Characterization of Lipoprotein EnvA in Chlamydia psittaci 6BC

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The primary sequence of the small cysteine-rich protein (EnvA) of Chlamydia psittaci 6BC has been shown to possess a potential lipid modification/signal peptide II-processing site, and the mature protein was labeled by a [3H]palmitic acid precursor. We further characterized the mature EnvA, showing that it lacks the N-terminal methionine of the primary peptide, is hydrophobic despite a peptide sequence that is predicted to be hydrophilic, and appears to be lipid modified at an N-terminal cysteine in a manner analogous to that of murein lipoproteins of gram-negative bacteria. We also report the fatty acid content of the small cysteine-rich proteins of C. psittaci and Chlamydia trachomatis L2 as determined by combined gas chromatography-mass spectrometry.

Chlamydiae are obligate intracellular bacteria which possess a gram-negative cell envelope that appears to lack peptidoglycan (3, 8). The genes encoding three envelope proteins, the major outer membrane protein and the large and small cysteine-rich proteins (CRPs), have been cloned and sequenced from several strains of chlamydiae, including Chlamydia psittaci 6BC (6, 7). In the nondividing, infectious, elementary body form of chlamydiae, the CRPs and the major outer membrane protein form a disulfide cross-linked supramolecular complex, which may be the functional equivalent of peptidoglycan (11, 13, 14, 21). The major outer membrane protein is not cross-linked, and the CRPs are not made in the logarithmically dividing reticulate body form of chlamydiae (11, 13, 14, 21).

The small CRPs of Chlamydia trachomatis (Omp3) and C. psittaci (EnvA) appear to be lipoproteins: the primary sequences of Omp3 and EnvA contain potential lipid modification/signal peptide II recognition sites (1, 7), and the C. psittaci small CRP is labeled from a [3H]palmitic acid precursor (7). The present study was undertaken to further characterize the small CRPs of chlamydiae. We have determined the fatty acid content of EnvA of C. psittaci and the small CRP of C. trachomatis, and we present evidence that EnvA is lipid modified at a cysteine residue in a manner similar to that of the murein lipoprotein of gram-negative bacteria.

MATERIALS AND METHODS

Purification of EnvA. Sodium dodecyl sulfate (SDS)-insoluble envelope protein complexes containing EnvA were prepared from Renografin-purified elementary bodies of C. psittaci 6BC and C. trachomatis LGV434 serotype L2 as described previously (7), except that noncovalently associated lipids were removed from the insoluble complexes by five extractions with 20-ml aliquots of methanol-chloroform-water (10:5:4 [vol/vol]). The complexes were treated with 20 mM dithiothreitol and alkylated in 50 mM iodoacetamide following the method of Glazer et al. (9). In some experiments, EnvA was purified from envelope protein complexes by immunoprecipitation with an anti-EnvA monoclonal antibody (7) and protein A-Sepharose CL-48 (Sigma Chemical Co., St. Louis, Mo.). EnvA for combined gas chromatography-mass spectrometry (GC-MS) was separated from other proteins in chloroform-methanol-extracted envelope protein complexes by SDS-polyacrylamide gel electrophoresis (PAGE) (17) by using a step gradient gel consisting of 1 cm of 10% acrylamide and 15 cm of 15% acrylamide. The EnvA, with a mass of between 15 and 6 kDa, was excised and eluted from crushed gel by the addition of two 20-ml aliquots of deionized water over a period of 24 h. The eluate was filtered through a 0.45-μm-pore-size cellulose nitrate filter (Nalgene, Rochester, N.Y.), placed in 1-kDa-molecular-mass-cutoff tubing (Spectrapor, Los Angeles, Calif.), dialyzed at 4°C against a total volume of 10 liters of water with several changes over a period of 2 days, and lyophilized.

Determination of methionine content of mature EnvA. Infected cells (1.3 × 10⁷) were pulsed between 24.5 and 25 h postinfection in the presence of cycloheximide (100 μg/ml) with either 1.0 mCi of [35S]methionine in methionine-free medium or 1.0 mCi of [35S]cysteine in cysteine-free medium (MEM Select Amine Kit; Gibco BRL, Grand Island, N.Y.). An identical number of cells were pulsed between 23 and 25 h postinfection with 2 mCi (60 Ci/mmol) of [3H]palmitic acid in serum-free HB101 medium (Irvine Scientific, Santa Ana, Calif.) containing cycloheximide. Envelope complexes were prepared at 25 h postinfection, and EnvA was purified by immunoprecipitation.

Triton X-114-phase partitioning. A monolayer of 3 × 10⁶ infected cells was labeled with 0.2 μCi of [35S]cysteine in cysteine-free medium containing cycloheximide between 25.5 and 26 h postinfection. The cells were then harvested and subjected to phase partitioning by a modification of the method of Radolf et al. (22). The infected cell pellet was suspended in 500 μl of chilled (4°C) 2% Triton X-114 (Sigma) in 50 mM phosphate-buffered saline (PBS) (pH 8.0). The suspension was sonicated until warm (approximately 1 min) and then placed on ice for the micelle phases to become homogeneous. The mixture was heated in a boiling-water bath to allow the phases to separate and was subjected to centrifugation for 10 min in a microcentrifuge at 37°C. The upper aqueous phase (90% fluid volume) and lower phase (10% fluid volume)
were carefully removed with micropipets and reextracted with either 100 μl of 10% Triton X-114 in PBS (final concentration, 2%) or 500 μl of PBS, respectively. Ten microliters of the aqueous phase and 1 μl of the Triton X-114 phase (approximately equal proportions of the chlamydial) were added to Laemmli SDS solubilization buffer (17) and subjected to SDS-PAGE and autoradiography.

**Analysis of proteinase K-treated EnvA and envelope protein complexes.** To prepare cysteine-labeled EnvA, infected cells (2 × 10⁷) were pulsed between 23 and 25 h postinfection with 0.5 μCi of [³⁵S]cysteine in cysteine-free medium containing cycloheximide (100 μg/ml) and then incubated in medium M199 plus 5% fetal calf serum (Gibco BRL) containing cycloheximide (0.5 μg/ml). At 50 h postinfection, EnvA was purified from envelope protein complexes by immunoprecipitation. To prepare palmitic acid-labeled EnvA, infected cells (4 × 10⁷) were labeled in serum-free medium between 20 and 50 h postinfection with 4.0 mCi of [³¹P]labeled palmitic acid. Envelope protein complexes were extracted with methanol-chloroform-water (10:5:4 [vol/vol]), reduced, alkylated, dialyzed, and concentrated by lyophilization. EnvA and protein complexes were treated with proteinase K (2 μg/ml; Sigma) in 50 mM ammonium bicarbonate for 5 h at 37°C, frozen, and lyophilized. The samples were dissolved in methanol-chloroform-water (10:5:4 [vol/vol]), spotted on precoated silica gel G channeled thin layer chromatography (TLC) plates (Analtech, Newark, Del.), which were developed in n-butanol-water-acetic acid (4:5:1 [vol/vol]) (10). Individual channels on the developed plates were scanned for radioactivity with a BIOSCAN (Washington, D.C.) System 200 imaging scanner.

**Analysis of acid- and base-hydrolyzed EnvA by TLC.** Cells were labeled with 4.0 mCi of [³¹P]labeled palmitic acid from 20 to 50 h postinfection, and lipid-extracted envelope protein complexes were prepared as described above. Portions of the complexes were subjected to SDS-PAGE, and gel bands (0.2 g) containing EnvA were excised and either acid hydrolyzed by the addition of 200 μl of 6 M HCl and incubation under nitrogen at 110°C overnight or base hydrolyzed by the addition of 200 μl of 0.2 M NaOH and incubation at 37°C for 4 h. Methanol, chloroform, and water (3 ml of each) were added to the acid hydrolysate, the mixture was incubated overnight at room temperature with shaking, and the lower chloroform phase was removed and evaporated under a stream of nitrogen. Deionized water (2 ml) was added to the base hydrolysate, incubated for an additional hour, and then acidified to below pH 7 by the addition of 1 M HCl. Equal volumes of methanol and chloroform were added, the mixture was agitated overnight, and the chloroform phase was removed and evaporated. As a control, 50 μCi of [³¹P]labeled palmitic acid in 10 μl of ethanol was treated with acid (100 μl of 6 M HCl at 110°C overnight) or base (100 μl of 0.1 M NaOH at 37°C for 4 h) and reduced in volume by lyophilization. Evaporated chloroform phases and palmitic acid controls were each dissolved in a small volume of methanol-chloroform-water (10:5:4 [vol/vol]) and spotted on channeled silica G plates which were developed in hexane-ether-acetic acid (60: 40:1 [vol/vol]) (2). Individual channels were scanned with a BIOSCAN imaging scanner.

**GC-MS analysis.** EnvA of *C. psittaci* was purified by SDS-PAGE, and lipid-extracted envelope complexes of *C. trachomatis* were prepared from elementary bodies grown in 5 × 10⁷ cells. Samples were hydrolyzed in 6 M HCl at 110°C overnight, the hydrolysates were esterified under nitrogen with BF₃ in methanol (20), and the fatty acid methyl esters were isolated by phase separation in pentane before being separated and analyzed by GC-MS. The data were acquired over a GC temperature gradient of 165 to 280°C for 30 min with a DB-1 fused silica capillary column (30 m by 0.25 mm inner diameter); J & W Scientific, Folsom, Calif.) installed on a Hewlett Packard (Walnut Creek, Calif.) 5970 series mass selective ion detector. Branched chain fatty acids were identified partially on the basis of their slightly shorter retention times compared with those of the corresponding straight chain acids. The MS fragmentation pattern of the identified chlamydial material was typical of fatty acid methyl ester fragmentations (19), with predominant fragments occurring at m/z ratios of 74 and 87, and a regularly spaced series reflecting the loss of methylene (-CH₂)₂ units.

**RESULTS**

**Signal processing and hydrophobic nature of mature EnvA.** The presence of a predicted hydrophobic signal peptide and a signal peptidase II recognition site (25) suggests that the primary EnvA peptide of *C. psittaci* is cleaved between serine 19 and cysteine 20 to yield a mature peptide of 68 amino acids containing 15 cysteine and no methionine residues (7). To determine whether a signal peptide is removed from EnvA, we examined the relative cysteine and methionine contents of the mature protein. When infected host cells were labeled with equal amounts of either [³⁵S]methionine or [³⁵S]cysteine, incorporation of cysteine, but not methionine, could be detected in a band of immunoprecipitated EnvA comigrating on SDS-PAGE with mature EnvA, even when 15 times as much material from the methionine-labeled preparation was loaded onto the gel (Fig. 1). The N-terminal methionine residue has been shown to be cleaved from primary peptides lacking positively charged neighboring amino acids (15, 24). However, EnvA contains lysine residues at positions 2 and 3, suggesting that the entire signal peptide, rather than a single methionine, has been posttranslationally removed. Attempts to determine the N-terminal sequence of mature EnvA by the Edman
Triton) was insufficient, the putative signal cation is molecular chlamydiae, on the procedure globomycin (postinfection) h SDS-PAGE and described in Methods. A preparation of EnvA labeled with a 3-min pulse of [35S]cysteine and purified by immunoprecipitation (lane labeled Ipp) is shown for reference (arrowhead).

Degradation procedure were unsuccessful, suggesting modification of the N-terminal amino acid of the mature protein.

Lipoproteins have been characterized, in part, by the inhibitory effect of globomycin on signal peptidase II cleavage of lipid-modified proteins in Escherichia coli (5). However, globomycin (40 μg/ml) had no effect on EnvA synthesized by chlamydiae, on the basis of its failure to alter the apparent molecular weight of EnvA in SDS-PAGE (data not shown). It is possible that chlamydial signal peptidase II is not susceptible to globomycin or that the antibiotic fails to penetrate host cells or inclusion membranes within which chlamydiae multiply.

When EnvA was subjected to Triton X-114-phase partitioning, it was found in the hydrophobic phase rather than in the aqueous phase (Fig. 2). Because the primary sequence of EnvA beyond the putative signal processing site is highly hydrophilic (data not shown), the hydrophobicity of mature EnvA is most likely due to its modification with lipid.

**Lipid modification of cysteine in EnvA.** In an attempt to identify the amino acid residue modified by lipid addition, we analyzed by TLC the peptides generated by treatment of alkylated [3H]palmitic acid- and [35S]cysteine-labeled EnvA preparations with proteinase K. Cysteine-labeled EnvA was purified by gel electrophoresis from lipid-extracted SDS-insoluble envelope complexes; however, only palmitic acid-labeled envelope complexes were examined in these experiments because the amount of label present in the purified preparations was insufficient for analysis. In a previous investigation (7), it was shown that EnvA is the only lipid-labeled protein in chloroform-methanol-extracted SDS-insoluble complexes. The solvent system used permits the migration of amino acids and small peptides; large peptides fail to migrate, and small hydrophobic substances, such as fatty acids, migrate rapidly (10). Undigested preparations of EnvA and envelope complexes failed to move from the origin (Fig. 3A and B), and free palmitic acid (Fig. 3C) migrated close to the solvent front. Alkylated, free cysteine residues migrated as several broad peaks, the most prominent of which was centered at Rf of approximately 0.6, with a sharp spike at Rf of 0.45 (Fig. 3E). The spike at Rf of 0.45 was also noted when unalkylated.
cysteine was analyzed (Fig. 3D) and is believed to be a contaminant of the radioactive cysteine preparation. The multiple bands in the alkylated cysteine preparation presumably reflect the various chemical modifications of cysteine incurred during the alkylation process. When proteinase-treated, cysteine- and palmitic acid-labeled preparations of EnvA were examined by TLC, comigrating peaks with Rf values of 0.85 and 0.70 were noted in both preparations (Fig. 3F and G). The minute amounts of material in these peaks prohibited chemical identification. However, on the basis of the migration rates (faster than free, alkylated cysteine but slower than free palmitic acid), the peaks at Rf of 0.85 were most likely lipid-modified cysteine and the peaks at Rf of 0.70 were most likely lipid-modified cysteinyl-cysteine dipeptide. In addition to the peaks at Rf values of 0.85 and 0.70, a broad peak between Rf values of 0.45 and 0.65 was noted in the cysteine-labeled preparation (Fig. 3F) and a relatively sharp peak at Rf of 0.60 was observed in the palmitic acid-labeled sample. The material in these peaks probably represents larger peptides that resulted from the incomplete hydrolysis by proteinase K. The use of larger amounts of proteinase K and longer periods of incubation failed to resolve labeled material into single bands (data not shown). Whatever the specific chemical composition of the various peaks observed in the experiment for which the results are shown in Fig. 3, the comigration of cysteine- and palmitic acid-labeled material supports the conclusion that EnvA is lipid modified at a cysteine residue. Modification of other residues, however, cannot be ruled out.

**Acid and base hydrolysis of fatty acids associated with EnvA.** The principal posttranslational modification of bacterial lipoproteins occurs at a cysteine residue, either by amide linkage of a fatty acid or by thioether linkage of a glycerol which may be esterified with fatty acids or by both (25). We examined the acid and base hydrolysates of EnvA by TLC in an attempt to distinguish between these types of linkage. Acid hydrolysis of lipoproteins releases amide- and ester-linked fatty acids whereas treatment with a base releases only esterified fatty acids (16). Both acid and base treatments of [3H]palmitic acid-labeled EnvA released label (Fig. 4). Successful base hydrolysis indicates that at least some of the label was covalently associated with EnvA through ester linkage of fatty acids. Because of the differential loss of material in the hydrolysis procedures, it was not possible to compare the amount of fatty acids released by base hydrolysis with the amount released by acid hydrolysis.

**Identification of fatty acids associated with EnvA.** To characterize the fatty acid content of EnvA, the methyl esters of the fatty acids released from acid-hydrolyzed purified EnvA, whole *C. psittaci*, and envelope complexes prepared from *C. trachomatis* L2 were analyzed by GC-MS (Table 1). The most abundant fatty acids in purified EnvA were 16:0, 18:0, 15:0 branched, and 17:0 branched, in a relative proportion of 6:5:3:4. The same fatty acids predominated in acid hydrolysates of whole *C. psittaci* 6BC but in a proportion of 4:4:5:5. These results suggest that the pool of fatty acids used in the synthesis of EnvA is not identical to the general pool used for synthesis

**TABLE 1.** Fatty acids released from purified EnvA, whole *C. psittaci*, and the envelope protein complex of *C. trachomatis* by acid hydrolysis

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>% Total fatty acids released from:</th>
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<tbody>
<tr>
<td></td>
<td>EnvA</td>
</tr>
<tr>
<td>16:0</td>
<td>32</td>
</tr>
<tr>
<td>18:0</td>
<td>27</td>
</tr>
<tr>
<td>15:0 br&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>17:0 br&lt;sup&gt;1b&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>17:0 br&lt;sup&gt;2b&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>14:0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>19:0 br&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
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<tr>
<td>20:0</td>
<td>0</td>
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<td>Total</td>
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<sup>a</sup> Fatty acids were identified as described in Materials and Methods.

<sup>b</sup> br, branched chain. Two peaks (br1 and br2) of differing mobility were noted for the 17:0 fatty acid methyl ester of EnvA and whole *C. psittaci*, indicating branch points at two sites.

<sup>c</sup> Identification was based on retention time and fragmentation pattern only. A molecular ion could not be identified.
FIG. 5. Structural model of EnvA. We propose that an N-terminal cysteine (indicated by a dot in the amino acid sequence) is modified by amide linkage with a fatty acid residue and by thioether linkage with a glycerol fatty acid in a manner analogous to that of murein lipoproteins. The first 28 amino acids of the 87-amino-acid proprotein are shown below the model.

of other chlamydial lipids. For comparative purposes, the fatty acid content of the lipid-extracted envelope protein complex of C. trachomatis L2 was determined by GC-MS analysis. The major fatty acids found in the complex were 16:0, 18:0, 15:0 branched, and 17:0 branched, in a proportion of 5:1:3:1. These values reflect the fatty acid content of the small CRP since it is the only component in extracted complexes that is labeled by 3H]palmitic acid (data not shown).

**DISCUSSION**

The structure of the lipid moiety of only a few bacterial lipoproteins has been chemically analyzed. Analyses have included differential release of ester- and amide-linked fatty acids, N-terminal protein sequence determination, fast atom bombardment MS analysis of the N-terminal peptide, and glycerol-cysteine identification. The practical problems associated with propagating more than milligram quantities of chlamydiae precluded the use of some of these analytical techniques and made the structural analysis of EnvA difficult in general. The evidence that we present here, therefore, supports but does not prove the structural model shown in Fig. 5. TLC analysis of proteinase K-generated peptides suggests that a cysteine residue of EnvA is lipid modified, most likely at cysteine 20, on the basis of the signal peptide II-processing site located between serine 19 and cysteine 20. Signal peptide processing is further supported by the failure to detect methionine in mature EnvA. Other residues may also be modified; however, modification of only cysteine has been observed in other bacterial murein lipoproteins (23). The release of fatty acids following treatment with a base suggests that at least some of the fatty acids are ester linked to the EnvA peptide. By analogy to other bacterial lipoproteins, the fatty acids are predicted to be attached to a glycerol molecule which is linked by a thioether bond to cysteine. Amide linkage of fatty acid to cysteine 20 is less certain, although the resistance of the N terminus of EnvA to Edman degradation protein sequencing supports this conclusion.

The small size (87 amino acids) and the general structure of EnvA proposed in Fig. 5 are reminiscent of the murein lipoprotein of gram-negative bacteria (12, 23). The lipid portion of murein lipoprotein has been shown to be embedded within the outer membrane, with the peptide portion extending into the periplasm where it can be covalently linked to peptidoglycan (12, 18). An unusual feature of EnvA that is not shared with other murein lipoproteins is its high cysteine content (17% in the proposed mature form). EnvA has been previously shown to be disulfide bond cross-linked in elementary bodies of C. psittaci, although the spatial relationship between EnvA and other chlamydial disulfide cross-linked proteins has not been established (11, 13, 14, 21). The presence of a lipid moiety on the otherwise hydrophilic EnvA suggests that the lipid portion of EnvA is anchored in a membrane, with the peptide portion extending into an aqueous environment. Because EnvA does not appear to be surface exposed (4), this extension must be into either the cytoplasm or the periplasm. One intriguing possibility is that the function of EnvA is roughly analogous to that of the murein lipoprotein, extending from the outer membrane into the periplasmic space where it may be cross-linked with other periplasmically exposed proteins to form an osmotically stable supramolecular complex.

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**REFERENCES**


