Proteomic Analysis of the Drosophila Larval Hemolymph Clot*

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Components of the insect clot, an extremely rapid forming and critical part of insect immunity, are just beginning to be identified (1). Here we present a proteomic comparison of larval hemolymph before and after clotting to learn more about this process. This approach was supplemented by the identification of substrates for the enzyme transglutaminase, which plays a role in both vertebrate blood clotting (as factor XIIIa) and hemolymph coagulation in arthropods. Hemolymph proteins present in lower amounts after clotting include CG8502 (a protein with a mucin-type domain and a domain with similarity to cuticular components), CG11313 (a protein with similarity to prophenoloxidase-activating proteases), and two phenoloxidases, lipophorin, a secreted gelsolin, and CG15825, which had previously been isolated from clots (2). Proteins whose levels increase after clotting include a ferritin-subunit and two members of the immunoglobulin family with a high similarity to the small immunoglobulin-like molecules involved in mammalian innate immunity. Our results correlate with findings from another study of coagulation (2) that involved a different experimental approach. Proteomics allows the isolation of novel candidate clotting factors, leading to a more complete picture of clotting. In addition, our two-dimensional protein map of cell-free Drosophila hemolymph includes many additional proteins that were not found in studies performed on whole hemolymph.

Drosophila is a useful model system for the study of innate immunity (3, 4). The humoral activity and induction of antimicrobial peptides in adults have attracted much attention (5). Increasingly though, cellular reactions are being studied thanks to the availability of cell lines with similarity to hemocytes and the establishment of high throughput techniques and large scale mutagenesis projects (6–8).

Whole genome induction studies have confirmed the presence of several immune induction pathways, which differentially contribute to responses against different classes of microorganisms, different phases of an immune reaction, and cellular versus humoral immunity (9–11). Genes involved in antimicrobial responses can be grouped according to their induction pattern using cluster analysis (11, 12). More recently, the systemic immune response has been studied at a posttranscriptional level using proteomics (13–16), summarized in Ref. 17. These studies were facilitated by the establishment of two-dimensional protein maps of the larval hemolymph (18, 19). Hemolymph samples at different times after infection or the addition of immune elicitors were compared with unchallenged controls. Known immune proteins as well as candidate immune proteins were identified, including proteins with similarity to vertebrate complement components (thiol ester motif-containing proteins) (13, 14), a pattern recognition protein (β-glucan binding protein) (14), several serine proteases, serine protease inhibitors (14), and metabolic proteins (13–15), summarized in Ref. 17.

Here we describe a proteomic approach for the identification of proteins involved in the Drosophila larval hemolymph clotting reaction, which is essential for both sealing wounds to avoid loss of hemolymph (hemostasis) and preventing dissemination of microbes into the hemocoel. Proteomics provides a particularly suitable means to study clotting that involves the fast interaction of cellular and humoral proteins, resulting in the precipitation of an insoluble matrix independent of and preceding changes in gene expression. This precipitation leads to a soft clot, which is hardened through the activity of cross-linking enzymes (summarized in Ref. 1). Candidates for cross-linking enzymes include phenoloxidase and transglutaminase. The formation of the soft clot is independent of hemolymph phenoloxidase (2), but the enzyme participates at later stages, leading to visible melanization of the clot (summarized in Ref. 1). Transglutaminases mediate covalent cross-linking between primary amines (e.g. a lysine residue) and glutamine residues (20). Clotting factor XIIIa, which is a transglutaminase, cross-links the vertebrate blood clot. Transglutaminases are also involved in clotting in non-insect arthropods (1, 21).

Our experimental approach in this study was to compare larval hemolymph before and after clotting by proteomics. In contrast to previous (immune) proteomic studies, we did not challenge larvae with microbial elicitors, and we removed hemocytes to focus on changes in the hemolymph after clotting. In addition to this comparison, we also identified transglutaminase substrates as a means to highlight clotting factors. Our results confirm and extend previous studies in the lepidopteran Galleria mellonella (21) and Drosophila melanogaster (2) that involved direct isolation of the clot and subsequent identification of its components. They provide further molecular insight into clotting and the events in hemolymph after wounding.
These events are the first line of defense against intruding microbes and set the stage for subsequent immune reactions.

**EXPERIMENTAL PROCEDURES**

Flies—Drosophila w1118 cultures were kept at 18 °C in a 12-h light and 12-h dark cycle on standard cornmeal sucrose medium. Third instar larvae were used in this study.

Pull-out Assay—To obtain a non-clotted plasma fraction (Fig. 3A), 10 animals were bled directly into anticoagulant Ringer’s solution (Drosophila Ringer’s solution containing 0.02 mM EDTA and no CaCl₂). In a first attempt to obtain clotted serum, animals were opened, left for 30 s, and then placed on an electron microscope grid (90-μm pore width), which acted as a filter to remove the clot. The flow-through fraction (serum) was collected in a capillary placed underneath the grid. Both samples were subsequently centrifuged for 10 min, first at 2000 × g to remove hemocytes and then at 11,000 × g and prepared for PAGE analysis. Comparison between the samples revealed that the differences between them were not sufficiently reproducible to allow a proteomics approach. Therefore, clotting was performed as part of a pull-out reaction (2) followed by centrifugation as described above. Briefly, the pull-out assay relies on the incorporation of paramagnetic beads into the clot and the subsequent removal of the clot with a magnet. The proteins in the remaining supernatant (serum) were precipitated with ice-cold chloroform: methanol and washed twice with ice-cold methanol. The pellet was dried in a vacuum centrifuge and dissolved in two-dimensional sample buffer (2-D Sample Preparation for Insoluble Proteins, catalog number 89886, Pierce). After 1 h the sample was desalted with the two-dimensional sample kit according to kit instructions.

Protein spots were matched automatically across three gels from ages Analyzer LAS 1000 plus and analyzed with the PDQuest software. Further confimation of protein identifications was obtained using Profound software. Searches were performed using the Mascot (Matrix Science; www.matrixscience.com/) and ProFound (prowl.rockefeller.edu/) engines.

![Fig. 1. The Drosophila hemolymph clot. A and B, hemolymph was prepared as described (2) and examined using phase-contrast microscopy (A) and labeling with fluorescein isothiocyanate-conjugated peanut agglutinin (B). C, the pull-out method. Proteins binding to the beads prepared by the pull-out method were analyzed using PAGE on a 10% gel and Coomassie stained. The supernatant from the first pull-out was added to new beads in three subsequent reactions that were analyzed both for their capacity to aggregate (aggr) beads (+) indicates a strong reaction and (+) a very weak aggregation in the second pull-out) and by PAGE. An aliquot of the remaining supernatant after the last reaction was collected in the gel (rest). The presence of several protein bands correlates with bead aggregation (the CG15825 product that was also identified by proteomics (this work) as a clot component is indicated by an asterisk; see (2) for details).](http://www.jbc.org/)

in-gel with sequencing grade-modified trypsin (Promega V511A) as described in (24), except that the reduction and alkylation steps were omitted because cysteines were carbamidomethylated on the equilibration step of two-dimensional gel electrophoresis. Coomassie-stained samples were destained in 0.025 M NH₄HCO₃, dehydrated in CH₃CN, and centrifuged in a vacuum centrifuge to complete dryness. Peptides were extracted in 50% CH₃CN and 5% CF₃COOH, dried in a vacuum centrifuge, and resuspended in 50% CH₃CN and 0.1% CF₃COOH. Silver-stained samples were prepared according to Gharahdaghi et al. (25) with some modifications. In brief, samples were desalted in a 1:1 solution of 0.03 M K₃Fe(CN)₆ and 0.1 M Na₂S₂O₃ and then washed once in water and once in 50% CH₃CN and 0.025 M NH₄HCO₃ followed by dehyrdation with 100% CH₃CN. Finally, samples were dried in a SpeedVac. Peptides in-gel digested as described above. Peptides were extracted in 50% CH₃CN and 5% CF₃COOH and dried completely in a SpeedVac. If necessary, the recovered peptides were purified and concentrated on C18 ZipTips (Millipore) according to the manufacturer's instructions. Prior to mass spectrometry, peptides were resuspended in 70% CH₃CN and 0.1% CF₃COOH. Mass spectra were recorded in positive reflection mode by using an Applied Biosystems MALDI-TOF Voyager-DE STR mass spectrometer equipped with delayed ion-extraction technology. α-Cyano-4-hydroxy cinnamic acid was used as the matrix. External calibration was performed using the Sequazyme peptide mass standard kit (Angiotensin I and ACTH, clips 1–17, 18–39, and 7–38; PerSeptive Biosystems), and autodigestion peaks of bovine tryspin were used for internal calibration. Peptide mass profiles were analyzed using Mascot (Matrix Science; www.matrixscience.com/) and ProFound (prowl.rockefeller.edu/). Searches were performed using the NCBI data base. Search parameters for monoisotopic peptide masses included allowance for one missed enzymatic cleavage and accepted the carbamidomethylation of cysteine residues and the oxidation of methionine as modifications. The results of protein identification by Mascot were confirmed based on molecular mass/PI values, and further confirmation of protein identifications was obtained using ProFound software.
CG numbers of the genes and predicted protein function were obtained from FlyBase (flybase.bio.indiana.edu/).

Transglutaminase Labeling of Hemolymph Proteins—Labeling of hemolymph proteins with 5-(biotinamido)pentylamine was performed essentially as described elsewhere (26). Briefly, larvae were bled into a buffer containing 0.05 M Tris-HCl, pH 7.5, 0.01 M dithiothreitol, and 0.005 M 5-(biotinamido)pentylamine (Pierce). After 10 min of incubation at 37°C, the reaction was stopped by adding EDTA to a final concentration of 0.05 M and centrifuged at 760 g. The supernatant was dialyzed against 0.1 M Na2HPO4, pH 7.2, containing 0.15 M NaCl, centrifuged at 15000 g, and analyzed in Western blots using peroxidase-conjugated streptavidin at a concentration of 0.2 μg/ml (Sigma).

RESULTS

Preparation of Drosophila Serum—A dense fibrous clot forms immediately when Drosophila larvae are bled. The clot can be visualized by phase-contrast microscopy or fluorescence microscopy using lectins that bind to the glycoprotein-rich clot (Fig. 1, A and B) (1). Although the reaction appears quite extensive, comparison of hemolymph before (plasma) and after clotting (serum) showed only a partial depletion of clotting factors, making a systematic proteomics comparison difficult (see “Experimental Procedures”). To allow more extensive clot formation and to quantitatively deplete clotting factors, we decided to use a method we had established previously for isolation of clot components (2). This method involves the incorporation of paramagnetic beads into the clot and the subsequent magnetic removal of the clot. Clotting factors can be extracted from the beads after extensive washing (Fig. 1, lane I). When the supernatant of the reaction was applied sequentially to new batches of beads, almost no bead aggregation was observed in the second incubation, and no aggregation at all was seen in subsequent incubations (Fig. 1C, lanes 2–4). Thus, most clotting factors are depleted during the first pull-out reaction. In accordance with this observation, few proteins bind to the beads in the second and subsequent pull-out reactions (Fig. 1C). The pull-out method can thus be used to quantitatively deplete clotting factors from plasma, providing a serum fraction that is not capable of further clotting.

Comparison between Plasma and Serum—To identify proteins that differ between plasma and serum, two-dimensional gel electrophoresis was used. We decided to first establish a two-dimensional reference map of plasma proteins, because available maps are all derived from complete hemolymph, which includes hemocytes. As shown in Fig. 2 and Table I, our map includes previously identified Drosophila hemolymph proteins as well as many proteins that had not been identified previously. A limited set of differences was observed comparing plasma and serum (see Fig. 3). Some differences could not be reproduced between replicas of the same treatment and were discarded. To avoid artifacts, we decided to only consider protein spots that differed at least 5-fold between serum and plasma (exceptions are indicated; see below and Table II). The results are shown in Fig. 3 and summarized in Table II. Several proteins were almost completely depleted from hemolymph after clotting. This included two isoforms of the CG15825 product, an immune-induced protein with two sets of internal repeats but no strong sequence homology to other known or predicted proteins (Fig. 3, spots a and b, and Fig. 4A). This protein had been isolated previously with the pull-out method...
### Table I
Proteins identified in Drosophila plasma

Proteins that have been identified previously by Vierstraete et al. (18) and Guedes et al. (19) in whole hemolymph are indicated. The spot numbers are indicated in Fig. 2. The sequence coverage represents the percentage of coverage by tryptic peptides, and the pI (theoretical isoelectric point) and theoretical molecular mass are also indicated.

| Sample no. | Gene identity | Protein name | Theoretical Mr | Theoretical pI | Sequence coverage | Molecular function | Biological process |
|------------|---------------|--------------|----------------|----------------|--------------------|-------------------|--------------------|
| 01         | CG6206-PA     | 123,292      | 5.54           | 18             | α-Mannosidase activity, hydrolysis | Carbohydrate metabolism |
| 02         | CG14526-PA    | 79,578       | 5.69           | 24             | Endothelin-converting enzyme activity | Proteolysis, peptidolysis, signal transduction |
| 03         | CG14476-PB    | 106,064      | 6.05           | 24             | α-Glucosidase activity | Polysaccharide metabolism |
| 04         | CG6953-PA     | 87,171       | 6.38           | 30             | Structural molecule activity | Cytoskeleton organization and biogenesis, ectoderm development |
| 05         | CG4725-PA     | 86,345       | 5.84           | 26             | Endothelin-converting enzyme activity | Proteolysis, peptidolysis, signal transduction |
| 06         | CG5779-PA     | 79,441       | 6.14           | 46             | Monophenol monoxygenase activity | Melanization, defense response |
| 07         | CG5779-PA     | 79,441       | 6.14           | 46             | Monophenol monoxygenase activity | Melanization, defense response |
| 08         | CG5779-PA     | 79,441       | 6.14           | 46             | Monophenol monoxygenase activity | Melanization, defense response |
| 09         | CG6186-PA     | 72,964       | 6.69           | 47             | Ferric ion binding, iron ion transporter activity, carrier activity | Iron ion homeostasis |
| 10         | CG8193-PA     | 79,520       | 6.49           | 39             | Monophenol monoxygenase activity | Defense response |
| 11         | CG8193-PA     | 79,520       | 6.49           | 39             | Monophenol monoxygenase activity | Defense response |
| 12         | CG8193-PA     | 79,520       | 6.49           | 39             | Monophenol monoxygenase activity | Defense response |
| 13         | CG8193-PA     | 79,520       | 6.49           | 39             | Monophenol monoxygenase activity | Defense response |
| 14         | CG15825-PA    | 56,671       | 5.97           | 25             | | |
| 15         | CG15825-PA    | 56,671       | 5.97           | 25             | | |
| 16         | CG15825-PA    | 58,112       | 6.45           | 27             | | |
| 17         | CG58210-PB    | 50,706       | 7.99           | 39             | Chitinase-like, growth factor activity | |
| 18         | CG1780-PA     | 48,802       | 7.65           | 41             | Idg activity | Cell-cell signaling, signal transduction |
| 19         | CG4559-PA     | 49,417       | 7.12           | 34             | Idg activity, NOT chitinase activity, hydrolase activity | Cell-cell signaling, signal transduction |
| 20         | CG1780-PA     | 48,802       | 7.65           | 35             | Monophenol monoxygenase activity | Melanization, defense response ferric ion binding, iron ion transporter activity, carrier activity |
| 21         | CG1106-PA     | 98,500       | 5.11           | 19             | Actin binding, structural constituent of cytoskeleton | Cytoskeleton organization and biogenesis |
| 22         | CG1313-PA     | 41,881       | 8.28           | 37             | Monophenol monoxygenase oxidoreductase activity, trypsin activity | Proteolysis and peptidolysis |
| 23         | CG5058-PH     | 39,850       | 7.60           | 67             | Fructose-biphosphate aldolase activity | Glycolysis |
| 24         | CG6953-PB     | 35,748       | 6.27           | 51             | Oxidoreductase activity | Glycolysis |
| 25         | CG502-PB      | 37,148       | 6.38           | 42             | Structural constituent of larval cuticle | |
| 26         | CG502-PB      | 37,148       | 6.38           | 42             | Structural constituent of larval cuticle | |
| 27         | CG502-PB      | 33,811       | 6.36           | 42             | Structural constituent of larval cuticle | |
| 28         | CG502-PB      | 33,811       | 6.36           | 42             | Structural constituent of larval cuticle | |
| 29         | CG5177-PA     | 31,291       | 6.63           | 39             | Trehalose phosphatase activity | Disaccharide metabolism |
| 30         | CG8193-PA     | 33,680       | 6.02           | 40             | Calcium ion binding | Anterior/posterior specification, calcium-mediated signaling |
| 31         | CG10467-PA    | 39,888       | 6.10           | 73             | Aldose-1 epimerase activity | Monosaccharide metabolism |
| 32         | CG2031-PD     | 40,126       | 6.04           | 61             | Arginine kinase activity | Phosphorylation |
| 33         | CG5896-PB     | 37,991       | 7.51           | 40             | Serine-type endopeptidase activity, trypsin activity | Proteolysis, peptidolysis |
| 34         | CG1780-PA     | 48,802       | 7.65           | 38             | Idg activity | Cell-cell signaling, signal transduction |
degree of glycosylation of the mucin domain. When the sequence of the CG8502 product was analyzed for mucin-type glycosylation (27), 19 significant consensus sites for O-glycosylation were identified, all of which are clustered in the mucin domain shown in Fig. 4B. In addition to predicted proteins from both Anopheles gambiae and Drosophila, CG8502 shows sequence similarity to several known cuticular proteins from other insect orders (28). The alignment with the best score is

(Fig. 2 and Ref. 2), supporting our proteomic approach. Four proteins depleted in serum were identified as different isoforms of the CG8502 product, a composite protein with a mucin-domain and a domain with similarities to cuticular proteins (Fig. 3, spots d–g, and Fig. 4B). Two splice variants of CG8502 have been identified (see FlyBase), accounting for some of the isoforms we observed. Additional variation may be due to differences in posttranslational modification, for example in the

### Table I—continued

| Sample no. | Gene identity | Protein name | Theoretical $M_r$ | Theoretical pI | Sequence coverage | Molecular function | Biological process |
|------------|---------------|--------------|------------------|----------------|------------------|-------------------|-------------------|
| 35         | CG4475-PA     | Chain A, crystal structure of Idgf2 | 47,096           | 6.09           | 48               | Idgf activity, NOT chitinase activity, hydrolase activity, hydrolyzing N-glycosyl compounds | Cell-cell signaling, signal transduction |
| 36         | CG17654-PA    | Enolase      | 54,589           | 8.69           | 40               | Phosphokynurate hydratase activity | Glycolysis |
| 37         | CG5154-PA     | Idgf5        | 50,361           | 6.32           | 37               | Chitinase activity, hydrolase activity, hydrolyzing N-glycosyl compounds, growth factor activity | Cell-cell signaling, polysaccharide metabolism, signal transduction |
| 38         | CG8063-PA     |              | 51,225           | 7.05           | 27               | Intramolecular isomerase activity, depachrome isomerase activity, receptor binding, structural molecule activity | Ectoderm development, insole derivative biosynthesis |
| 39         | CG15293-PA    |              | 36,733           | 7.77           | 37               | Calcium ion binding | Anterior/posterior specification, calcium-mediated signaling |
| 40         | CG1809-PC     | Regucalcin   | 33,860           | 6.02           | 32               | Calcium ion binding | Anterior/posterior specification, calcium-mediated signaling |
| 41         | CG3050-PA     | ProPO-AE     | 40,447           | 5.99           | 48               | NOT serine-type endopeptidase activity | Metalloendopeptidase activity, metalloprotease activity, metalloprotease D activity, carboxypeptidase E activity, lysine carboxypeptidase activity |
| 42         | CG4122-PF     | Carboxypeptidase D | 48,987 | 5.87 | 38               | Metallocarboxypeptidase activity | Carboxypeptidase D activity, metallocarboxypeptidase activity, metallocarboxypeptidase D activity, carboxypeptidase D activity, lysine carboxypeptidase activity |
| 43         | CG11395-PA    |              | 48,869           | 6.23           | 30               | NOT serine-type endopeptidase activity | Monophenol monooxygenase activator activity, trypsin activity, serine carboxypeptidase activity |
| 44         | CG8586-PA     |              | 50,855           | 5.66           | 25               | NOT serine-type endopeptidase activity | Defense response, proteolysis, peptidolysis |
| 45         | CG3065-PA     | ProPO-AE-binding protein 44a | 44,070 | 6.18 | 28               | Monophenol monooxygenase activator activity, trypsin activity, serine carboxypeptidase activity | Defense response, proteolysis, peptidolysis |
| 46         | CG1548-PA     | Cathepsin D precursor | 42,878 | 5.9 | 44               | Cathepsin D activity | Proteolysis, peptidolysis |
| 47         | CG2915-PA     | α-Amylase B precursor | 51,310 | 5.76 | 28               | Arginine kinase activity | Phosphorylation |
| 48         | CG18730-PA    | α-Amylase B precursor | 54,274 | 5.47 | 19               | Arginine kinase activity | Phosphorylation |
| 49         | CG17377-PA    | Serine protease inhibitor 5 | 52,539 | 5.43 | 54               | Arginine kinase activity | Phosphorylation |
| 50         | CG18525-PA    | Serine protease inhibitor 5 | 48,659 | 5.37 | 54               | Arginine kinase activity | Phosphorylation |
| 51         | CG5390-PA     | Serine protease inhibitor 5 | 45,851 | 5.32 | 29               | Arginine kinase activity | Phosphorylation |
| 52         | CG40160-PA    | LD1329p | 46,644           | 5.46           | 34               | Arginine kinase activity | Phosphorylation |
| 53         | CG22021-PD15,19 | Arginine kinase | 40,126 | 6.04 | 58               | Arginine kinase activity | Phosphorylation |
| 54         | CG2216-PA     | Ferritin 1 heavy chain homolog | 20,597 | 5.42 | 42               | Ferrous iron binding | Iron ion homeostasis |
| 55         | CG1469-PA     | Ferritin 2 light chain homolog | 25,489 | 5.90 | 63               | Ferrous iron binding | Iron ion homeostasis |
| 56         | CG2227-PD15,19 | GIP-like | 29,189           | 6.07           | 39               | Isomerase activity | Carbohydrate metabolism |
| 57         | CG4306-PA     | Ferritin 2 light chain homolog | 24,570 | 6.21 | 52               | Ferrous iron binding | Iron ion homeostasis |
| 58         | CG1469-PA     | Ferritin 2 light chain homolog | 25,489 | 5.90 | 42               | Ferrous iron binding | Iron ion homeostasis |
| 59         | CG2297-PA     | Ferritin 2 light chain homolog | 16,155 | 8.22 | 41               | Ferrous iron binding | Iron ion homeostasis |
| 60         | CG1469-PA     | Ferritin 2 light chain homolog | 25,455 | 5.90 | 56               | Ferrous iron binding | Iron ion homeostasis |
| 61         | CG1314-PA     | Ferritin 2 light chain homolog | 17,896 | 7.49 | 38               | Ferrous iron binding | Iron ion homeostasis |
| 62         | CG32031-PD18,19 | Arginine kinase | 17,997 | 7.55 | 52               | Ferrous iron binding | Iron ion homeostasis |
| 63         | CG8160-PA     | Ferritin 2 light chain homolog | 20,819 | 8.91 | 49               | Arginine kinase activity | Phosphorylation |
| 64         | CG1469-PA     | Ferritin 2 light chain homolog | 25,455 | 5.90 | 49               | Arginine kinase activity | Phosphorylation |
| 65         | CG1469-PA     | Ferritin 2 light chain homolog | 40,126 | 6.04 | 33               | Arginine kinase activity | Phosphorylation |

#### Notes
- The alignment with the best score is used for further analysis.
- The mucin domain shows significant similarity to known cuticular proteins from other insect orders.
- The sequence similarity suggests potential roles in protein folding, protein targeting, and defense response.
- The alignment with the best score is used for further analysis.
shown in Fig. 4B. Although some cuticular proteins extend beyond the aligned region shown in Fig. 4B, none of them contains a mucin domain similar to the CG8502 product, making this a unique protein.

A role for phenoloxidase in clotting is supported by two observations. 1) Two proteins that are slightly less abundant in serum (spots 08 and 09 in Fig. 2, which consistently differed between serum and plasma; see “Experimental Procedures”) were identified as phenoloxidases. These proteins include an isoform of phenoloxidase CG8193 (spot j in Fig. 3) and two members of the immunoglobulin family (CG11314 and CG11315; spots j and k in Fig. 3). The ferritin may be cleaved, because a second isoform at the expected mass (spot h in Fig. 3) disappeared when the faster migrating spot appeared. Different masses have been observed for the CG1469 product (14). The two Ig-type proteins show the highest similarity with members of a family with a single Ig domain (MD-2-related molecules), which have been implied in lipid metabolism and lipopolysaccharide recognition in mammals (29).

**Table II**

| Spot no. | Gene identity |
|----------|---------------|
| A        | CG15825-PA    |
| B        | CG15825-PA    |
| C        | CG11313-PA    |
| D        | CG8502-PC     |
| E        | CG8502-PC     |
| F        | CG8502-PA     |
| G        | CG8502-PA     |
| H        | CG4306 + CG1469 |
| J        | CG11315-PA    |
| K        | CG11314-PA    |
| L        | CG1469-PA     |

| Spot no. | Gene identity |
|----------|---------------|
| A        | Unknown, repetitive |
| B        | Unknown, repetitive |
| C        | Serine-protease |
| D        | Cuticle, mucin |
| E        | Cuticle, mucin |
| F        | Cuticle, mucin |
| G        | Cuticle, mucin |
| H        | Unknown + ferritin 2 light chain |
| J        | Ig member |
| K        | Ig member |
| L        | Ferritin 2 light chain |

| Spot no. | Gene identity |
|----------|---------------|
| A        | BCG11313-PA   |
| B        | BCG11315-PA   |
| C        | BCG11313-PA   |
| D        | BCG11313-PA   |
| E        | BCG11315-PA   |
| F        | Unknown, repetitive |
| G        | Unknown, repetitive |
| H        | Unknown, repetitive |
| J        | Unknown, repetitive |
| K        | Unknown, repetitive |
| L        | Unknown, repetitive |

| Spot no. | Gene identity |
|----------|---------------|
| A        | Unknown, repetitive |
| B        | Unknown, repetitive |
| C        | Unknown, repetitive |
| D        | Unknown, repetitive |
| E        | Unknown, repetitive |
| F        | Unknown, repetitive |
| G        | Unknown, repetitive |
| H        | Unknown, repetitive |
| J        | Unknown, repetitive |
| K        | Unknown, repetitive |
| L        | Unknown, repetitive |

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**Proteins that are present in different amounts in plasma versus serum**

All spots differed at least 5-fold between serum and plasma except spot h. This spot is nevertheless indicated, because a putative processing product of CG1469 (spot l) differs >5-fold.

**Proteins depleted from hemolymph during clotting.** Shown are two-dimensional gels of plasma and serum (10 individuals each). Spots that differ between samples are indicated by small letters (see Table II). Note that some differences (for example, a spot marked with an asterisk that is located below and to the right of spot h differs between the samples shown here) were not observed in all pairwise comparisons and were therefore disregarded. Protein gels were treated as described in Fig. 2, except that 7-cm NL-IEF strips were used followed by 10% gels.

**Proteins depleted from hemolymph during clotting.** Shown are two-dimensional gels of plasma and serum (10 individuals each). Spots that differ between samples are indicated by small letters (see Table II). Note that some differences (for example, a spot marked with an asterisk that is located below and to the right of spot h differs between the samples shown here) were not observed in all pairwise comparisons and were therefore disregarded. Protein gels were treated as described in Fig. 2, except that 7-cm NL-IEF strips were used followed by 10% gels.
B and C. All BP-labeled spots that also labeled strongly with Coomassie were discarded. This left five proteins that met our criteria for a transglutaminase substrate; i.e., they were not labeled in a sample incubated without BP, and they labeled strongly in the presence of BP despite their low abundance. These proteins include two spots that we were unable to identify (Fig. 5B, spots d and e), a subfraction of lipophorin, CG15825 (see above), and a secreted form of gelsolin.

**DISCUSSION**

We have identified factors involved in the coagulation of *Drosophila* larval hemolymph by proteomic comparison of plasma and serum and by BP labeling of transglutaminase substrates. Care had to be taken to ensure that clotting was extensive, because our attempts to deplete clotting factors from the resultant serum were not sufficient for reliable proteomic detection until we added a pull-out assay (2). This procedure may contribute to the differences between our results and those from other studies on changes in larval hemolymph following immune challenge (13–15). Similar to a proteomic study of immune-induced proteins (14), we decided to only consider proteins that differed at least 5-fold between serum and plasma to be confident that the observed differences resulted from coagulation. 

Proteomics and BP labeling supplement and expand pull-out results. Pull-out facilitated the identification of large proteins, which are less accessible in two-dimensional gels (i.e., the abundant fat body protein 1 was found neither in our map nor in two previous maps of *Drosophila* hemolymph proteins). However, a pull-out assay can only detect proteins in the clot and not changes in the hemolymph that activate or accompany clot formation. In addition, some proteins in the clot may be covalently cross-linked, preventing their characterization in protein gels. Because proteomics relies on the comparison of whole hemolymph, these problems are avoided. Limitations of proteomics studies are the detection limit and the fact that some physiologically relevant changes may remain unnoticed because of the high variation between samples (see “Experimental Procedures”). This fact is particularly true for abundant proteins, of which only minor fractions are incorporated into the clot. We were able to identify proteins in this category by virtue of their being substrates for transglutaminase, leading to a shift in their migration behavior in two-dimensional gels after the incorporation of BP. Proteins identified this way include gelsolin and lipophorin, which is abundant in hemolymph and has been implied in clotting. From this and a previous study in *Galleria* (21), it appears that insect transglutaminase is not as specialized as vertebrate factor XIIIa and acts on several substrates. In addition to the glutamine-containing substrates identified using BP, there may be other substrates that contain lysine. The existence of several substrates is suggested by our findings and results from horseshoe crab, one of the best-studied models for non-insect arthropod hemolymph clotting (30).
pull-out were expected to identify partially overlapping subsets of clotting factors. Proteins identified directly in the clot (2) and indirectly by proteomics (this work) include lipophorin, phenoloxidase CG8193, and the CG15825 protein product. Novel proteins identified here include phenoloxidase CG5779, a candidate for a prophenoloxidase-activating protease, gelsolin, the CG8502 product, a ferritin subunit and two members of the Ig family. Somewhat surprisingly, only one (CG8193) of the two phenoloxidases that differ between serum and plasma was isolated by pull-out (2). We speculate that the different phenoloxidases may play different roles during coagulation. Other proteins identified as part of the clot include a secretory form of gelsolin (31). Gelsolin has been implied in the formation of amyloids in vertebrates (32), which fits with a potential function as a clotting factor. Gelsolin can also act as a crystallin (33), and crystallins may play a role in insect hemolymph clotting (21). Ferritins have been shown to be immune-regulated (13–15).

Interestingly, the different CG8502 isoforms have two domains, which may locate them at the border between the clot and the cuticle. Mucin domains have been identified in proposed clotting factors including hemomucin (34), a Galleria mucin (21), and a Drosophila 150-kDa mucin (35). The other domain is similar to insect cuticular proteins, suggesting that the CG8502 product may help seal wound edges by anchoring the clot to the cuticle. Mutations in this gene will make this prediction testable.

Finally two immunoglobulin superfamily members, CG11314 and CG11315, were found to be elevated during clot formation. Both proteins belong to a subfamily of Ig-like proteins, which contain an ML (MD-2-related lipid recognition) domain (29). CG11314 is activated in hemocytes upon the expression of an activated form of the ras oncogene product (36). Our data suggest that the two proteins are released from hemocytes during clot formation. MD-2, the vertebrate members of the ML family, has recently been shown to bind lipopolysaccharides and participate in immune activation via the Toll-like receptor 4 (37). Similarly the two Drosophila proteins may participate in immune recognition events. Alternatively, their capacity to bind lipids may recruit them for clot formation.

In summary, we have compared hemolymph protein levels before and after clotting to identify candidate clotting factors. We also identified substrates for the enzyme transglutaminase, the only orthologue of a vertebrate clotting factor in the Drosophila genome. The results of these two methods and the pull-out method to directly identify clot factors were compared. The identification of several proteins by very different methods is strong evidence that they play a role in the clot. For example, the CG15825 product, which was identified by proteomics, is a substrate for transglutaminase and was also isolated directly in the clot by pull-out. In addition, the corresponding RNA has been shown to be induced during immune reactions in whole genome induction studies with slow (replenishment?) kinetics, supporting a role in immunity (9). Other proteins identified here, e.g. gelsolin and the different CG8502 mucin isoforms, were not found by pull-out. This is probably due to inherent limitations in the pull-out technique. Our identification of additional putative clotting factors such as these suggests that coagulation is likely to involve redundant proteins. This may explain our recent
finding that strongly reducing the levels of hemolecin (a protein with domains shared with vertebrate clotting factors) (38) isolated by pull-out (2) does not noticeably reduce the ability of Drosophila larvae to survive wounding, although the clotting ability of the mutant larvae is strongly reduced (2, 39). Some of the proteins we identified best fit the picture of structural components of a clot involved in sealing wounds. Other proteins have been implied in immune reactions in Drosophila (phenoloxidase and ferritin) or in mammals (the small Ig members). Our proteomic analysis has thus substantially improved and expanded our ideas about coagulation. Combining results from proteomics and pull-out with miRNA interference knockdown, genetic tools will enable us to gain better insight into the sequence of events during hemolymph clotting and understand how clotting contributes to immune defense.

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