Proteomic Analysis of the Systemic Immune Response of *Drosophila*

Francine Levy‡, Philippe Bulet‡§, and Laurence Ehret-Sabatier‡¶

Improvements in two-dimensional gel electrophoresis, mass spectrometry, and bioinformatics provide new tools to characterize proteins involved in a physiological process, such as the immune response of the insect model *Drosophila melanogaster*. Profiling of the proteins present in the hemolymph (insect blood) of noninfected flies versus flies infected with bacteria or fungi was performed by two-dimensional gel electrophoresis, silver or Coomassie staining, and image analysis. Through this differential analysis, more than 70 out of 160 spots were up- or down-regulated by at least 5-fold after microbial infection. Coomassie staining, in-gel digestion, and database searches yielded the identity of a series of proteins that are directly involved in the *Drosophila* immune system. This included proteases, protease inhibitors, and recognition molecules such as prophenol oxidase-activating enzymes, serpins, and Gram-negative binding protein-like. Proteins with a potential function in the immune response were also identified, such as an odorant binding protein, peptidylglycine α-hydroxylating monooxygenase, and transferrin, allowing new candidates for further investigation of innate immune mechanisms. Moreover, several molecules resulting from the cleavage of proteins were detected after the fungal infection. Altogether, this first differential proteomic analysis of the immune response of *Drosophila* paves the way for the study of proteins affected during innate immunity. *Molecular & Cellular Proteomics* 3: 156–166, 2004.

Innate immunity is the first line of defense against infectious pathogens. From invertebrates to vertebrates, characterization of innate immune mechanisms in multicellular organisms revealed striking similarities, suggesting a common evolutionary ancestry (1). Through its particularly convenient genetics, the fruit fly, *Drosophila melanogaster*, has rapidly emerged as a powerful model system to study these first-line defense mechanisms. To combat microbial infections, *Drosophila* has developed a large range of strategies based on cellular and humoral reactions. This includes phagocytosis by macrophage-like blood cells, activation of proteolytic cascades leading to localized melanization and coagulation, and synthesis of potent antimicrobial peptides (AMPs) by the fat body. The study of regulation of AMPs has attracted the most attention over the past few years. It has been established that antimicrobial activity in *Drosophila* is regulated by two distinct intracellular signaling pathways, Toll and immune deficiency (Imd), which are activated by different pathogens. The Imd pathway responds strongly to infection by Gram-negative bacteria and results in the expression of several antibacterial peptide genes, including *Diptericin*. In contrast, the Toll pathway is triggered by fungi and Gram-positive bacteria, regulating the expression of several AMP genes, such as *Drosomycin* (for a review of both pathways, see Ref. 2). A key point of the Toll pathway is an extracellular proteolytic cascade leading to the cleavage of the protein Spaetzle, the ligand of the transmembrane receptor Toll (3). In the case of fungal activation, the cascade is negatively regulated by the blood serpin Necrotic (Nec) (4), inhibiting the serine protease Persephone (Psh) (5). On the other hand, Toll activation by Gram-positive bacteria involves two extracellular proteins thought to be in a complex after the infection, a peptidoglycan recognition protein, PGRP-SA (6), and a Gram-negative binding protein, GNBP 1 (61). Although much has been learnt about *Drosophila* immunity through genetic studies, many aspects remain unclear, in particular the identity of the molecules that are recruited in the hemolymph during the activation of the signaling cascades. In order to investigate which peptides are induced in the hemolymph after a microbial challenge, earlier peptidomic approaches combined matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and liquid chromatography. These studies led to the detection of 24 *Drosophila* immune-induced molecules (DIMs) after bacterial or fungal injury (7), and one molecule, pherokine-2, induced after a viral infection (8). All these peptides have a molecular mass below 15 kDa, and their

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function in the host defense is still under investigation. In parallel, the transcriptional profiles of the Drosophila immune response have been examined using DNA microarrays (9, 10). This strategy uncovered a large number of differentially regulated genes, directing the choices for future mutant studies. Nevertheless, several groups have reported a poor correlation between mRNA level and protein abundance in the cell (11–13), indicating that there is a clear interest in directly monitoring protein levels. It is also important to remember that most proteins undergo post-translational modifications that can affect their function, half-life, or distribution (14). In contrast to other methodologies, proteomics permits the analysis of such modifications in relation to a particular physiological state.

To carry out protein profiling in the context of the systemic immune response of Drosophila, a proteomic approach combining two-dimensional (2D) gel electrophoresis, MS, and data-bank searches was performed. This method allowed the qualitative and quantitative comparison of proteins with a molecular mass greater than 15 kDa that were present in the hemolymph of Drosophila before and after immune challenge, followed by the identification of proteins regulated by the infection. In the present differential proteomic analysis of Drosophila hemolymph, at least 70 proteins were revealed to be up- or down-regulated 5-fold or more after a bacterial or fungal challenge. Among these, we identified proteins from many functional classes such as proteases, serpins, enzymes involved in energy metabolism, or molecules involved in iron metabolism. This paper is the first report of a differential proteomic analysis that identifies proteins involved in the Drosophila immune response.

**EXPERIMENTAL PROCEDURES**

Apparatus and chemicals were supplied by Bio-Rad (Hercules, CA) unless otherwise indicated.

**Sample Preparation**

Cinnabar brown flies (cn bw) were raised in tubes containing a standard cornmeal medium. Experimental infections were performed on 3- to 4-day-old adult males.

**Inoculation with Bacteria**—Flies were inoculated into the thorax using a sharp tungsten needle that had been dipped into a concentrated culture of Micrococcus luteus (Gram-positive bacteria) and/or Escherichia coli 1106 (Gram-negative bacteria). For the control group flies, a clean needle was used to create a nonseptic injury in the fly thorax.

**Natural Infection with Fungus**—Anesthetized flies were shaken in a Petri dish containing a sporulating culture of the filamentous fungus Beauveria bassiana. Flies were then transferred to fresh fly-medium tubes and were kept at 29 °C until the hemolymph was collected. Noninfected flies were used as a control group.

**Hemolymph of Drosophila** (from 30 to 600 individuals) was collected 6 h post bacterial infection and 72 h following fungal infection using a microcapillary set up on a Nanoject apparatus (Drummond Scientific, Broomall, PA). To solubilize proteins, the hemolymph was directly diluted in a buffer containing 8 M urea (Q-Biogene, Carlsbad, CA), 4% (w/v) 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS; Sigma, St. Louis, MO), 0.5% (v/v) Triton X-100 (Sigma; deionized on AG50W-X8 resin), 0.5% (w/v) carrier ampholytes 3–10 or 5.5–6.7 (Biolytes®). Dithiothreitol (DTT) was added to the buffer prior to use to give a final concentration of 20 mM. Protein concentration was determined by RC/DC™ protein assay, using bovine serum albumin (Sigma) as standard protein. The recommendations of the manufacturer were followed with an additional sonication step (4 min) during the resuspension of the pellet.

**2D Electrophoresis**

Sample volumes were adjusted in order to analyze the same amount of protein (from 15 to 300 μg) for each set of experiments (control/infected). Samples were loaded on linear immobilized pH gradient (IPG) strips (ReadyStrips IPG Strips, pH range 3–10, 5–8, or 5.5–6.7). In this study, two different sizes of strips were used, 7 cm (15 μg of protein in 150 μl sample buffer) for establishment of experimental conditions and 17 cm (100, 200, or 300 μg of protein in 350 μl sample buffer) for detailed analyses. Active rehydration and isoelectric focusing (IEF) were performed at 20 °C using a protean IEF cell. After active rehydration (50 V) for at least 10 h, IEF was performed following three steps: conditioning (15 min at 250 V), voltage ramping (250–4,000 V in 2 h for 7-cm strips and 250–10,000 V in 5 h for 17-cm strips), and focusing (5 h at 4,000 V for 7-cm strips and 8 h at 10,000 V for 17-cm strips). The current was limited to 50 μA/strip.

Samples were subjected to reduction/alkylation prior to second dimension electrophoresis. Strips were incubated for 15 min in an equilibration buffer containing 6 M urea, 2% (w/v) SDS, 375 mM Tris (Sigma), pH 8.8, 20% (w/v) glycerol, and 2% (v/v) DTT, then for 20 min in the same solution substituting DTT with 2.5% (w/v) iodoacetic amide (Sigma). The second dimension was carried out on an 11% polyacrylamide gel (SDS-PAGE using piperazine diacrylamide as cross-linker). Electrophoresis was performed for 30 min at 20 V and 1 h at 150 V on a Mini-Protein II for 7-cm IPG strips or for 30 min at 32 mA, then 5 h at 48 mA, on a Protean II XL for 17-cm IPG strips.

Spots were either detected by silver staining (15) or colloidal Coomassie brilliant blue (CBB) G-250 staining (16) following the modifications reported by Matsui et al. (17).

**Image Analyses**

Gel images were captured using the GS-800™ Calibrated Densiometer at a resolution of 63 × 63 pixels and were analyzed with the PDQuest software according to the protocols provided by Bio-Rad. This included an automated spot detection and matching function, followed by manual designation of spots as landmarks for alignment of the gels. A master gel was constructed summarizing all the observed spots. Spot intensities (spot volumes) were normalized according to the mode “total of all valid spots,” and analyses were performed using the quantitative and qualitative modes. The data obtained from comparison of the normalized spot volumes were exported into a data base, and Excel was used to calculate the variation rate of each spot. For induction or suppression of spots, a comparison was made between the intensity of the spots present on one of the gels and the background level of the other gel (determined after a normalization step). The confidence limit for detecting meaningful apparition or disappearance of spots was defined as 10-fold of the appropriate background level (recommended by the manufacturer). Following reproducibility tests for up- and down-regulation of protein spots, the confidence threshold for these phenomena was set at 5-fold below or above the spot intensity seen in controls.

**In-gel Digestion, MS Analysis, and Identification of Proteins**

Spots of interest were excised from the gels within 6 h after staining and digested using trypsin (Promega, Madison, WI) according to a protocol described in Jensen et al. (18). When silver staining was used in place of CBB, a destaining step described by Gharahdaghi et al. (19) was performed at the beginning of the protocol. The tryptic
peptides were extracted with 70% acetonitrile (Merck, Darmstadt, Germany) and 3% formic acid (Prolabo, Paris, France). After 10 min of sonication, peptides were desalted using ZipTip C18 microtips (Millipore, Bedford, MA) and eluted in 4 μl of 50% acetonitrile.

Tryptic peptides were analyzed on a Biflex III MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). This instrument was used at a maximum accelerating potential of 19 kV and was operated in reflector mode, using delay extraction (200 ns). Samples were prepared according to the "sandwich method" (20) with matrix/cyano-4-hydroxycinnamic acid (Sigma). The calibration was performed in internal mode using fragments from trypsin autolysis with monoisotopic (M-H) at m/z 842.5100, 1045.5462, and 2211.1046. Protein identification relied on the comparison of the measured mass of the tryptic peptides with the predicted masses from databases (Genpept, MSDB, or NCBI) with a tolerance of 50 ppm. These comparisons were carried out using MS-Fit (prospector.ucsf.edu) or Mascot (www.matrixscience.com) tools.

RESULTS

2D Electrophoresis of Drosophila Hemolymph: Establishing the 2D Maps

One of the most decisive steps in 2D electrophoresis is the solubilization of the proteins, which varies greatly from one sample type to another. As few studies have been made on 2D analysis of hemolymph of Drosophila (21, 22), our initial experiments were focused on the optimization of the solubilizing buffer. Various conditions were investigated on 7-cm IPG strips (15 μg of protein per gel) to optimize and standardize procedures before using the 17-cm IPG strips. The first experiments were performed according to protocols provided by the manufacturer (Bio-Rad) using one detergent at a time (0.5–2% Triton X-100 or 2–4% CHAPS) or in combination. A mixture composed of 0.5% Triton X-100 and 4% CHAPS was found to be the most efficient for solubilization of proteins. In order to improve the solubility of molecules, urea was replaced by a mixture of thiourea/urea (2 M/7 M), reported to have a much higher power of solubility (23), and DTT was replaced by tributylphosphine (5 mM), a more powerful reducing agent (24). However, these reagent modifications had no obvious benefit for the 2D maps. From these results, the solubilizing buffer used for all the experiments was 8M urea, 0.5% Triton X-100, 4% CHAPS, and 20 mM DTT.

The first experiments were performed on IPG strips with the broadest range of pH commercially available (3–10). All almost all the spots were located in a pH range greater than 5 (data not shown), pH range from 5 to 8 was selected to improve resolution of the largest number of spots. After comparison of the hemolymph protein profiles from female versus male flies (data not shown), we chose to use males to minimize interference from the proteins involved in oogenesis in females.

To improve sensitivity and resolution, the use of 17-cm IPG strips was tested with 100 μg of hemolymph collected from 200 Drosophila. The conditions of sample preparation determined on 7-cm strips were found to be completely compatible with the use of 17-cm strips. An example of a control hemolymph 2D map is shown in Fig. 1A. Following silver staining, around 350 spots can be detected on the 2D map obtained with 17-cm strips between pH 5 and 8.

Reproducibility of 2D Maps and Threshold Levels

Once the experimental methodology had been determined, the reproducibility of 2D maps from three independent control samples was explored. For 80% of the spots, the variation in intensity, as measured by PDQuest, was less than 2-fold between control samples, and this value rose to 98% of all spots at the 5-fold level. To be confident that variations observed were due to the experimental treatments, it was there-
fore decided to only consider differences between control and infected samples at 5-fold or more as differentially expressed.

**Differential Analysis After an Experimental Infection**

A comparison was made between hemolymph proteins from control and immune-challenged flies. The first infections were performed by inoculating the flies with a mixture of Gram-positive (*M. luteus*) and Gram-negative (*E. coli*) bacteria, known to efficiently initiate the immune response of the fly (7). A 2D differential time-course study, from 30 s up to 24 h postinfection, indicated that most of the alterations in protein expression occurred 6 h after challenge (data not shown). This time point was therefore the only one used for further bacterial infection studies. The analysis of silver-stained 2D maps of control versus infected flies revealed that more than 50 spots are regulated (data not shown). These experiments showed that the comparison of 2D gels was adapted for detecting regulation of protein spots during the immune response of *Drosophila*, in terms of resolution and sensitivity.

To study the specificity of protein alterations, flies were challenged with different types of microorganisms: Gram-positive bacteria, Gram-negative bacteria, or filamentous fungus. For bacterial infections, the protein profile of hemolymph was analyzed 6 h postinfection. For fungal infections, time-course studies up to 72 h revealed that before this time no significant changes were detected (data not shown). This is in agreement with the low level of AMPs detected by MS for times less than 72 h after fungal infection (7).2 Fig. 1 shows the 2D maps of proteins seen in the hemolymph of control flies (Fig. 1A) versus flies naturally infected with *B. bassiana* (Fig. 1B). While there were differences in spot regulation between fungal and bacterial treatments, the 2D maps obtained were comparable (data not shown). Following qualitative and quantitative analysis of these maps using PDQuest, the number of spots regulated by each infection was obtained (Table I). Whatever the inducer used, the number of positively regulated spots was almost the same as the number of those which were negatively regulated. However, fungal infection induced more changes than bacterial challenge. As the sampling time after fungal infection (72 h) was much longer than that for bacteria (6 h), it is tempting to correlate this with the higher number of differentially regulated spots. Nevertheless, as previously indicated, the time-course studies performed after bacterial or fungal infections showed that the selected time points corresponded to the times of optimum variation in protein expression.

After 2D mapping, one area had a noticeably higher density of regulated spots than was seen in other areas of the gel (Fig. 1B; boxed zone). Increasing sample loading (200 μg) and, at the same time, using narrow-range IPG strips (pH 5.5–6.7) increased the resolution in this area and allowed the analysis of spots that were either too faint or not well resolved on 5–8 2D maps (Fig. 2, spots A–E).

![Fig. 2. Selected area of a narrow-range 2D map of proteins present in *Drosophila* hemolymph following a natural fungal infection. Two hundred micrograms of protein (hemolymph from about 400 adult male flies) were loaded on a narrow-range 2D gel (IEF on pH 5.5–6.7 17-cm IPG strips followed by 11% SDS-PAGE). The pH and molecular mass ranges shown correspond to the boxed area in Fig. 1B. Labeled spots (A–E) are regulated spots highlighted by quantitative PDQuest analysis.](image)

|                   | *B. bassiana* | *M. luteus* | *E. coli* |
|-------------------|---------------|-------------|-----------|
| **Induced**       | 23            | 22          | 14        |
| **Up-regulated**  | 15            | 7           | 8         |
| **Total of positively regulated spots** | 38            | 29          | 22        |
| ** Suppressed**   | 31            | 6           | 10        |
| **Down-regulated** | 10            | 18          | 10        |
| **Total of negatively regulated spots** | 41            | 24          | 20        |

2 L. Ehret-Sabatier, unpublished data.
Identification of Altered Proteins

Regulated spots from silver-stained gels were analyzed using a classical MALDI fingerprint approach (25). Unfortunately, only 15% of the altered proteins could be identified in this way. Failure of the identification procedure was mainly due to a lack of signal in MALDI analysis. In order to improve the protein identification, sample loading was further increased, thus permitting the use of colloidal CBB staining. In agreement with the lower sensitivity of CBB versus silver staining, fewer spots were observed in the 2D maps, for both control (Fig. 3A, 160 spots) or infected flies (B. bassiana (B); M. luteus (C)). Results for E. coli and clean injury are not shown. Quantitative analysis revealed that 70 spots were up- or down-regulated by at least 5-fold after bacterial or fungal infection. Of all the spots excised, most were unambiguously characterized (summary of regulated spots so far identified in Table II). Proteins were classified according to the information given in Flybase (August 2003, flybase.bio.indiana.edu). As can be seen, the proteins belong to many different functional classes, including energy metabolism, iron metabolism, defense mechanisms, and others.

DISCUSSION

This work describes the first differential proteomic analysis of the immune response of Drosophila, paving the way for the study of proteins affected during this process. Previous transcriptomic and peptidomic approaches allowed the analysis of differential regulation of genes or molecules with molecular masses of less than 15 kDa, respectively. In order to analyze proteins with higher molecular masses, a strategy based on 2D gel electrophoresis was developed. Transcriptomic analyses of the Drosophila immune response have been carried out using DNA microarrays (9, 10). In both studies, a variation threshold of plus or minus 2-fold control levels was used to determine the differential regulation of genes. In the present proteomic study, a variation threshold of 2-fold did not provide enough confidence to eliminate intersample differences and after testing the reproducibility of spot intensity, a threshold of 5-fold variation was retained. As a consequence of such differences, quantitative comparisons between transcriptomic and proteomic analyses should be carefully considered. Indeed, in this study, comparisons were only performed for the most important variations.

The optimization of sample preparation led to a well-re-
solved 2D map for the proteins present in the hemolymph of adult male *Drosophila* (see Fig. 1A). Following gel silver staining, this map showed an average of 350 spots with molecular masses of between 15 and 150 kDa (11% SDS-PAGE), within a 5–8 pH range. This map may now serve as a reference adult 2D map, complementary to the recently published map from larval hemolymph analyzed on a 12–14% linear gradient polyacrylamide gel and within a 4–7 pH range (26). Moreover, our

### Table II

Protein identification of 2D gel spots from Figs. 2 and 3

| Function                      | Gene identity<sup>a</sup> | Identity/homology<sup>c</sup> | Spot labels from Figs. 2 and 3<sup>d</sup> | Expression<sup>e</sup> | B. bassiana | M. luteus | E. coli |
|-------------------------------|---------------------------|--------------------------------|--------------------------------------------|------------------------|-------------|-----------|---------|
| Calcium binding               | CG15848                   | Sarcoplasmic calcium-binding protein 1 | 1                                        | i                      | ND          | ND        | ND      |
|                               | CG17876                   | α-amylase distal               | 2                                          | + + (5)   | ++ (5)   | +         |
| Chaperon/Heat Shock           | CG14207                   | HSP20-like chaperone           | 3                                          | +                      | ++ (7)   | ++ (9)    |
| Defense/Immunity              |                           |                                |                                            |                        |             |           |         |
| Serpins                       | CG1857                    | Nec                            | 4                                          | + + (6)   | =        | =         |
|                               | CG6687                    | Nec-like                       | D                                          | i                      | ND        | ND        | ND      |
|                               |                           | Nec-like                       | E                                          | i                      | ND        | ND        | ND      |
| Serine proteases              | CG16705                   | ProPO-AE                       | 5                                          | + + (6)   | =        | =         |
|                               | CG1102                    | ProPO-AE                       | C/6                                        | i                      | ND        | ND        | ND      |
| Binding                       | CG13422                   | GNBP-like                      | 7                                          | i                      | =        | =         |
|                               | CG10363                   | TEPP                           | D                                          | i                      | ND        | ND        | ND      |
| DNA                           | CG7780                    | DNase II                       | 8                                          | + + (12)  | =        | =         |
|                               | CG7780                    | DNase II                       | 9                                          | i                      | =        | =         |
| Enzymes                       |                           |                                |                                            |                        |             |           |         |
| Amidation                     | CG3832                    | PHM                            | B                                          | i                      | ND        | ND        | ND      |
| Energy metabolism             |                           |                                |                                            |                        |             |           |         |
| Dehydrogenases                | CG9042                    | Sn-glycerol 3 phosphate dehydrogenase | 10                                         | s                      | ND        | ND        | ND      |
|                               | CG9244                    | Sn-glycerol 3 phosphate dehydrogenase | 11                                         | s                      | ND        | ND        | ND      |
|                               | CG3752                    | Aconitase (mitochondrial)      | 12                                         | ND         | i         | ND        |         |
|                               | CG12055                   | Glyceraldehyde 3 phosphate dehydrogenase | 14                                         | +                      | =         | =         |         |
|                               | CG17654                   | Enolase                        | 15                                         | s                      | ND        | ND        |         |
|                               |                           | Enolase                        | 16                                         | -- + (6)  | ND        |           |         |
|                               | CG6058                    | Fructose 1,6 bisphosphate aldolase | 17                                         | +                      | =        | ++ (6)    |
| Glycolysis                    |                           |                                |                                            |                        |             |           |         |
|                               | CG4929                    | Arginine kinase               | 18                                         | i                      | ND        | ND        | ND      |
| Kinases                       | CG4475                    | IDGF2                          | 20                                         | s                      | ND        | ND        |         |
| Proteases                     | CG4549                    | IDGF3                          | 21                                         | s                      | ND        | ND        |         |
|                               | CG11154                   | ATP synthase b chain           | 23                                         | =                      | i        | i         |         |
| Hydrogen transport            | CG6030                    | ATP synthase subunit d         | 24                                         | s                      | ND        | ND        | ND      |
| Iron metabolism               | CG6186                    | Tsf1 entire form               | 25                                         | + + (10)  | =        | =         |         |
|                               |                           | Tsf1 entire form               | 26                                          | + + (15)  | =        | =         |         |
|                               |                           | Tsf1 cleaved form              | 27                                          | i                      | ND        | ND        |         |
|                               |                           | Tsf1 cleaved form              | 28                                          | i                      | ND        | ND        |         |
|                               |                           | Tsf1 cleaved form              | 29                                          | i                      | ND        | ND        |         |
|                               |                           | Tsf1 cleaved form              | 30                                          | i                      | ND        | ND        |         |
|                               |                           | Tsf1 cleaved form              | 31                                          | i                      | ND        | ND        |         |
|                               |                           | Tsf1 cleaved form              | 32                                          | i                      | ND        | ND        |         |
|                               |                           | Tsf1 cleaved form              | 33                                          | i                      | ND        | ND        |         |
|                               |                           | Tsf1 cleaved form              | 34                                          | -- -- (22)  | + + (6)  | ++ (11)   |
|                               |                           | Tsf1 cleaved form              | 35                                          | -- -- (17) | =        | =         |         |
|                               |                           | Tsf1 cleaved form              | 36                                          | s                      | =        | =         |         |
|                               |                           | Tsf1 cleaved form              | 37                                          | s                      | =        | +         |         |
|                               |                           | Tsf1 cleaved form              | 38                                          | -- -- (13) | =        | =         |         |
|                               |                           | Tsf1 cleaved form              | 39                                          | +                      | -- (5)   | =         |         |
|                               |                           | Tsf1 cleaved form              | 40                                          | ++ (7)   | =        | =         |         |
|                               |                           | Tsf1 cleaved form              | 41                                          | ND        | i        |         |         |
| Olfactory proteins            | CG7584                    | Obpd90c                        | 40                                          | + + (7)   | =        | =         |         |
| Others                        | CG17919                   | PEBP                           | 41                                          | ND        | i        |         |         |

<sup>a</sup> Functional categories imposed by Flybase.

<sup>b</sup> CG number applied to each gene following from complete sequencing of the *Drosophila* genome by Celera.

<sup>c</sup> Function as described in Flybase for fully identified gene products or proposed function and/or homology for the others.

<sup>d</sup> Spot labels: letters according to those in Fig. 2; numbers according to those in Fig. 3.

<sup>e</sup> Level of expression for each spot is determined thanks to PDQuest analysis as described in “Experimental Procedures.” i, protein induced after the infection; s, protein suppressed after the infection; + + (x), protein that expression level increased more than 5-fold after the infection (the variation rate is indicated in brackets); -- (x), protein that expression level decreased more than 5-fold after the infection (the variation rate is indicated in brackets); +, protein that expression level increased after the infection with a variation rate lesser than 5-fold; --, protein that expression level decreased after the infection with a variation rate lesser than 5-fold; =, protein that expression level was unchanged after the infection; ND, not detected on the gel.
study showed that the use of narrow-range strips, such as pH 5.5–6.7, significantly increased resolution and sensitivity, allowing the study of proteins present at a low concentration in adult *Drosophila* hemolymph (see Fig. 2).

While silver staining is very sensitive and revealed a large number of spots, silver-stained proteins can be difficult to identify due to a lack of signal in MALDI-MS studies. To overcome this problem, we chose to increase sample loading. This increased loading also allowed the use of colloidal CBB staining, which is less sensitive than silver staining (160 spots detected versus 350) but more compatible with MS analysis afterward (27).

Analyses of CBB gels revealed that 70 of the 160 detected proteins were differentially expressed by at least 5-fold after a fungal or bacterial challenge. Some of these proteins were fully induced or totally suppressed, indicating major effects of bacterial or fungal challenge. Furthermore, the majority of the spots were specifically regulated by one pathogen, whereas only few spots corresponded to proteins altered in all cases of infection. Proteins that were identified are given in Table II.

Proteins that were identified are given in Table II. The identified proteins that fall into these categories will not be further discussed as they act in general metabolic processes. However, it is noteworthy that some of them are regulated at the mRNA level during aging or after oxidative damages (30). The remainder of the discussion will focus on proteins more specifically related to the immune response.

### Proteases and Protease Inhibitors

Two putative prophenoloxidase activating enzymes (proPO-AEs) [CG1102 (see Fig. 2, spot C, and Fig. 3, spot 6)](https://doi.org/10.1074/mcpr.3.2.162) and CG16705 (see Fig. 3, spot 5), were found to be up-regulated upon fungal infection. ProPO-AEs are serine proteases that activate the precursor prophenoloxidase into active phenoloxidase (31). This reaction leads to the production of melanin, which is then deposited on the pathogen. Interestingly, the *Drosophila* genome encodes 11 genes for putative proPO-AEs, but only five were found to be up-regulated after an immune challenge (10). In this proteomic analysis, the expression level of two of the proPO-AEs was strongly increased in the hemolymph after the fungal infection. Lack of observed variation in the products of the other proPO-AE genes previously seen as regulated may indicate a weak variation (less than 5-fold), a concentration into the hemolymph below the limit of detection, or an absence of correlation with mRNA changes. Surprisingly, proPO-AEs CG1102 and CG16705 were not found to be altered by either the bacterial infections or by the clean injury. This may be explained by a local melanization close to the site of inoculation, without spread of the secreted molecules throughout the hemolymph. In contrast to this, as already mentioned, natural fungal infection induces severe lesions everywhere in *Drosophila*, which may contribute to expansion of the immune response.

For the prophenoloxidases, three genes are present in the *Drosophila* genome but only proPO A₁ was observed during this proteomic analysis, and its concentration remained constant whatever the microbial challenge tested.

The expression level of two serpins is altered by the fungal infection. The first one corresponds to Nec (CG1857; see Fig. 3, spot 4), an essential component of the serine protease cascade that leads to the activation of Spaetzle, the Toll ligand. Nec is clearly overexpressed after the natural fungal infection but not after bacterial challenge. This result differs from the previously published DNA microarray data, where overexpression of the corresponding mRNA was observed for the three types of infection. Interestingly, a parallel Western blot analysis using an anti-Nec antibody (4) allowed the detection of a second immunopositive spot after fungal infection. Using MS analysis, this spot was identified as a truncated form of Nec, in accordance with its observed molecular
mass of 45 kDa. Comparison between mass fingerprints of the entire and truncated forms of Nec revealed that the cleavage occurred at the N-terminal part, although the precise cleavage site remains to be elucidated. This result agrees with recent data from our laboratory.3 A second serpin (CG6687; see Fig. 2, spots D and E) was found to be induced by fungal infection, and these spots may also correspond to N-terminal truncated forms. Indeed, the observed molecular masses on 2D gels (around 40 kDa) are lower than that calculated for the full-length molecule (49 kDa), and the first 100 residues were not seen after MS analysis of these spots.

**Recognition Molecules**

In this study, by far the most strongly induced spot seen after fungal infection corresponded to a GNBPs-like, CG13422 (see Fig. 3, spot 7). This molecule belongs to a family of putative pattern recognition proteins, the GNBPs/β-1,3-glucan recognition proteins, but differs from the other GNBPs by the lack of a β-glucanase domain. The first GNBPs was originally named after its isolation from the silkmoth Bombyx mori as a protein binding to Gram-negative bacteria (32). However, further studies showed that some members of this family are not exclusively implicated in Gram-negative recognition (33), while others have completely different specificities.2 In Drosophila, recent genetic data have shown that two GNBPs (GNBP 1 and GNBP 3) were clearly involved in the Toll pathway at the level of pathogen recognition. A mutation in the Osiris gene, which encodes GNBP 1 (CG6895), blocks the activation of the Toll pathway in response to Gram-positive infections but not to fungal nor to Gram-negative infections.2 A mutation in GNBP 3 (CG5008), encoded by a gene called Hades, is required for the activation of the Toll pathway after fungal infection.4 Our results suggest that a second member of GNBP family, CG13422, is involved in fungal recognition. In agreement with a function similar to that of GNBP 3, CG13422 is phylogenetically closer to it than to other GNBP s. The implication of GNBP proteins in fungal sensing fits with a capacity to bind glucan, a component of the fungal cell wall.

Among the putative pathogen recognition proteins, only imaginal disc growth factor (IDGF)-2 and IDGF-3 are affected by an immune-challenge. IDGF proteins contain a putative chitin-binding lectin domain, but their role in the immune function remains to be established.

**Complement-like Protein**

The last protein clearly related to the immune response and highlighted by this study is a complement-like protein called thiolester-containing protein 4 (TEP4; see Fig. 2, spot A), belonging to the complement C3/α2-macroglobulin superfamily. α2-Macroglobulins are evolutionary ancient protease inhibitors, which react by a unique mechanism involving the proteolytic cleavage of the target protease and its physical entrapment within the folds of the inhibitor (reviewed in Ref. 34). In Drosophila, six TEP-encoding genes have been identified, four of which have a thiolester site perfectly conserved. Three of them, TEP1, 2, and 4, are transcriptionally up-regulated after an immune challenge (9, 10, 35, 36). In Anopheles gambiae, it was shown that aTEP1 serves as a complement-like opsonin and promotes bacterial phagocytosis in the mosquito hemocyte-like cell line 5.1 (37). Moreover, the binding of aTEP1 to bacteria occurs through the thiolester bond, leading to the cleavage of aTEP1. Interestingly, our results showed the appearance of a TEP4 fragment in the hemolymph of Drosophila after fungal infection. The observed molecular mass on 2D gels was 97 kDa, whereas the full-length TEP4 was expected to be 168 kDa. Sequence coverage observed by MS analysis was from residue Tyr-1 to Trp-639, indicating that the fragment corresponded to the N-terminal part of the protein. No tryptic fragment corresponding to peptides following Trp-639 was seen in MS measurements, preventing the exact localization of the cleavage site. However, we can conclude from the molecular mass of the fragment observed in the 2D gels that cleavage should occur further toward the C-terminal than this residue. The use of other enzymes during in-gel digestion would give another peptide fingerprint and help to define the cleavage site more precisely.

**Molecules with Putative Roles in Immune Response**

**Odorant-binding Protein**—This proteomic study allowed the identification of a protein that belongs to the insect odorant-binding protein (OBP) family, Obp 98c, as immune-responsive (see Fig. 3, spot 40). Based on their tissue-specific expression, these proteins are thought to participate in sensing odors and/or pheromones. In addition, OBP family members have been found in nonsensory tissues where they may act as transporters for other types of hydrophobic molecules (38). Recently, two pherokines related to the OBP family were shown to be induced by viral and bacterial infections, Phk-2 and Phk-3, respectively, suggesting a role for these molecules in the recognition and/or neutralization of invading microorganisms (8). Our results support this hypothesis, showing that another member of the OBP family is specifically overexpressed after fungal infection. In contrast to this overexpression, Obp 99c appeared to be repressed after bacterial infections. Such specificity in the alteration of Obp 99c indicates the merit of further studies of these proteins in the context of the immune response.

**Peptidylglycine α-Hydroxylating Monoxygenase**—After fungal infection, the peptidylglycine α-hydroxylating monooxygenase (PHM; see Fig. 2, spot B) was found to be induced. PHM is the rate-limiting enzyme for C-terminal α-amidation, a specific and necessary modification of numer-

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3 N. Pelte and J. M. Reichhart, manuscript in preparation.
4 M. Gottar and D. Ferrandon, manuscript in preparation.
ous secretory peptides (39). In relation to the immune response, many AMPs from different structural families share C-terminal amidation (40–43). In Drosophila, for example, cecropin exhibits this post-translational modification. Drosophila diptericin is thought to be C-terminal amidated, as seen in Phormia (44). Several studies report an increased activity of the AMPs following amidation and a decrease in activity when the amide is removed. Examples of positive effects of C-terminal amidation include the increase in dermaseptin S3 potency against pathogens (45) and the restoration of activity of a synthetic peptide derived from the antimicrobial bovine seminal plasmin (46). One of the most important roles attributed to C-terminal amidation is a higher stability against proteolysis, for example via carboxypeptidase. In Drosophila, among the DIMs secreted into the hemolymph after a septic injury, several occur in a carboxyamidated form (DIM 1, 2, and 4; Swiss-Prot accession numbers P82706, O77150, and P82705, respectively) (7). These molecules are not directly antimicrobial but are suspected to serve as chemokines during the immune response. The strong variation of PHM seen during this proteomic analysis leads us to propose an essential role for this enzyme in the maturation of effector molecules.

Phosphatidylethanolamine Binding Protein—Another of the regulated molecules found in our study and induced upon a Gram-positive infection was the CG17919 gene product (see Fig. 3, spot 41), which contains a phosphatidylethanolamine binding (PEB) domain. The PEB protein (PEBP) family is a highly conserved group of proteins with homologues in a wide variety of organisms (47). Despite its widespread expression, little is known about the role of this protein family. Various functions have been suggested for these molecules, such as binding of lipids (48), inhibition of serine proteases (49), or acting as Drosophila OBPs (50). It has been shown that a rat PEBP, Raf-1 kinase inhibitor protein (RKIP), acts as a negative regulator of the mitogen-activated protein kinase cascade, which controls mitogenesis and cell differentiation (51). RKIP was also found to act as an inhibitor of NF-κB activation, the transcriptional factor required for the up-regulation of a large number of genes in response to infection, viral or bacterial infections, and other stress stimuli (52). The data suggest an important role for PEBP family, particularly in coordinating regulation of the various signaling pathways. The strong regulation of CG17919 shown in this study after a Gram-positive bacterial infection, associated with the large similarities between components of the Drosophila Toll pathway and the mammalian NF-κB-dependent pathway, suggest a potential role of PEBPs in the innate immune response of both invertebrates and vertebrates.

Transferrin and Ferritin—Variations in expression of two molecules involved in iron metabolism, transferrin (see Fig. 3, spots 25 and 26) and ferritin (see Fig. 3, spots 35 and 38), were observed during this study. Transferrin (Tsf) was detected at a basal level in control 2D gels as a series of several post-translationally modified forms (72 kDa, pI 6.8–7.5). All these forms were overexpressed after fungal infection. In contrast, ferritin levels were found to be decreased by fungal challenge. Tsf is a major transporter for iron in vertebrate blood. It is classified as an acute-phase protein, the concentration of which is highly modified under conditions of stress or infection. In Drosophila and Aedes, Tsf was shown to be up-regulated after bacterial infection (53, 54) and in mosquitoes during the encapsulation of filarial worms (55). These data support the idea that this transporter may sequester iron from invading organisms, depriving them of an essential growth element. Furthermore, lactoferrin, a related bactericidal and bacteriostatic molecule, was also implicated in the prevention of Pseudomonas aeruginosa biofilm development at concentrations below those that kill or prevent growth of the bacteria (56). Our results constitute the first example of the regulation of an insect Tsf after a fungal infection, indicating that this iron-binding protein may also be involved in the immune response against fungi. On the pathogen side, iron-binding proteins are suspected to participate in host-bacteria interactions. For example, genes encoding iron transport proteins were found to be essential for the in vivo virulence of Serratia marcescens invading Caenorhabditis elegans (57).

In addition to the increase in Tsf production, fungal infection also induced proteolytic cleavage of Tsf (see Fig. 3, spots 27–33). Several spots corresponding to either N-terminal or C-terminal fragments were identified on 2D gels from B. bassiana-infected Drosophila. As fungi are known to release chitinases and proteases during host penetration, it cannot be excluded that such proteases of fungal origin may be involved in these cleavage events. However, preliminary results indicated that the cleavage of Tsf occurred without any fungal challenge in a Drosophila mutant overexpressing the endogenous protease Psh. As the same fragments of Tsf were observed in fungally infected wild-type flies and in noninfected psh mutant flies, it is reasonable to assume that the cleavage of Tsf, observed on 2D gels after a natural fungal infection, resulted from the action of a Drosophila protease that may be or not Psh. Up to now, involvement of Tsf cleavage products in the immune response of invertebrates had not been described, while two studies mention a potential link between Tsf fragments and the immune response in vertebrates. In one study, OTAP-92, a cationic fragment of hen ovotransferrin, was shown to possess antimicrobial activity, killing Gram-negative bacteria through a membrane damage mechanism. OTAP-92 has a structural motif similar to that of insect defensins (58). In a second recent study, Belosevic et al. showed that truncated forms of Tsf induced the production of nitric oxide by lipopolysaccharide-stimulated goldfish macrophages, whereas the full-length Tsf did not (59). The authors put forward a model of fish macrophage activation mediated by a noncytokine host protein (i.e. Tsf) (60). These data, in combination with the induction of Tsf fragments in Drosophila hemolymph, lead us to propose their involvement.
in the innate immune response, either as antimicrobial peptides or as inducers. Further studies, using Drosophila mutants altered at different levels in their immune response, are underway to investigate these hypotheses.

In conclusion, this study led to a well-resolved reference map for the proteins present in the hemolymph of adult Drosophila. Qualitative and quantitative differential analyses, after bacterial or fungal challenge, showed the regulation of 70 out of 160 spots, with a 5-fold variation threshold. As compared with transcriptional profiling, where a maximum of 6.5% of the genes were found to be regulated by a microbial challenge with a 2-fold variation threshold (9, 10), the results presented here show the potential of the proteomic approach to identify new molecules in the Drosophila immune response.

In this study, proteins belonging to families already linked with the Drosophila immune response were identified, such as proPO-AEs or serpins. Other molecules were also highlighted, such as Obp 99c, PHM, or Tsf, affording new candidates for further investigation of innate immune mechanisms. Finally, a particularly striking point, which could not have been observed using a transcriptional analysis, was the detection of processed forms of proteins (truncated serpines, truncated TEP4, and Tsf fragments) after the fungal infection. All these results underline the necessity of combining transcriptional and proteomic approaches to get a clear insight into a process as complex as innate immunity.

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† To whom correspondence should be addressed. Tel.: 33-(0)-388-41-70-62; Fax: 33-(0)-388-60-60-22; E-mail: L.Sabatier@ibmc.u-strasbg.fr.

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