Analysis of Signal-dependent Changes in the Proteome of Drosophila Blood Cells During an Immune Response*

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Innate immunity is based on the recognition of cell-surface molecules of infecting agents. Microbial substances, such as peptidoglycan, lipopolysaccharide, and β-1,3-glucans, produce functional responses in Drosophila hemocytes that contribute to innate immunity. We have used two-dimensional gel electrophoresis and MS to resolve lipopolysaccharide-induced changes in the protein profile of a Drosophila hemocytic cell line. We identified 24 intracellular proteins that were up- or down-regulated, or modified, in response to immune challenge. Several proteins with predicted immune functions, including lysosomal proteases, actin-binding/remodeling proteins, as well as proteins involved in cellular responses to oxidative stress, were affected by the immune assault. Intriguingly, a number of the proteins identified in this study have recently been implicated in phagocytosis in higher vertebrates. We suggest that phagocytosis is activated in Drosophila hemocytes by the presence of microbial substances, and that this activation constitutes an evolutionarily conserved arm of innate immunity. In addition, a number of proteins involved in calcium-regulated signaling, mRNA processing, and nuclear transport were affected, consistent with a possible role in reprogramming of gene expression. In conclusion, the present proteome analysis identified many proteins previously not linked to innate immunity, demonstrating that differential protein profiling of Drosophila hemocytes is a valuable tool for identification of new players in immune-related cellular processes. Molecular & Cellular Proteomics 3:796–808, 2004.

Drosophila, like other insects, relies on innate immune reactions to protect itself against infections. Invertebrates and vertebrates have very similar intracellular signaling pathways that regulate innate immune responses (1). Exposure to bacterial lipopolysaccharide (LPS), a component of the outer cell wall of Gram-negative bacteria, represents a model for studying the activation and output of the innate immune system. Several studies have described the gene expression profiles using DNA microarrays of Drosophila larvae and flies in response to microbial infections (2, 3), the cultured Drosophila hemocyte-like Schneider cell line 2 (SL2) (4), and mbn-2 cells after LPS exposure. Although the DNA microarray technology provides a powerful technique for identification of genes that are up- or down-regulated in response to microbial challenge, many cellular processes are regulated at the translational and post-translational level and are therefore not detected by DNA microarray analysis. Direct comparisons of data from microarray and proteomic analyses on the same biological material have revealed significant differences in the ratio between the mRNA and the corresponding protein product for many genes (5, 6). Thus, in many systems no strict correlation exists between the mRNA and protein concentrations, and protein expression levels cannot be easily predicted from quantitative mRNA data (7). Proteomics is a valuable tool for assessing changes in cellular protein expression and modification, providing additional information about cellular signal regulation.

In contrast to gene expression studies, the proteomic approach has so far not been extensively used for analysis of Drosophila cells or tissues, albeit the well-annotated Drosophila genome makes it an ideal organism for protein identification through peptide mass fingerprinting. Recent studies reported the use of two-dimensional gel electrophoresis (2-DE) and MS to identify constituents of Drosophila larval hemolymph secretome (8, 9). Similar approaches were used to study how infection affects the hemolymph secretome, and a number of proteins were identified whose expression and/or secretion were immune responsive (10–12).

In the present comparative proteome analysis, we used the immunocompetent hemocyte-like Drosophila melanogaster mbn-2 cell line, which has been established from larvae of the mutant malignant blood neoplasm (mbn) (13). These cells

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have retained their capacity to phagocytose bacteria and respond to microbial substances by Rel/NF-κB mediated activation of genes coding for antimicrobial peptides (14, 15). The intracellular proteome of Drosophila mbn-2 cells was analyzed following LPS challenge and the identity of intracellular targets of LPS-induced signaling was deduced. We resolved proteins that were post-translationally modified, as well as proteins that changed in abundance, reflecting either redistribution between different cellular compartments or synthesis and degradation in response to signaling. The great benefit of using an organism with a well-annotated genome for proteome analysis is reflected by the fact that we could deduce the identity of more than 90% of the proteins analyzed by MALDI-TOF MS. This shows that Drosophila cells constitute a very good system for proteome analysis and for characterization of signaling events at the post-translational level. Furthermore, our study indicates new roles for many of the identified proteins in the activation and functional implementation of immune processes.

EXPERIMENTAL PROCEDURES

Cell Culture—The Drosophila mbn-2 cell line (13, 16) was grown in Schneider’s medium (Pan Biotech GmbH, Aidenbach, Germany) with 5% FCS, 6× Glutamax I (Life Technologies, Inc., Grand Island, NY), 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamycin at 25 °C. To obtain whole cellular lysate, the cells were washed twice in PBS and solubilized in the lysis buffer 8 m urea, 4% CHAPS, 4 mg/ml DTT, 2% IPG buffer 3–10 NL, 300 U/ml endonuclease. To inhibit protease, kinase, and phosphatase activities, a complete protease inhibitor mixture (Boehringer-Mannheim, Mannheim, Germany), 10 mM NaF, and 1 mM Na3VO4 (Sigma, St. Louis, MO) was added to all buffers. The suspension was sonicated, incubated at 4 °C for 1 h, and then centrifuged to sediment cell debris.

To stimulate immune reactions, 10 μg/ml LPS (Escherichia coli O55:B5; Sigma) was added to the culture, and the cells were collected after 30 min and 6 h challenge. Commercially available LPS preparations have been shown to contain low amounts of other microbial substances, such as peptidoglycan fragments, and it cannot be ruled out that some of the effects monitored upon LPS treatment were due to such contaminating microbial substances. The cells were harvested by centrifugation at 2,500 rpm for 10 min. To take advantage of the compartmentalization of a eukaryotic cell, we used subcellular fractionation and prepared cytoplasmic and nuclear fractions from subconfluent mbn-2 cells cultures. The cellular pellet was suspended in cold buffer A: 10 mM HEPES, pH 7.8, 15 mM KCl, 2 mM MgCl2 and centrifuged at 2,500 rpm for 10 min. The cells were resuspended in buffer A plus 1 mM DTT and lysed in a glass dounce homogenizer, followed by addition of 1/10 volume of buffer B (50 mM HEPES, pH 7.8, 1 mM KCl, 30 mM MgCl2, 1 mM DTT) to make the homogenate isotonic. The cell homogenate was centrifuged at 6,580 × g for 20 min, and the obtained pellet and supernatant were designated as nuclear and cytoplasmic fractions accordingly. A copurification of cytoskeletal and endoplasmic reticulum (ER) proteins is difficult to avoid as the nucleus is connected to the cytoskeleton of the cells and the outer nuclear membrane is continuous with the ER (17), therefore the nuclear fraction is designated as nuclear ††. Lipids from the nuclear †† fraction were removed with chloroform/methanol, and precipitated proteins were solubilized in lysis buffer containing 8 m urea, 4% CHAPS, 4 mg/ml DTT, 2% IPG buffer 3–10 NL, complete protease inhibitor mixture, 10 mM NaF, and 1 mM Na3VO4. Proteins in the cytoplasmic fraction were precipitated with cold acetone and solubilized in the same buffer. The protein concentration in the samples was determined by a detergent-compatible Coomassie Plus protein assay reagent (Pierce, Rockford, IL).

Two-dimensional Polyacrylamide Gel Electrophoresis—IEF was performed using 13-cm ready-made IPG strips with nonlinear pH 3-10 gradient and IPGphor focusing system (Amersham Biosciences, Piscataway, NJ). Fifty micrograms of protein extract was used for an analytical gel and 300 μg of protein extract for a preparative gel. Rehydration solution (8 μl urea, 4% CHAPS, 4 mg/ml DTT, 1% IPG buffer 3–10 NL, bromophenol blue) was added to the protein extracts in lysis buffer to a final volume of 250 μl. The IPG strips were rehydrated for 12 h, and IEF was performed for 25,000–30,000 Vh. Before SDS-PAGE, the IPG strips were first equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, 6 μl urea, 30% glycerol, 2% SDS, 10 mg/ml DTT, and then for 15 min in the same buffer with 25 mg/ml iodoacetamide instead of DTT. After equilibration, the strips were placed on the top of 12% gels and embedded in 1% hot low-melt agarose in SDS electrophoresis running buffer 25 mM Tris, 192 mM glycine, 0.1% SDS. SDS-PAGE was performed in Hoefer SE600 gel electrophoresis unit in 1 mm thick 16 × 14-cm gels at 20 mA per gel. The gels were stained with silver by using commercial silver staining kit PlusOne and protocol from Amersham Biosciences. The samples were prepared from two independent cell populations, and two gels were run for each preparation, totaling four gels for control and four gels for treated samples.

For analysis of the electrophoretic mobility of calmodulin, 2 μM EDTA or 0.1 mM CaCl2 was added to the SDS electrophoresis running buffer and polyacrylamide gels. The gels were stained with Brilliant blue G-colloidal (Sigma).

Image Analysis of Two-dimensional Gels—Silver-stained gels were digitized using Luminescent Image analyzer LAS-1000plus (Fuji Film,
### Table I

The proteins from mbn-2 cells of *Drosophila melanogaster* identified by MS

| Spot no. | Gene identity | Protein name | Theoretical Sequence coverage (%) | Theoretical pl | MW (Da) | Sequence coverage (%) |
|----------|---------------|--------------|-----------------------------------|----------------|---------|----------------------|
| 2        | CG4264        | Heat shock 70-kDa protein cognate 4 (Hsc70-4) | 5.36 71,372 | 45          |
| 3        | CG3842        | Heat shock 70-kDa protein cognate 5 (Hsc70-5) | 5.80 74,248 | 43          |
| 4        | CG12101       | Heat shock protein 60 (Hsp60) | 5.38 60,885 | 56          |
| 5        | CG7033        | T-complex protein 1, subunit β | 5.68 58,223 | 49          |
| 6        | CG3231        | Chaperonin ATPase | 6.18 58,495 | 38          |
| 7        | CG3720        | Hsp70/Hsp90 organizing protein homolog (Hop) | 6.24 55,920 | 34          |
| 11       | CG8983        | Protein disulfide isomerase (ERp60) | 5.62 55,547 | 38          |
| 16       | CG5809        | Protein disulfide isomerase (CaBP1) | 5.48 47,179 | 31          |
| 20       | CG1837        | Heat shock protein | 5.46 25,824 | 30          |
| 27       | CG4164        | Heat shock protein | 5.57 13,049 | 48          |
| 36       | CG1803        | Calreticulin (Crc) | 4.39 47,122 | 37          |
| 43       | CG19999       | Inositol triphosphate receptor, MIR domain | 6.29 23,847 | 53          |
| 53       | CG4254        | Protein disulfide isomerase | 5.16 47,362 | 54          |
| 55       | CG3595        | Myosin II regulatory light chain, nonmuscle (sqh) | 4.21 17,827 | 55          |
| 63       | CG1890        | Tubulin-specific chaperone | 4.31 40,585 | 29          |
| 64       | CG1341        | Component of 19S proteasome (Rpt1) | 5.75 48,910 | 47          |
| 29       | CG8882        | Translation initiation factor (Trip1) | 5.17 36,365 | 33          |
| 31       | CG4634        | Nucleosome remodeling factor (Nurf-38) | 5.43 32,858 | 39          |
| 32       | CG14792       | Ribosomal protein S2 (stbarista) | 4.76 30,266 | 39          |
| 33       | CG3644        | Bicaudal-transcription factor (bic) | 6.85 17,727 | 62          |
| 35       | CG12030       | UDP-galactose 4-epimerase | 6.31 30,266 | 39          |
| 42       | CG1721        | Phosphoglycerate mutase (Pglym78) | 6.62 28,612 | 52          |
| 47       | CG6030        | ATP synthase, subunit d (ATPsyn-d) | 6.10 20,245 | 56          |
| 49       | CG11981       | Proteasome endopeptidase 3 (Pros3) | 5.28 23,618 | 40          |
| 51       | CG3644        | Bicaudal-transcription factor (bic) | 6.85 17,727 | 62          |
| 52       | CG2952        | Cyclophilin-peptidyl-prolyl cis-trans isomerase | 6.69 22,185 | 54          |
| 56       | CG3186        | Eukaryotic initiation factor 5A (eIF-5A) | 4.98 17,922 | 58          |
| 58       | CG11271       | 40S ribosomal protein S12 (RPS12) | 5.93 15,501 | 44          |
| 60       | CG4087        | 60S acidic ribosomal protein (RPLP1) | 4.30 11,550 | 50          |
| 62       | CG11001       | FK506-binding protein-peptidyl-prolyl cis-trans isomerase (FK506-bp2) | 7.85 11,716 | 68          |
Tokyo, Japan). Two-dimensional gel imaging and analysis software PDQuest 7.1 (Bio-Rad, Hercules, CA) was used for quantification of protein spots, gel-to-gel matching, and identification of differences between the control and treated samples. Gel images were normalized so that the total quantity in valid spots on analyzed gels was made equal. Protein spots with changes greater than 2-fold in magnitude, as observed by comparing four gels of control and four gels of LPS-treated cells, were analyzed further. The LPS-induced change in integrated intensity of these spots was subjected to statistical analysis with a Student's t test. The spots with statistically significant changes (p < 0.05) were excised from preparative gels (300 μg of total protein), and the proteins were identified by peptide mass fingerprinting.

**MALDI-TOF MS Analysis and Protein Identification—** A silver staining method, which is compatible with MS, was used. This method omits the use of glutaraldehyde in the sensitization step and formaldehyde in the silver impregnation step. The stained protein spots were excised from preparative gels using stainless steel blades. A modified sample preparation method was used, which incorporates a destaining step to remove silver prior to in-gel digestion with trypsin. Briefly, silver ions were removed with a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. Next, the gel pieces were washed with water, then with 50% ACN in 25 mM ammonium bicarbonate and finally dehydrated with 100% acetonitrile. Proteins were in-gel digested with sequencing grade-modified trypsin (Promega V511A; Madison, WI) as described in (20), except that reduction and alkylation steps were omitted because cysteines were carbamidomethylated on the equilibration step of 2-DE. The resultant peptides were extracted with 50% ACN/5% TFA and dried in the vacuum centrifuge. If necessary, the recovered peptides were purified and concentrated on C18ZipTips (Millipore, Bedford, MA) according to the manufacturer’s instructions.

Mass spectra were recorded in positive reflection mode by using an Applied Biosystems MALDI-TOF Voyager-DE STR mass spectrometer (Foster City, CA) equipped with a delayed ion extraction technology. α-Cyano-4-hydroxycinnamic acid was used as the matrix. The TOF was measured using the following parameters: 20 kV accelerating voltage, 200 ns delay, low mass gate of 700 Da, and acquisition mass range 800–4,000 Da. External calibration was performed using the Sequazyme Peptide Mass Standard kit with Angiotensin I (1,296.6853 Da) and ACTH clips 1–17 (2,093.0876 Da), 16–39 (2,465.1989 Da), 7–38 (3,657.9294 Da) (PerSeptive Biosystems, Foster City, CA), and for internal calibration autodigestion peaks of bovine trypsin were used.

The peptide mass profiles produced by MS were analyzed by using the programs Mascot (www.matrix sciences.com), ProFound (prowil.rockefeller.edu), and PeptideIdent (www.expasy.org/tools/peptideIdent.html). The monoisotopic peptide masses were compared with the theoretical masses derived from the NCBInr database for *Drosophila melanogaster*. Known masses of trypsin autodigest products were excluded from the searches. Search parameters included allowed mass accuracy of 0.1 Da, more than four peptide mass hits required for a protein match, consideration of one missed enzymatic cleavage, pl range of 3.0–10.0, and molecular mass range of 1–200 kDa. Accepted modifications included carbamidomethylation of cysteine residues and methionine in oxidized form. The program FindMod (www.expasy.org/tools/findmod/) was used to find modifications for analysis of unmatched peptide masses. Gene identity (CG number) and predicted protein function are found at the FlyBase Report (www.flybase.bio.indiana.edu).

**RESULTS**

The Establishment of a *Drosophila* mbn-2 Cell Two-dimensional Electrophoresis Map—High-resolution 2-DE was used for separation of complex protein mixtures of *D. melanogaster* hemocyte-like mbn-2 cells and two-dimensional gel images were analyzed to visualize the protein content of these cells. The established *Drosophila* mbn-2 cell reference map is shown in Fig. 1. There are ~1,200 spots detected on the map using the Bio-Rad PDQuest image analysis software. In an initial phase of protein identification, we randomly excised protein spots from a preparative gel to create the map. Sixty-five proteins were identified by MALDI-TOF on the basis of peptide mass matching, following in-gel digestion with trypsin. The analysis of peptide mass fingerprinting data is summarized in Table I. Among the identified proteins there are well-known cytoskeletal proteins and antioxidants, and a large number of proteins falls into the categories of chaperones, enzymes, and Ca^{2+}-binding proteins. Comparison of our mbn-2 cell proteome data and the published maps of *Drosophila* larval hemolymph revealed the presence of several common functional groups as heat shock proteins and chaperons, antioxidant defense components, structural proteins, and metabolic enzymes (8, 9). Some of these overlaps probably stem from the fact that hemocytes make up about 10% of the total hemolymph volume, explaining why several intracellular proteins were detected in the hemolymph samples (8, 9). In addition, some proteins are expressed both as intracellular and secreted forms.

**LPS-induced Changes in the mbn-2 Cell Proteome—** Microbial challenge of *Drosophila* cells elevates innate immune responses within 30 min, with activation remaining high for several hours (2–4, 14). The proteome of nuclear^{2+} and cytoplasmic fractions were analyzed after 30 min and after 6 h of LPS treatment using replicate two-dimensional gels. It is
known that effector molecules, like antimicrobial peptides and proteins, are up-regulated in mbn-2 cells in response to microbial challenge and secreted into the surrounding medium (14). The present study focused on the intracellular proteome of hemocyte-like cells, and the pre-electrophoresis cell washes removed secreted proteins. The results of computer-assisted image analysis of two-dimensional gels revealed a number of protein spots that were up- or down-regulated, or modified in response to the LPS treatment. The targets of the LPS response disclosed in this analysis included proteins involved in mRNA processing, nuclear transport, signal transduction, antioxidant reactions, cytoskeleton regulation, protein folding (Tables II and III).

Early Responses to LPS—To analyze early changes in response to signaling, we focused our analysis on the nuclear +fraction of cells treated with LPS for 30 min, as shown in Fig. 2. The rapid changes observed in the nuclear + fraction of LPS-treated cells compared with untreated control cells reflect most likely regulated post-translational modifications and traffic over the nuclear membrane, rather than induced gene expression and de novo protein synthesis. Among the affected proteins (see Table II) are several RNA processing and chromatin binding proteins, signaling molecules, nuclear pore complex protein, and lamin Dm. As has been explained in “Experimental Procedures,” the nuclear + fraction contains also cytoskeleton-related and ER proteins.

Beneath the inner nuclear membrane there is a fibrillar network called the nuclear lamina, which also is attached to chromatin and nuclear pores (21). The nuclear lamins are involved in a number of functions including nuclear envelope support and assembly, DNA synthesis, transcription, and apoptosis. Drosophila contains two lamin genes, lamin C and lamin Dm 0, but the term lamina refers solely to protein products encoded by the Dm 0 gene (22). The expression of this gene in Drosophila yields numerous lamin polypeptides that may be the result of post-translational modifications or variation due to alternative splicing. We identified accumulation of several lamin isoforms after LPS treatment (arrows in Fig. 3), indicating that the nuclear lamina takes part in cellular changes induced by the immune activation. This is consistent with a suggested role of nuclear lamins in regulation of gene expression (23).

LPS treatment modulated proteins that normally participate in protein transport and ligand binding (Table II). Drosophila CG6251 or Nup62 is a glycoprotein in the nuclear pore complex (NPC). The increased nuclear concentration of Nup62 after 30 min of LPS treatment (Figs. 2 and 5, spot 70) possibly reflects an activation of protein transport to the nucleus in response to immune challenge. We found that the abundance of α-glucosidase (similar to protein kinase C substrate) and the Drosophila homolog of the receptor of activated protein kinase C (RACK1) increased after 30 min of LPS treatment (Table II, Fig. 2, spots 67 and 73). RACK1 is an adaptor molecule that binds the activated form of protein kinase C (PKC) and directs the localization of the activated enzyme to distinct cellular compartments (24). α-Glucosidase, a highly acidic 60-kDa protein, contains negatively charged glutamic acid repeats that contribute to the anomalously slow migration of this molecule in SDS-PAGE as 80-kDa protein (25). α-Glucosidase (Figs. 2 and 5, spot 67), a protein with a Ca 2+ -binding EF-hand domain, is involved in protein folding and quality control of N-glycosylated proteins (26).

Interestingly, the LPS treatment of mbn-2 cells caused modifications of another calcium binding protein, the pluripotent Ca 2+ sensor calmodulin (CaM). We observed an altered migration pattern of CaM both after 30 min and 6 h of LPS challenge (Figs. 2, 4, and 5, spot 54). Partial sequencing of

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**Table II**

| Spot no. | Gene identity | Protein name (Gene name/symbol) | Theoretical pl | M r (Da) | Sequence coverage (%) | Function | Character of regulation/modification |
|----------|---------------|---------------------------------|---------------|---------|----------------------|----------|-------------------------------------|
| 66       | CG10689       | RNA helicase                     | 6.12          | 102,834 | 25                   | Pre-mRNA splicing factor | Increased concentration |
| 67       | CG6453        | α-1Glucosidase                   | 4.37          | 62,414  | 20                   | Involved in protein folding control of glycoproteins | Increased concentration |
| 68       | CG6944        | Lamin Dm (Lam)                  | 6.10          | 71,371  | 54                   | Nuclear matrix | Increased concentration of several isoforms |
| 69       | CG9373        | RNA binding protein             | 7.66          | 66,862  | 32                   | mRNA processing | Increased concentration |
| 70       | CG6251        | Nuclear pore p62 (Nup62)        | 4.70          | 40,659  | 31                   | Component of nuclear pore complex | Increased concentration |
| 71       | CG1837        | Protein disulfide isomerase     | 5.16          | 47,363  | 54                   | Chaperone | Increased concentration of one isoform |
| 72       | CG10159       | Boundary element-associated factor of 32 kDa (BEAF-32) | 6.10          | 31,985  | 54                   | DNA binding, transcription regulation | Increased concentration |
| 73       | CG9066        | Hypothetical protein with hem/ steroid binding domain | 4.60          | 28,018  | 65                   | Receptor activity | Increased concentration |
| 74       | CG7111        | Receptor of activated protein kinase C1 (Rack1) | 7.14          | 36,109  | 61                   | Regulation of phosphorylation | Increased concentration |
| 74       | CG3501        | Hypothetical protein            | 5.65          | 22,995  | 63                   | Unknown function | Up-regulation of one isoform |
| 54       | CG8472        | Calmodulin (Cam)                | 4.09          | 16,669  | 64                   | Control of a large number of enzymes by Ca 2+ | Altered migration pattern |
CaM by ESI-MS/MS showed that the N-terminal peptide has an acetylated alanine without an N-terminally deduced methionine (ADQLTEEQIAEFK). Notably, this analysis also revealed that the N-terminal and C-terminal fragments of CaM were identical from control and treated samples, verifying that the N-terminal and C-terminal fragments of CaM were not the result of proteolysis.

An overwhelming amount of data implicates cytoskeletal proteins and their regulators as central to various functions in immune cells. Several actin-binding/remodeling proteins, like profilin and coflin/tropomyosin type, were up-regulated following 6 h of LPS exposure (Table III, Figs. 4 and 5, spots 64 and 81). Cofilin (twinstar) has two isoforms with different pl: the main form, which showed good accordance between experimental and theoretical pl 6.7 (Figs. 1 and 4, spot 53a), and a minor form with decreased apparent pl 5.5, as determined for regulation through phosphorylation (29). Up-regulation of the phosphorylated form of cofilin (twinstar) was observed after LPS treatment (Figs. 4 and 5, spot 53b).

Oxidative stress, an indispensable attribute of LPS action in mammalian cells, disrupts the balance between production of reactive oxygen species and their removal by endogenous antioxidants. Treatment of the mbn-2 cells with LPS affected proteins that are involved in the cellular response to oxidative stress. After 6 h of LPS action, we observed an alteration of the 1-Cys peroxiredoxin pattern. The two-dimensional map of the cytoplasmic fraction showed two isoforms of 1-Cys peroxiredoxin, and in control samples the spot with low pl and several isoforms were observed after LPS treatment (Figs. 4 and 5, spot 53b).

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| Spot no. | Gene identity | Protein name | Theoretical pl | Sequence coverage (%) | Function | Character of regulation/modification |
|----------|---------------|--------------|----------------|-----------------------|----------|---------------------------------------|
| 5        | CG7033        | T-complex protein 1, β subunit | 5.68 | 71 | Chaperone for tubulin and actin folding | Up-regulation |
| 75       | CG8439        | T-complex protein 1, ε subunit (Cct5) | 5.78 | 36 | Chaperone for tubulin and actin folding | Up-regulation |
| 76       | CG10843       | Cytochrome P450 (Cyp4p3) | 6.07 | 19 | Monooxygenase, antioxidant | Up-regulation |
| 77       | CG3902        | Acyl-CoA dehydrogenase | 6.28 | 33 | Fatty acid metabolism | Down-regulation |
| 28       | CG1548        | Cathepsin D (cathD) | 5.90 | 33 | Aspartic protease | Down-regulation |
| 33       | CG6692        | Cathepsin L (Cp1) | 6.12 | 36 | Cysteine protease | Down-regulation |
| 78       | CG5730        | Annexin IX (AnnIX) | 4.92 | 27 | Actin remodeling, Ca-binding protein | Up-regulation |
| 79       | CG8947        | Cathepsin K (26–29 kDa proteinase) | 6.26 | 22 | Cysteine protease | Down-regulation |
| 80       | CG12405       | 1-Cys Peroxiredoxin (Pnx2540–1) | 5.97 | 84 | Antioxidant | Down-regulation of one isoform |
| 53a-53b  | CG4254        | Coflin (twinstar) | 6.74/5.6 | 57 | Actin remodeling | Up-regulation of phosphorylated isoform |
| 54       | CG8472        | Calmodulin (Cam) | 4.09 | 64 | Control of a large number of enzymes by Ca\(^{2+}\) | Altered migration pattern |
| 81       | CG6891        | Cofilin/tropomyosin type | 4.90 | 51 | Actin remodeling | Up-regulation |
| 82       | CG6878        | FABP | 5.56 | 55 | Transporter of hydrophobic molecules | Up-regulation |
| 64       | CG9553        | Profilin (chickadee) | 5.20 | 73 | Actin remodeling | Up-regulation |
mechanism against oxidative stress, by reducing the generation of free radicals (30). On the other hand, up-regulation of lipocalin-related fatty acid binding protein (FABP) following LPS exposure (Figs. 4 and 5, spot 82) could be part of a removal system of fatty acids. The FABPs are a family of proteins that act as intracellular transporters of hydrophobic metabolic intermediates and take parts in modulation of signal transduction and gene transcription (31). It is worth mentioning that LPS treatment of the murine monocyte/macrophage cell line also led to significant changes in the concentration of a lipid-binding protein (32), as well as in LPS-treated Drosophila larvae (11).

Cytochrome P450, which is responsible for metabolism of xenobiotics, was also an LPS up-regulated target (Table III, Figs. 4 and 5, spot 76). Cytochrome P450s support the oxidative, peroxidative, and reductive metabolism of a wide variety of xenobiotics and endogenous substrates (33) and are responsible for metabolic resistance to insecticides (34). The transcriptional induction of the cytochrome P450 gene was also revealed at a genome-wide transcriptome analysis of Drosophila immune responses (2).
Several proteins with important functions in protein destination were affected in mbn-2 cells by the LPS treatment: two subunits of the chaperonin-containing T-complex and cathepsins (Table III). The transcriptional induction of T-complex protein 1 was also identified in a microarray analysis of *Drosophila* immune responses (2). The aspartic protease cathepsin D, which is an important component of the lysosome, was presented in at least seven isoforms in mbn-2 cells. LPS treatment provoked disappearance of four of these isoforms (Figs. 4 and 5, spots 28), indicating different modes of processing and modifications of this enzyme after LPS treatment. Interestingly, we also found that the lysosomal cysteine proteases cathepsin L (Figs. 4 and 5, spot 33) and cathepsin K (26/29 proteinase) (Figs. 4 and 5, spot 79) diminished after 6 h of LPS treatment. These proteases are synthesized as inactive pro-enzymes that are activated by proteolytic cleavage (35, 36). In trypsin digests of the 37-kDa cathepsin L, we identified the peptide corresponding to residues 91–102, which is located up-stream of the predicted cleavage site at Leu-135 of the mature enzyme (37), demonstrating that spot 33 corresponds to the unprocessed, pro-form of the protease. Therefore it is possible that the observed decrease of the 37-kDa form of cathepsin L reflects processing of the pro-enzyme into the active form in response to LPS treatment. Similar results were observed in *Drosophila* SL2 cells with phagocytosed beads (36). Taken these data together indicate that some lysosomal proteases are processed in response to LPS treatment and stimulation of phagocytosis.

**DISCUSSION**

The present study demonstrates that 2-DE integrated with MS is a useful approach in consolidating protein changes in *Drosophila* immune cells associated with functional responses to LPS. In total, about 90 protein spots were analyzed in this study with MALDI-TOF MS, and the protein identity for more than 90% of these could be identified unambiguously. This demonstrates that the genome of *Drosophila* is well-annotated and that this is a very suitable organism for proteome analysis. We identified 24 proteins whose variations in concentration, as well as modifications, were the result of specific reactions in these cells, activated by the LPS challenge.

**Ca²⁺-binding Proteins**—Not much is known about Ca²⁺-dependent regulation of innate immunity in *Drosophila*. It was therefore interesting to note that several Ca²⁺-binding proteins were affected by LPS treatment of the mbn-2 cells. CaM

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**Fig. 4.** Two-dimensional maps of cytoplasmic fractions from control and LPS-treated mbn-2 cells. Fifty micrograms of total protein was loaded, and ~900 spots were detected. Proteins regulated after 6 h of LPS treatment are numbered. Spot numbers refer to identification in Table III.
is a highly conserved acidic Ca$^{2+}$-binding protein presented in all eukaryotic organisms, which has been implicated in a myriad of cellular signaling processes (38). CaM is involved in the regulation of transcription not only indirectly through CaM-dependent kinases and phosphatases (39), but also directly by interaction with transcription factors. There is evidence that some mammalian Rel/NF-$\kappa$B proteins are regulated through interaction with CaM (40). It is tempting to speculate that CaM is involved in transducing Ca$^{2+}$-dependent changes in gene expression in response to immune signaling. Further analysis of the cause and consequences of Ca$^{2+}$ alterations should help to understand which role CaM and other Ca$^{2+}$-binding proteins might play in innate immune reactions.

Annexin IX, which was up-regulated in cytoplasmic fractions of LPS-treated mbn-2 cells (Figs. 4 and 5, spot 78), was previously identified among the immune-regulated Drosophila genes using whole genome oligonucleotide microarrays (2, 3). Its functional role during an immune response is not yet understood. Annexins are defined as a family of soluble, hydrophilic proteins that bind to negatively charged phospholipids in a Ca$^{2+}$-dependent manner. They have been implicated in the regulation of phagocytosis, cell signaling, and membrane-associated cytoskeleton (41). A number of annexins bind to F-actin and also modify the profilin effect on actin polymerization. Some annexins have been linked to exocytotic processes, more specifically post-trans-Golgi network events in the biosynthetic pathway. Translocation of different annexins to the membrane of maturing phagosomes has been observed in a number of phagocytic cell types and has been taken as an indication for the involvement of these proteins in phagocytosis. It is thus possible that annexin IX plays a role in phagocytosis, but this hypothesis awaits experimental evidence.

**Protein Transport and Ligand Binding**—Drosophila Nup62 is the homologue of the well-known human nuclear porin p62 and yeast NSP1. A direct comparison of the best-studied
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| Spot No. | Protein name | 2-DE patterns | Spot volumes |
|----------|--------------|---------------|--------------|
| 5        | TCP 1β subunit | ![image](image1.png) | ![image](image2.png) |
| 76       | Cytochrome P450 | ![image](image3.png) | ![image](image4.png) |
| 77       | Acyl-CoA dehydrogenase | ![image](image5.png) | ![image](image6.png) |
| 28       | Cathepsin D | ![image](image7.png) | ![image](image8.png) |
| 33       | Cathepsin L | ![image](image9.png) | ![image](image10.png) |
| 78       | Annexin IX | ![image](image11.png) | ![image](image12.png) |
| 79       | Cathepsin K | ![image](image13.png) | ![image](image14.png) |
| 80       | 1-Cys peroxiredoxin | ![image](image15.png) | ![image](image16.png) |
| 53b      | Cofilin (twinstar) phosphorylated form | ![image](image17.png) | ![image](image18.png) |
| 81       | Cofilin/ tropomyosin | ![image](image19.png) | ![image](image20.png) |
| 64       | Profilin | ![image](image21.png) | ![image](image22.png) |
| 82       | Fatty acid binding protein | ![image](image23.png) | ![image](image24.png) |

Fig. 5—continued
NPCs from yeast and Xenopus revealed that the NPC structure is evolutionarily conserved and has a common fundamental architecture (42). It has been suggested that NSP1/Nup62 does not have a fixed position in the nuclear pores and that it is directly involved in nuclear export/import (43). The accumulation of Nup62 in the nuclei of LPS-treated mbn-2 cells may reflect a more active protein transport between the cytoplasm and the nucleus in response to LPS treatment (Figs. 2 and 5, spot 70). This would be consistent with the well-studied nuclear accumulation of the Rel/NF-κB and STAT transcription factors in response to immune induction in insects. In addition, it has been shown that the nuclear transport factor 2 (NTF-2), which is essential for nuclear import of the Rel/NF-κB proteins during an immune response in Drosophila, interacts physically with Nup62 (44, 45). Thus the complex of NTF-2 and Nup62 may facilitate the passage of material from the cytoplasm into the nucleus and may be crucial for the import of regulatory proteins like Rel/NF-κB in response to LPS signaling. In a two-hybrid-based protein-interaction analysis of the fly proteome Nup62 was identified in association with 21 proteins (46). One of these was the nucleoporin, dNup88 (members only, mbo), which has been shown to be selectively required for the nuclear translocation of the Rel family transcription factors Dorsal and Dif (47). In conclusion, there is a clear link between several NPC proteins and the nuclear import of Rel/NF-κB proteins in Drosophila. The accumulation of Nup62 in response to LPS treatment corroborates this connection even further and indicates a possible role of Nup62 in selective import/export of regulatory proteins during an immune response.

The Drosophila RACK1, which was up-regulated after 30 min of LPS treatment (Fig. 2, spot 73), is an important component of the PKC-mediated signaling pathway. It binds activated PKC to the cytoskeleton, anchors other signaling enzymes, and plays an essential role in the Jak/Stat pathway (48, 49). The possible role of this protein in innate immune response is unresolved. But in a two-hybrid-based protein-interaction analysis of the fly proteome, RACK1 was identified in association with 27 proteins (46), and among them was the protein product of the immune deficiency (imd) gene, which controls antibacterial defense in Drosophila (50).

Actin-remodeling Proteins and Phagocytosis—Phagocytosis is an essential part of the innate immune response against microorganisms. Recent investigations have revealed a pivotal role for two coordinated cellular processes, cytoskeletal alterations and membrane trafficking, in the phagocytic events (51). Phagocytosis by neutrophils, macrophages, and other professional vertebrate phagocytes requires rapid remodeling of actin (52). The mbn-2 cell line was established from Drosophila larval hemocytes with the capacity of phagocytosis (13), and it has kept this function (53). It was previously shown in a microarray study by Boutros et al. (4) that LPS treatment of the Drosophila hemocyte-like cell line SL2 stimulates expression of a number of cytoskeletal proteins in a JNK pathway-dependent manner. In addition, a recent microarray study of the mbn-2 cell line2 has revealed up-regulation of several genes (spire, singed, inscutable, and enabled) with different functions in cytoskeletal organization.

In the present study, LPS treatment led to up-regulation of profilin and cofilin/tropomyosin type. Profilin and cofilins are the very important players in multiple steps in the cycle of actin polymerization and depolymerization (54, 55). Profilin has been suggested to be directly involved in phagocytosis in both Drosophila and Dictyostelium (56). In a search for phagocytosis mutants in Drosophila, a profilin mutant was isolated (53). Interestingly, in this mutant, called chicadee (chic), phagocytosis was increased, suggesting that profilin plays a negative regulatory role in phagocytosis in Drosophila. Profilin is a complex, Janus-like protein involved both in up-regulation and down-regulation of actin polymerization depending on its concentration and the cellular conditions (57). Cofilin (twinstar) has two isoforms, and the phosphorylated form was up-regulated after LPS treatment (Figs. 4 and 5, spot 53b). Phosphorylation of cofilin (twinstar) inactivates its actin-depolymerizing activity and enhances the formation of filamentous actin (58). Increased concentration of actin 5C and cofilin (twinstar) was also observed in the hemolymph of LPS-challenged Drosophila larvae (11).

Using a random RNAi-based screen in Drosophila SL2 cells, 34 gene products involved in phagocytosis were identified (59). This analysis indicated a possible involvement of the cell-surface receptor, peptidoglycan recognition protein PGRP-LC in phagocytosis of Gram-negative bacteria. Three different isoforms of PGRP-LC are expressed by differential splicing, and it has been suggested that different PGRP-LC forms are targets for peptidoglycan and LPS, respectively (60). The chemical nature of the ligand that would bind to PGRP-LC and stimulate phagocytosis is not known, but link
ing our data with the finding that PGRP-LC promotes phagocytosis of Gram-negative bacteria makes it tempting to speculate that LPS is the active ligand.

A comprehensive analysis of the phagosome proteome has been carried out with the J774 mouse macrophage-like cell line, and more than 140 proteins associated with latex bead-containing phagosomes were identified (61). This analysis revealed a close association of ER with phagosomes. The authors showed that ER-resident proteins were genuine components of phagosomes and proposed a new model of ER-mediated phagocytosis. Interestingly, several proteins affected in Drosophila mbn-2 cells after LPS challenge (lamin, protein disulfide isomerase (PDI), peroxiredoxin, annexin, cathepsins D and L, T-complex protein β- and ε-subunits) were mentioned in this new concept of ER-mediated phagocytosis in mouse macrophages (61, 62). Thus, although designated functions of these proteins are not obviously connected with innate immune functions, they fall into the category of proteins involved in ER-mediated phagocytosis in mammals. It remains to be proven whether all of these proteins also play a role in phagocytosis in Drosophila. This could be effectively studied by RNAi-based silencing of the corresponding genes.

In conclusion, the analysis of Drosophila mbn-2 cells after LPS treatment has established a number of interesting targets involved in intracellular processes linked to immune functions such as Ca2+ signaling, nuclear transport, phagocytosis, and cytoskeleton remodeling. Future studies will be designed to increase our understanding of the role of these proteins during the activation of innate immunity.

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