that many clotting substrates in fact are species-specific, simply because the required features for quick protein aggregation are so easy to acquire throughout evolution. This hypothesis might be confirmed by the alpha-crystallin like protein from *Galleria* and the *Drosophila* gene product of CG 15825. Alternatively, the different methods used (production of the anti-clot antiserum in *G. mellonella* and the bead assay followed by mass spectrometry analysis in *D. melanogaster*) may favour or prevent the detection of certain proteins: The clot used for production of the *Galleria* antiserum was formed *in vitro* by a cell-free haemolymph sample (Li et al., 2002). Therefore cellular coagulogens of *G. mellonella* may be missed by this antibody, while the *Drosophila* clot formed in the pull-out assay was allowed to form during approximately 20 min in the presence of haemocytes. However, also the pull-out assay might miss some clotting factors: The anti-clot antiserum may even identify clot components cross-linked by transglutaminase or phenoloxidase activity, these may be so strongly bound to the beads of the pull-out assay that they cannot be removed from them prior to gel electrophoresis and might thus not be recognised as *Drosophila* coagulation proteins.

Finally I would like to address the question why hemolectin, which is obviously important for *Drosophila* haemolymph clotting, is not represented with a homologous

protein identified in the *Galleria* coagulum. Genes with similarity to *Drosophila* hemolectin (Goto et al., 2001) exist not only in the related lepidopteran species *B. mori* (Kotani et al., 1995) and *M. sexta* (Scholz, Dissertation 2002), but also in the recently completed honey-bee genome (gene accession number "XP_395067", http://www.ncbi.nhlm.nih.gov). Therefore it appears valid to assume that a hemolectin homologue is present in *Galleria* as well. A protein with a molecular weight similar to hemolectin is a major component of the *Galleria* clot and its binding requires calcium (Li et al., 2002, Figure 3A). If this putative "*Galleria* hemocytin/hemolectin" is involved in early steps of haemolymph clotting, it might be quickly covered by interacting factors, rendering it inaccessible to antibody recognition. According to this assumption the putative *Galleria* hemolectin homologue may have been present in the *in vitro* clot, but not be recognisable by the anti-clot antiserum.

4.6 Regulation of haemolymph coagulation – preliminary results

I tested several mutants of known immune genes (for example *spätzle*, *Toll* and *necrotic*) with the pull-out assay. No obvious qualitative differences concerning bead aggregation and bead-associated proteins were observed in any of these cases (data not shown). This is not too surprising, since activation of clotting should be quick and therefore probably does not involve molecules implied in gene induction. The actual clot proteins and enzymes are rather expected to be constitutively present in the haemolymph or stored in and released from haemocyte granules. Although a wound-induced **induction of hemolectin** had been reported (Goto et al., 2001), the re-investigation described in the present study (Figure 28) led to contrary results. At all included time points (1, 6, 12 and 24 h) after poking no significant up-regulation of hemolectin as described by Goto et al. (2001) had occurred, although the same primer sequences were used in both studies. Several explanations can be given for this discrepancy: It is quite difficult to normalise the wound size of poked larvae and the resulting effect on transcriptional regulation. Therefore a comparably strong poking intensity might have led to an effective depletion of hemolectin resulting in a strong induction in the work by Goto et al., while this process was delayed or absent in the larvae used in the present study. Furthermore it remains to be shown, in which time

window hemolectin is induced. It could be assumed that if there is an induction of hemolectin transcription, this does probably not occur during the first hours after poking and can thus be considered a haemostatic regulation of haemolymph levels.

In an extensive study the effect of several vertebrate blood clotting inhibitors on the morphological appearance of insect haemolymph coagulation has been tested (Grégoire, 1953). An important side result of this investigation is the observation that use of different surfaces like e. g. hydrophobic and non-adhesive material or coated glass slides did not have any impact on clot formation. Inhibitory effects of some of the tested chemicals should be interpreted carefully, since they caused damage of cellular structures in general. Furthermore clotting may still occur at wound sites *in vivo* as well as at the edges of a sample haemolymph drop or around an incorporated particle even in the presence of inhibitory substances, due to the enormous speed of the reaction (Beard, 1950 and personal observation). Nevertheless, some of the tested chemicals significantly reduced coagulation in relatively low concentrations. These compounds included reducing agents, chelators of calcium ions and organic esters of sulphuric acid. Since the morphological appearance of haemolymph clots is highly variable (Grégoire, 1953), it is difficult to standardise these results.

I aimed to test the effect of **putative coagulation inhibitors** on the protein composition of the clot retrieved with the pull-out assay, which is shown in Figure 29. In general calcium requirement for the clotting process seems to be a common feature conserved from invertebrates to vertebrates. This is also supported by the effect of calcium ionophores on microparticle formation (Figure 11). Similarly even *Galleria* haemolymph incubated in a buffer excluding calcium and including EDTA showed a less extensive cross-linking reaction leading to lower levels of high molecular weight complexes (Li et al., 2002). Therefore reaction buffers lacking calcium and including EDTA may be used to inhibit coagulation *in vitro*. Another compound with a clear inhibiting effect on insect haemolymph clotting is the antioxidant ascorbic acid (Figure 29 and Gawa Bidla, unpublished results). It was demonstrated earlier that the aminophospholipid translocase inhibitor and vertebrate anticoagulant suramin also obstructs insect haemolymph coagulation (Grégoire, 1953). Thus an involvement of inner leaflet phospholipids in initiation of the clotting process seems likely in insects as in vertebrates and horseshoe crabs (Nakamura et al., 1998). Preliminary results from

D. melanogaster and *G. mellonella* also point towards an activation of melanisation through phosphatidylserine exposure (Gawa Bidla, unpublished results). In vertebrates vWF stimulates phosphatidylserine exposure on platelets (Briede et al., 2003). It is tempting to speculate on a similar regulating mechanism for hemolectin as an initial regulator molecule for clotting. A localised higher concentration of activating factors or the cross-linking enzymes might represent a way to restrict coagulation to the wound site. Hemolectin as a large protein with many accessible interaction domains may furthermore serve a function as a reaction platform for other clotting factors and bring interacting molecules into close proximity.

Presently it is not yet possible to organise the different clotting proteins of insects into an array suggesting a regulation pathway. Another question concerns the biochemical nature of the regulation mechanisms that check coagulation in a temporal and spatial manner to prevent over-activation of the reaction. One theoretical possibility is the separation of a cellular and a plasma coagulogen, as proposed by Bohn (1986). An alternative explanation could be the presence of inactive pro-forms of the proteins that undergo proteolytic cleavage or conformational change during the initiation of clotting. As described earlier, **proteolytic cascades** regulated by protease inhibitors are employed by vertebrates and horseshoe crabs, while no such regulation has been found in crustaceans so far. Proteases involved in the different cascades might have formed through quick evolution including domain switching of protease domains (see chapter 4.7). Although there is no clear experimental evidence for insects, a proteolytic activation of insect clotting has been repeatedly assumed (Hoffmann & Reichhart, 2002, Tzou et al., 2002), probably because regulation of the prophenoloxidase cascade by serine proteases is already well accepted. Evidence for the general importance of proteases is the large number of serine protease genes in the *Drosophila* genome (Tzou et al., 2002). Activation of clotting by the serine protease scolexin has been proposed for *M. sexta* (Finnerty et al., 1999). The data collected during the present study did not lead to a clear conclusion about a possible role for proteases in *Drosophila* haemolymph coagulation. The pull-out assay was performed in the presence of a protease inhibitor cocktail in additional experiments, which did not lead to qualitative, but possibly some quantitative changes in the protein binding pattern on the beads (not shown).

Even if the primary clotting process is independent of proteolytic cleavage, observations in homozygous ***serpin-27A*** mutant larvae point at least towards an influence of proteases on coagulation. The gene product of *serpin-27A* negatively limits an activating enzyme in the prophenoloxidase cascade, thus restricting phenoloxidase activity to the wound (De Gregorio et al., 2002, Ligoxygakis et al., 2002). It is also required for establishment of the dorsoventral polarity in *Drosophila* embryos (Hashimoto et al., 2003), reminiscent of the dual function of the Toll receptor in immunity and embryonic development. Since there is an orthologue of *serpin-27A* in *M. sexta* (Zhu et al., 2003), prophenoloxidase regulation by this inhibitor might represent an evolutionary conserved feature. Haemolymph from homozygous mutant *serpin-27A Drosophila* larvae is able to form a clot *in vitro*, though its morphology is changed with a more "spread veil" structure compared to wildtype haemolymph samples (Gawa Bidla, unpublished results). ***Black cells (Bc)*** mutants of *D. melanogaster* lack haemolymph phenoloxidase activity due to inappropriate intracellular activation of the enzyme in the crystal cells. *Black cells* mutants must still possess cuticular prophenoloxidase and other phenoloxidase-like activities (like for example laccases), since cuticular sclerotisation is not affected. Wounds in the integument of *Black cells* larvae often melanise normally (personal observation). Binding of haemolymph proteins from single mutations of *serpin-27A* and *Bc* to the beads did not apparently differ from wildtype samples (Figure 30). Surprisingly a **double mutation *Black cells / serpin-27A*** had an impact on the aggregation ability of the beads (not shown) and led to a lower hemolectin level retrieved from them (Figure 30). Since the double mutation caused melanotic aggregation of haemocytes *in vivo* and an apparently lower haemolymph amount (personal observation), haemolymph from 30 larvae were used from this stock. Nevertheless the difference in the hemolectin amount is still visible. It is probable that proteolytic activation is not a common feature of all phenoloxidases, since the enzyme CG 8193 found in the pull-out clot does not possess a conserved cleavage site. If this is the case, the function of these differently activated phenoloxidases may be not affected in the *serpin-27A* mutant. In the double mutant the combined effect of two mutations causing over-reactive and delocalised phenoloxidase activity might thus result in a deregulated melanisation phenotype in the haemolymph influencing appearance of the coagulum and possibly

clot composition. This explanation is supported by microscopical observations of haemocyte preparations from the *Black cells* / *serpin-27A* double mutant, which show protein aggregation in proximity to haemocytes, but a less abundant fibre network in the plasma (personal observation). It is difficult to explain the strong effect of a double mutation *Bc/serpin-27A* on the amount of hemolectin isolated from the beads, especially considering the fact that other proteins of the clot seem to be largely unaffected (Figure 30). It could be hypothesised that this reflects a secondary effect of a generally reduced number of circulating haemocytes. A hypersensitive reaction at wound sites and intravascular coagulation caused by circulating clotting factors is avoided in vertebrates by anticoagulants. The serpin described here might represent such a regulating factor in insects.

Both phenoloxidase and transglutaminase activity seem to influence hardening of the clot. Interestingly the single transglutaminase gene in *Drosophila* displays its highest similarity not with the factor XIII in vertebrate blood clotting, but with the tissue type transglutaminase in vertebrates, which amongst others cross-links proteins during basement membrane formation. Vertebrate parasites express transglutaminases to cross-link host basement membrane proteins on their surfaces to block vertebrate host immunity ("molecular mimicry"). This enzymatic activity is crucial for the development of *Plasmodium malariae* oocysts (Adini et al., 2001) and parasitic nematodes (Chandrashekar & Mehta, 2000). While the exact mechanism of this host immune inhibition is not clear, it is reminiscent of the lipophorin deposition on endoparasitoid wasp eggs to block a defence reaction in insect hosts (Kinuthia et al., 1999). The cross-linking activities might therefore not only be important for clot hardening but furthermore block putative inflammatory characteristics of the coagulum to prevent an over-activation of the immune system. Vertebrate clotting factor XIII not only has a function in final cross-linking of fibrin, but also enhances phosphatidylserine exposure (Bratton, 1993) and stimulates phospholipase A_2 (Miele, 2003), thereby playing a key role in inflammation processes. Phospholipase A_2 is also induced in *Drosophila* after infections (Irving et al., 2001). As in vertebrates *Drosophila* transglutaminase may lead to phosphatidylserine exposure and eicosanoid production. The interplay between phenoloxidase and transglutaminase activity is not investigated so far. However, synthetic melanin was recently shown to inhibit a vertebrate transglutaminase *in vitro*

(Ikura et al., 2002). The authors of this report claim that the inhibition mechanism is universal for all transglutaminases. If this regulation should prove to be valid even for *Drosophila* and *in vivo*, phenoloxidase may act at later steps of cross-linking than transglutaminase, inhibiting inflammatory processes.

From the knowledge available so far I propose the following model for *Drosophila* haemolymph clotting: In an initiation phase the wound must be sensed to activate the clotting process (Figure 32 A). The activation needs to be quick and yet needs to possess a certain amount of specificity, avoiding haemolymph clotting throughout the body cavity. So far it can only be speculated, which factors may be required for this initiation. Since clotting occurs more frequently at the edges of cover slips on a haemolymph preparation than in the centre of a sample (personal observation), an involvement of oxygen seems likely. Alternatively released intracellular components and ruptured membranes of injured tissue as well as exposed negatively charged phospholipids on apoptotic haemocytes at the wound site might be recognised by clotting factors or additional haemocytes.

Clotting might then start by degranulating or rupturing haemocytes and probably primary aggregation of proteins at the wound site (Figure 32 B). In similarity to vWF, hemolectin has the ability to form large aggregates and may thus represent one of the primary clotting factors in *Drosophila*. In addition the hemolectin sequence includes an autocatalytic cleavage site GDPH (Figure 31), which is conserved in the honey-bee homologue (gene accession number "XP_395067", http://www.ncbi.nhlm.nih.gov) and also present in vertebrate mucins (Lidell et al., 2003). It was described that cleavage of this motif exposes a new C-terminal end of the mucins, which in turn attaches the proteins to surfaces and to each other (Lidell et al., 2003). One might speculate that wound reactions could cause a similar self-activation of hemolectin. However, vertebrate vWF and *B. mori* hemocytin lack this autocleavage site and at least for vWF a conformational change is known to lead to aggregation.

The primary clot may be strengthened and cross-linked by enzymatic activities like phenoloxidases and transglutaminase released from the haemocytes and thereby locally concentrated at the wound site (Figure 32 C).

Finally an unregulated clotting reaction would be detrimental to the organism. Therefore the process has to be terminated effectively, for which several possible regulatory mechanisms might be considered (Figure 32 D). An activating signal from the wound site could be weakened through the forming clot layers or the clotting factors and enzymes may be used up from the haemolymph. Cross-linking of clotting factors as an integral part of the reaction might terminate the reaction. However, wound scars are often not completely melanised (personal observation) and therefore a strict regulation of the clotting process solely through phenoloxidase activity is probably not the case. Additional clot-associating proteins may stop the process effectively as well, in analogy to anticoagulants in the vertebrate system. Possible candidates for such a regulation in insect haemostasis are annexins and lipophorin, which have been discussed earlier.

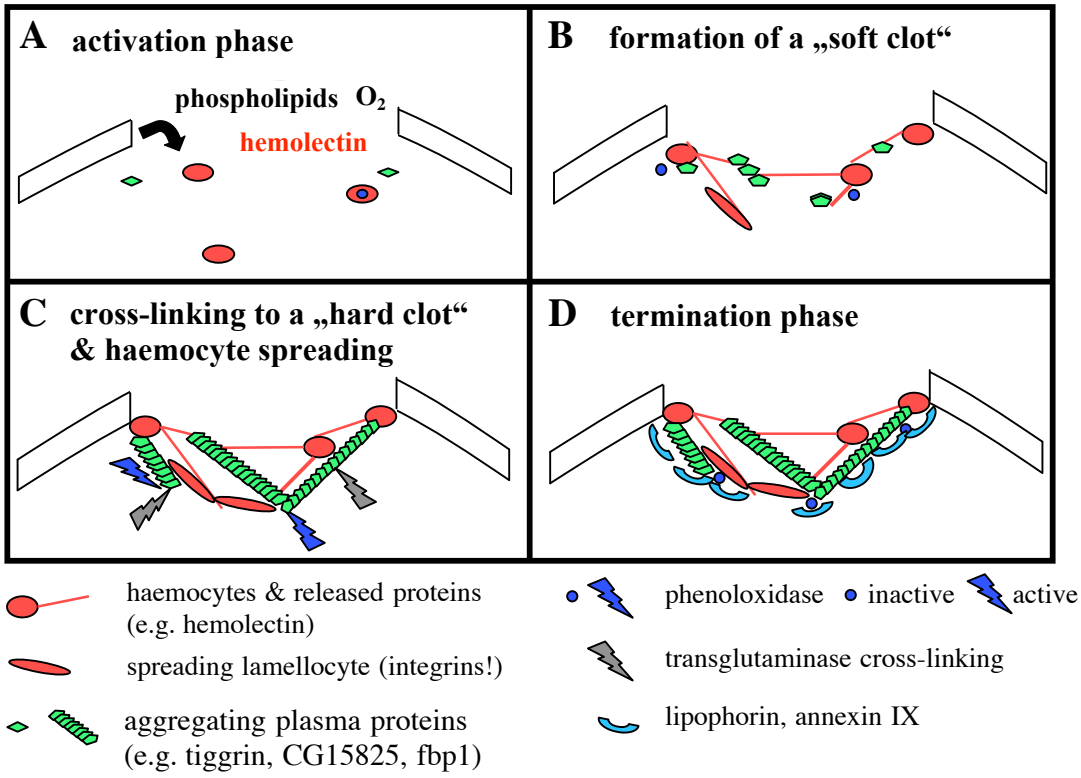

Figure 32: A four-step model for haemolymph coagulation in *Drosophila*
(for further explanation see body text)

4.7 Considerations regarding the evolution of clotting factors

At what point during evolution did the need for coagulation emerge? Which components did such an ancient clotting system include? Which characteristics of haemostasis proved to be successful and are present in different organism groups? Unicellular organisms possess powerful regenerative properties. These are not specialised for wound healing, but rather part of general cytokinetic processes and might be similar to actin polymerisation and tightening of "actomyosin purse strings" around "wounds" in single cells (Henson et al., 2002). Ancient multicellular organisms with low body pressure probably close wounds simply by contractions. Even in vertebrates myofibroblasts cause cellular contractions, which play a key role in an initial compaction of the wounded area (Racine-Samson et al., 1997). The formation of a new epidermis and spreading of epithelial cells may be an evolutionary old mechanism, as suggested by Galko & Krasnow (2004). As soon as a primary body cavity developed, these rather slow reactions were probably not sufficient anymore and injuries had to be sealed by cellular plugs or coagulated body fluid. The evidence available so far suggests that the cellular contribution to clot formation is more ancient and conserved than coagulation of plasma proteins, as pointed out by Aird (2003). In this context it should be noted once more that blood cell attachment is the primary wound reaction in both vertebrates and invertebrates and even occurs more immediately than protein aggregation. In leeches haemocytes and other cells simply stick together to close off wounds (de Euguileor et al., 1999). Both haemocyte aggregation and degranulation occur in molluscs, although the matrix around cell clumps is formed much later than observed in insects, namely 24 h after injury (Franchini & Ottaviani, 2000).

The requirement for quicker clotting of body fluids possibly occurred in connection with the evolution of the deuterocoele. This included the formation of smaller haemolymph vessels with higher internal pressure as derivates of the primary body cavity. At this point a simple protein interaction of a clotting enzyme and one or more substrates might have been sufficient. It is tempting to hypothesise that an already existing transglutaminase took over this function. Since the specificity of this enzyme is not restricted to any conserved sequence motif, the identity of its substrates might be highly variable as observed (Korayem et al., unpublished results). One could speculate

that an easy way of acquiring new transglutaminase substrates may be to make use of pre-existing abundant plasma proteins. General features of coagulation (phospholipids and microparticle formation, glycoproteins stored in intracellular granules) have probably been conserved throughout evolution, but the actual proteins incorporated into the clot seem to have evolved quickly by expansion of certain DNA elements, gene duplication, exon shuffling and domain switching between proteins.

Sequence comparison between clotting factors of the discussed arthropod groups and vertebrates does not reveal any orthologues except **transglutaminase**, which participates in cross-linking of the clot in vertebrates (Griffin et al., 2002, Jiang & Doolittle, 2003), horseshoe crabs, crustaceans and seemingly as well in insects (Korayem et al., unpublished results). Interestingly cross-linking of amino-groups of the plasma coagulogen with carboxamide groups of the haemocyte coagulogen of a cockroach by a transglutaminase-like activity has been described earlier (Ferstl et al., 1989). However, one has to keep in mind that the *Drosophila* genome includes only one transglutaminase gene, but eight genes are present in the human genome (Lorand & Graham, 2003). This suggests that the vertebrate clotting factor XIII has specialised to perform its action in blood clotting, while the invertebrate enzymes probably fulfil various physiological functions. Prophenoloxidases are present in insects and crustaceans and even the related hemocyanins of crustaceans, horseshoe crabs and a spider (Decker & Rinke, 1998) possess an intrinsic phenoloxidase activity. Hence it is possible, that in addition to transglutaminases phenoloxidases evolved to provide an alternative cross-linking activity in arthropod haemolymph.

Many vertebrate vascular proteins like vWF, fibronectin, vitronectin and fibrinogen have no orthologues in *Drosophila*, probably due to specialised requirements of the closed circulatory system of vertebrates (Hynes & Zhao, 2000). Other proteins involved in clotting in the reviewed organism groups share conserved sequence stretches like serine protease or vWF domains, but cannot be considered orthologues. Nevertheless, a conservation of all domains of a certain protein might not even be required for effective clotting. New transglutaminase substrates may easily emerge from an ancestral protein by insertion of **glutamine- and lysine-rich patterns**. The crayfish clotting protein, which is similar to some insects' female-specific vitellogenins, could be regarded a good example for this model. In addition to vitellogenin-like sequence stretches

(including vWF D-domains) the crustacean clotting protein contains lysine- and glutamine-rich segments. Although not regarded a clotting factor so far, a glutamine-rich "early stage encapsulation protein" was identified in the beetle *Tenebrio molitor* (Cho et al., 1999) and is a good candidate for a transglutaminase substrate. In the same insect species a vitellogenin-like protein with high glutamine content enhances phenoloxidase activity (Lee et al., 2000). Interestingly, in addition to its role as a yolk protein precursor vitellogenin was also proposed to be a clotting protein in sea urchins (Cervello et al., 1994). Thus it cannot be excluded that vitellogenins may have a conserved function in the coagulation process due to intrinsic aggregation properties. In crayfish an α_2-macroglobulin is linked to the clot by transglutaminase and regulates protease activity at the wound site (Hall & Söderhäll, 1994). Even the amino acid sequence of this protein includes numerous exposed lysine and glutamine residues. A protein with high similarity to α_2-macroglobulin is present in *Drosophila* as well (CG 7052) and might fulfil a similar functions, in case it proves to be an integral part of the coagulum.

Proteins suitable for clotting frequently form large multimers, enhancing their "sticky" character (Furlan, 2002). Sequences with similarity to the **vWF D-domain** are present in several crayfish coagulogens, the insect proteins hemocytin, hemolectin, lipophorin as well as vertebrate vWF itself. The vWF D-like domains in vertebrates are flanked by the CGLCG pattern that facilitates self-catalysed association of vWF (Mayadas & Wagner, 1992). A truncated GLCG motif was reported to account for the adhesive properties of the structural glue protein spiggin from a fish (Jones et al., 2001). Intriguingly five GLCG patterns are present in *Drosophila* hemolectin (Figure 31) and all five are conserved in the homologue from *Apis mellifera* (gene accession number "XP_395067", http://www.ncbi.nhlm.nih.gov). Three of these GLCG motifs are present in the similar proteins of the lepidopteran insects *M. sexta* and *B. mori* and the missing two are situated in the N-terminal end of the *Drosophila* protein, which is deleted in these species. The strong conservation of the pattern between three different insect orders suggests a functional aspect of this motif. A sequence GGLG(Y) with similar hydrophobic side chains accounts for "sticky" protein properties in mussel byssus threads, in chorion and other fibrillar proteins of insects as well as in spider dragline silk (Robson et al., 1993). The tendency of a protein to self-aggregate might be acquired

easily by mutations and duplications that lead to these sequence motifs. Similarly the **cell adhesion motifs RGD, KGD and LRE** of clotting proteins like hemolectin may have evolved quickly and enhance haemocyte adhesion and immune reactions at the wound site. Another remarkable feature of hemolectin is the high abundance of 305 cysteine residues exceeding 7,9 % of the complete sequence, compared to an average 1,7 % for all proteins (McCaldon & Argos, 1988). In both hemolectin and vWF cysteines represent the most abundant amino acid. Cysteine residues have been proposed to play an important role in vWF multimerisation (Mayadas & Wagner, 1992).

It was calculated that the aggregation ability of **vertebrate fibrinogen** developed during approximately 50 million years (Doolittle et al., 1997). Fibrinogen is the only self-associating and peptide-binding member of an otherwise lectin-like protein family, the ficolins, including horseshoe crab tachylectin (Kairies et al., 2001) and a number of fibrinogen-like proteins in *Drosophila* and *Anopheles* (Christophides et al., 2002; Zdobnov et al., 2002). Ficolins consist of a lectin-like and a collagen-like domain and are involved in the lectin-complement pathway in vertebrates (Fujita, 2002). In the mosquito *Armigeres subalbatus* a ficolin-like lectin has recently been implicated in insect immunity as a pattern recognition molecule (Wang et al., 2004). It might thus be speculated that the ancestor protein of fibrinogen was rather characterised by its ability to bind pathogen-associated glycodeterminants. Furthermore it is interesting to note that casein, responsible for milk clotting, though not phylogenetically related, shares structural similarity with fibrinogen (Jolles et al., 1978).

Clotting systems of vertebrates and horseshoe crabs independently evolved regulatory **serine protease cascades** to ensure a localised reaction. Evolution of the vertebrate clotting system by relatively recent gene duplication events can be assumed, since lower chordates (Jiang & Doolittle, 2003) and jawless vertebrates (Davidson et al., 2003) only possess a simplified cascade. An ancestral protease cascade conserved from protostomes to deuterostomes as proposed by Krem & Di Cera (2002) is thus unlikely from today's view. Even in insects evolution of serine proteases was calculated to have occurred in comparably short geological time periods (Ross et al., 2003). Moreover, the seemingly evolutionary ancient transglutaminase is the only protein in the vertebrate cascade that is not a serine protease. The crustacean clotting enzyme is not

proteolytically activated and whether insect coagulation involves such a regulation remains to be investigated. Nevertheless, a protease in connection with the insect prophenoloxidase cascade might regulate both clotting and melanisation, like in horseshoe crabs (Nagai & Kawabata, 2000).

Arthropods possess an open circulatory system and thus may afford a more extensive coagulation reaction than vertebrates, which have to keep clotting much more localised to avoid thromboses. Potentially quicker spreading of microorganisms through the haemolymph to inner organs might also lead to a stronger need for effective immobilisation of potential pathogens in arthropod clots (Theopold et al., 2004). Future studies on coagulation in invertebrates with closed circulatory systems like annelids or cephalopods would therefore be interesting from an evolutionary point of view. In general comparative investigations of clotting systems in different insect species might provide insights into the adaptation of coagulation to a certain environment. A first hint towards such diversity is given by the different degree of hemolectin conservation in the genome of the hymenopteran insect *Apis mellifera* and the dipteran *D. melanogaster* compared to the lepidopterans *B. mori* and *M. sexta*. It may be an attractive approach to compare clotting in water-living insects like larvae of dragonflies or *Chironomus tentans* with marine but non-insect species like horseshoe crabs and crustaceans to clarify the requirements of coagulation in an aqueous environment.

Finally some sawfly species (Tenthredinidae, Hymenoptera) have extremely sensitive integuments and bleed easily as part of an external defence mechanism by toxic haemolymph compounds (Boéve & Schaffner, 2003). Similarly in some insect orders "reflex bleeding" is characterised by localised sensitive regions of the integument (Holloway et al., 1993). It may be assumed that extensive bleeding requires an especially powerful regulation of the clotting system. In agreement with this theory easy-bleeding sawfly species suck back haemolymph into the body, heal wounds within minutes and recover their normal body weight even after repeated haemolymph loss (Boéve, unpublished results).

5 Conclusion and Future Perspectives

The aim of this dissertation was to identify and characterise haemolymph clotting factors in insects. Starting from an almost exclusively morphological description of the haemolymph coagulum, several putative clot proteins of two insect species, *Galleria mellonella* and *Drosophila melanogaster* have been studied.

A functional involvement of some molecules like *Drosophila* hemomucin remains largely unclear and has to be further investigated. The data collected in the present study underline earlier assumptions of broad biological functions for this protein in immunity (microparticle formation, pathogen entrapment in the clot) and development (e. g. possibly trachea formation). Therefore the approach chosen here to knock down hemomucin *in vivo* at different developmental time stages and in specific organs by RNA interference still seems valuable. Since the synthesis of a DNA-construct for *in vivo* RNAi of hemomucin was not successful, one can still only speculate about the specific roles of this protein.

Anti-clot antiserum and pull-out assay independently led to the identification of lipophorin and prophenoloxidase as clot components. Together with the enzyme transglutaminase these proteins might represent more conserved constituents in insect haemolymph coagulation. Even arylphorins of both species, are candidate clotting factors with analogous counterparts in *D. melanogaster* and *G. mellonella*, including the *Galleria* gallysin, *Drosophila* larval serum protein 1 (*lsp1*) and fat body protein 1 (*fbp1*). In contrast, relatively high variability may be expected for transglutaminase substrates in insects. In agreement with this idea, two species-specific putative substrates of the enzyme, the -crystallin-like *Galleria* protein and the *Drosophila* CG 15825 gene product, evolved independently. In summary, the evolution of insect clotting proteins seems to be characterised by both conservative traits (e. g. vWF D-like domains, transglutaminase) and quick development of new species-specific elements by evolutionary tinkering (glutamine residues in e. g. CG 15825; -crystallin).

The factors mentioned in the previous paragraph allow only vague assumptions for the molecular mechanisms underlying the initial steps of haemolymph coagulation. Self-aggregation of proteins due to certain sequence characteristics such as vWF domains and glutamine residues, seems to be a likely explanation for quick clot formation. *Drosophila* hemolectin is a good candidate for one of the first clot proteins attaching to a wound and its domain structure suggests interactions with various additional factors. An *in vivo* RNAi knock-down of hemolectin led to a clearly affected clotting process. *Drosophila* tiggrin and other RGD-containing proteins in the coagulum possibly represent the link to the haemocytes that assist in closing the wound and stimulate formation of the new epithelium.

In addition, it becomes increasingly clear that both transglutaminase and phenoloxidase are involved in later steps of clot formation and strengthen the primary coagulum by cross-linking of proteins. The interplay of these enzymatic activities is still largely unexplored and an investigation of their crosstalk may provide insights into their probably dissimilar roles in clot formation.

Drosophila represents an ideal model organism to study insect haemolymph clotting, since genetic tools and a huge number of available mutants will facilitate a detailed description of the coagulation process and its role in the immune system of the fly. Simultaneously evolutionary questions may rather be successfully addressed by comparing clotting proteins of different insect orders and species. In the near future the putative clotting factors described here may be investigated more specifically for their functions as well as for their interactions with each other. A redundancy of multiple possible ligands is already commonly accepted for components of the vertebrate coagulum (Furlan, 2002), but remains to be shown for insects. Similarly many clotting defects in insects may turn out to be of a quite moderate nature due to overlapping enzymatic cross-linking activities and possibly even flexibility in the spectrum of covalently linked substrate proteins.

5 Zusammenfassung und Zukunftsperspektiven

Die Identifikation und Charakterisierung von Gerinnungsfaktoren in der Hämolymphe von Insekten war Ziel der vorliegenden Dissertation. Auf Grundlage einer bisher nahezu ausschließlich morphologischen Beschreibung des Koagulums wurden mehrere potentiell an diesem Prozess beteiligte Proteine der beiden Insektenarten *Galleria mellonella* und *Drosophila melanogaster* näher studiert.

Die funktionelle Bedeutung einiger Moleküle wie z. B. *Drosophila* Hämomucin bleibt weiterhin weitgehend unklar. Da die Synthese eines DNA-Konstruktes für RNA-Interferenz *in vivo* nicht erfolgreich verlief, kann über Funktionen von Hämomucin weiterhin nur spekuliert werden. Die vorliegenden Daten unterstützen frühere Annahmen, nach denen dieses Protein an mehreren biologischen Funktionen wie Immunität (Mikropartikel-Bildung und Demobilisierung von Pathogenen im Clot) und Entwicklung (z. B. möglicherweise Bildung der Tracheen) beteiligt ist. Daher erscheint der gewählte Ansatz, die Expression von Hämomucin zu unterschiedlichen Entwicklungsstadien und in bestimmten Organen zu beeinträchtigen, weiterhin Erfolg versprechend.

Das Anti-clot-Antiserum und der Pull-out-Assay führten unabhängig voneinander zur Identifikation von Lipophorin und Phenoloxidase als Bestandteile eines Clots bei den beiden untersuchten Insektenarten. Zusammen mit Transglutaminase repräsentieren diese Proteine möglicherweise die konservierten Bestandteile der Hämolymph-Koagulation in Insekten. Auch Arylphorine sind mögliche Kandidaten für Gerinnungsfaktoren mit Analogen in unterschiedlichen Insektenarten, identifiziert wurden u. a. das *Galleria* Gallysin, sowie *Drosophila* Larvales Serumprotein 1 (*lsp1*) und Fettkörperprotein 1 (*fbp1*). Im Gegensatz hierzu kann für Transglutaminase-Substrate in Insekten eine relativ hohe Variabilität angenommen werden. In Übereinstimmung mit dieser Ansicht haben sich zwei vermutlich artspezifische Substrate des Enzyms unabhängig voneinander entwickelt, das den -Kristallinen ähnliche *Galleria* Protein und das Produkt des *Drosophila* CG 15825-Genes. Die Evolution von Clotting-Proteinen der Insekten scheint sowohl durch sowohl konservative Züge (z. B. vWF-D-ähnliche Domänen, Transglutaminase) als auch durch schnelle Entwicklung neuer artspezifischer Elemente durch hohe Variation innerhalb

kurzer Zeiträume (Expansion von Glutaminresten in CG 15825 und β-Kristallin) gekennzeichnet zu sein.

Die zuvor genannten Faktoren erlauben nur erste Annahmen über molekulare Mechanismen als Grundlage der ersten Schritte in der Hämolymph-Koagulation. Selbstaggregation von Proteinen aufgrund bestimmter Sequenzmerkmale, wie vWF-Domänen und Glutaminreste, scheint eine gute Erklärung für die schnelle Bildung eines Hämolymph-Clots zu sein. *Drosophila* Hämolektin ist ein guter Kandidat für eines der primären wund-assoziierten Clotting-Proteine und die Domänenstruktur des Proteins legt Interaktionen mit zahlreichen weiteren Faktoren nahe. RNA-Interferenz *in vivo* mit Hämolektin führte zu einer deutlichen Beeinträchtigung des Koagulationsprozesses. *Drosophila* Tiggrin und andere Proteine mit einem RGD-Motiv stellen möglicherweise die Verbindung des Clots zu den Hämozyten her, welche die Wundheilung fördern und die Neubildung des Epitheliums anregen.

Es wird zunehmend deutlich, dass Transglutaminase und Phenoloxidase an späteren Schritten der Clotbildung beteiligt sind und das primäre Koagulum durch Kreuzvernetzung von Proteinen verstärken. Das Zusammenspiel dieser Enzymaktivitäten bleibt weitestgehend unerforscht und eine nähere Analyse ihrer wechselseitigen Regulation wird möglicherweise Aufschluss über ihre vermutlich unterschiedlichen Rollen im Gerinnungsprozess geben.

Drosophila ist ein viel versprechender Modellorganismus für die Studie der Hämolymphgerinnung in Insekten, da genetische Methoden und eine große Anzahl verfügbarer Mutanten eine detaillierte Untersuchung des Gerinnungsprozesses und seiner Rolle im Immunsystem der Fliege ermöglichen. Durch weitere Arbeiten werden den hier beschriebenen Faktoren spezifischere Funktionen zugewiesen werden und ihre Interaktionen untereinander analysiert werden können. In Übereinstimmung mit dem hier präsentierten Konzept könnten viele Gerinnungsstörungen in Insekten aufgrund von leicht überlappenden Kreuzvernetzungsaktivitäten und möglicherweise ebenfalls Flexibilität ihrer Substrate von eher gemäßigter Natur sein. Eine Redundanz der vielfältigen möglichen Liganden ist für Bestandteile des Vertebraten-Koagulums bereits akzeptiert (Furlan, 2002).

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