Molecular Characterization and Subcellular Localization of Macrophage Infectivity Potentiator, a Chlamydia trachomatis Lipoprotein

NEFF, Laurence, et al.

Abstract

Macrophage infectivity potentiator (MIP) was originally reported to be a chlamydial lipoprotein from experiments showing incorporation of radiolabeled palmitic acid into native and recombinant MIP; inhibition of posttranslational processing of recombinant MIP by globomycin, known to inhibit signal peptidase II; and solubility of native MIP in Triton X-114. However, the detailed structural characterization of the lipid moiety on MIP has never been fully elucidated. In this study, bioinformatics and mass spectrometry analysis, as well as radiolabeling and immunochemical experiments, were conducted to further characterize MIP structure and subcellular localization. In silico analysis showed that the amino acid sequence of MIP is conserved across chlamydial species. A potential signal sequence with a contained lipobox was identified, and a recombinant C20A variant was prepared by replacing the probable lipobox cysteine with an alanine. Both incorporation of U-(14)C-esterified glycerol and [U-(14)C]palmitic acid and posttranslational processing that was inhibitable by globomycin were observed for recombinant wild-type MIP but [...]
Molecular Characterization and Subcellular Localization of Macrophage Infectivity Potentiator, a *Chlamydia trachomatis* Lipoprotein

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Macrophage infectivity potentiator (MIP) was originally reported to be a chlamydial lipoprotein from experiments showing incorporation of radiolabeled palmitic acid into native and recombinant MIP; inhibition of posttranslational processing of recombinant MIP by globomycin, known to inhibit signal peptidase II; and solubility of native MIP in Triton X-114. However, the detailed structural characterization of the lipid moiety on MIP has never been fully elucidated. In this study, bioinformatics and mass spectrometry analysis, as well as radiolabeling and immunochemical experiments, were conducted to further characterize MIP structure and subcellular localization. In-silico analysis showed that the amino acid sequence of MIP is conserved across chlamydial species. A potential signal sequence with a contained lipobox was identified, and a recombinant C20A variant was prepared by replacing the probable lipobox cysteine with an alanine. Both incorporation of U-14C-esterified glycerol and [U-14C]palmitic acid and posttranslational processing that was inhibitable by globomycin were observed for recombinant wild-type MIP but not for the recombinant C20A MIP variant. The fatty acid contents of native and recombinant MIP were analyzed by gas chromatography-mass spectrometry, and the presence of amide-linked fatty acids in recombinant MIP was investigated by alkaline methanolysis. These results demonstrated a lipid modification in MIP similar to that of other prokaryotic lipoproteins. In addition, MIP was detected in an outer membrane preparation of *Chlamydia trachomatis* elementary bodies and was shown to be present at the surfaces of elementary bodies by surface biotinylation and surface immunoprecipitation experiments.

Chlamydiae are obligately intracellular gram negative bacteria that are major human pathogens capable of causing a wide range of diseases (11, 12, 24, 34, 43). All chlamydiae share a characteristic biphasic cycle of development with infectious, spore-like elementary bodies (EB) and intracellular dividing reticulate bodies (RB) that are metabolically active and inhabit a nonfusogenic inclusion (46). The mechanisms by which chlamydiae induce diseases are poorly understood but may include a proinflammatory immune response to chlamydial antigen (22), even if this antigen has yet to be unequivocally revealed (62). In many bacterial diseases, lipoproteins play a prominent role in pathogenesis, with a ubiquitous presence as major constituents of the bacterial cell wall. However, little is known about the actual existence and role of lipoproteins in chlamydiae. One of the few lipoproteins characterized so far, in *Chlamydia trachomatis*, is macrophage infectivity potentiator (MIP), which was identified as a lipoprotein by incorporation of radiolabeled palmitic acid into the native and recombinant proteins (41), inhibition of posttranslational processing of recombinant MIP (rMIP) by globomycin (a specific inhibitor of signal peptidase II [31]), and solubility of native MIP in Triton X-114 (42). Other studies have shown that *C. trachomatis* MIP is a 27-kDa membrane protein located in both EB and RB (38), with a COOH-terminal region showing high homology with eukaryotic and prokaryotic FK506 binding proteins (FKBP) (41) and exhibiting peptidyl-prolyl cis/trans isomerase (PPIase) activity (40). However, no evidence supporting the presence of a classical lipoprotein feature in MIP, similar to the murein lipoprotein from *Escherichia coli* (25), the best characterized bacterial lipoprotein, has been shown. Since chlamydiae are notably very atypical bacteria, phylogenetically separated from other eubacteria (48), we have conducted a detailed structural characterization of MIP. The probable signal sequence was determined by in-silico analysis, and the cysteine in position 20 (cysteine20) was predicted to be the lipobox cysteine (33, 44). To assess the involvement of cysteine20 in lipid modification, an rMIP variant was prepared by replacing cysteine20 with alanine (C20A) using site-directed mutagenesis (17). To characterize the structure of the lipid moiety, U-14C-esterified glycerol and [U-14C]palmitic acid attachments to the lipobox cysteine, as well as peptide signal release, were examined for both wild-type (WT) rMIP and the C20A rMIP variant. The fatty acid contents of native and WT rMIP were analyzed by gas chromatography-mass spectrometry, and amide linkage of any fatty acid present was investigated by alkaline methanolysis for WT rMIP. To define its subcellular localization and possible surface exposure, the presence of native MIP was examined in a chlamydial outer membrane complex (COMC) preparation, and its presence at the surfaces of EB was investigated by surface biotinylation and surface immunoprecipitation experiments.
MATERIALS AND METHODS

Sequence alignments. The sequences of MIP or putative MIP were found in the Swiss-Prot/TREMBL database. To study homologies among five MIP sequences, the CLUSTAL W multiple sequence alignment program (69) (available at www.ebi.ac.uk/clustalw) was used with Gonnet 250 as a matrix. To study homologies between two sequences, the SIM binary sequence alignment program (29) (available at www.expasy.org/tools/sim-prot.html) was used with Blosum62 as a comparison matrix.

In silico analysis. Three different computer prediction programs were used to detect the pattern characterizing bacterial lipoprotein pre-cursors in MIP sequences. The pattern is a well-conserved cysteine-containing “lipobox” of four residues within the lipoprotein signal peptide sequence. The bioinformatics tools used were PROSITE (accession number PS000103; www.expasy.org/prosite/) (30), DOLOP (database of bacterial lipoproteins; available at www.mrc-lmb.cam.ac.uk/genes/dolop/) (44) with (L/V)(A/S/T/V/I)(G/A/S)C as the consensus pattern, and Lipop (accessible at the cbs.dtu.dk/services/LipoP) (33).

Homologues of proteins required for precursor export and lipoprotein biosynthesis in E. coli were searched for in the proteomes of five different chlamydial species. Complete proteomes deduced from genomic sequences (28, 35, 52, 53, 59, 63) were available at the HAMAP (high-quality automated and manual annotation of microbial proteomes) site (http://www.expasy.org/sprot/hamap/).

Bacteria, EB of C. trachomatis PV-L2 strain 434 (inactivated by a photochemical treatment affecting bacterial genomes) were either purchased from BioMedical Technologies (Cambridge, MA) or prepared with Tris-EDTA buffer treatment (45). Immune sera against synthetic peptides or recombinant lipoproteins were prepared as follows: 20 μl of recombinant lipoprotein in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail (Complete, EDTA-free; Roche Diagnostics AG, Rotkreuz, Switzerland; and disrupted by sonication before addition of an equal volume of buffer containing 1% SDS, 2% Triton X-100, and 20 mM β-mercaptoethanol. After 1 h of incubation at 4°C, insoluble material was pelleted by centrifugation at 20,000 × g for 5 min at 4°C. A Seize X protein A immunoprecipitation kit (Pierce, Perbio Science, Lausanne, Switzerland) was used according to the manufacturer’s instructions for the purification of native MIP. Briefly, purified IgG antibodies raised in rabbits against WT MIP were used to probe native MIP from the EB lysate. Antibodies were chemically cross-linked to a protein A gel with the bifunctional reagent disuccinimidyl suberate. The EB lysate was then incubated with the immobilized antibody to form the immune complex. After a wash, native MIP was eluted with 0.1 M glycine (pH 2.5) and immediately neutralized with Tris-HCl.

Identification of fatty acids associated with native and WT MIP after acid and base hydrolysis by gas chromatography–mass spectrometry analysis. To evaluate the proportions of ester-linked and amide-linked fatty acids, two types of hydrolysis were performed. Fatty acids bonded through ester or amide linkages were liberated by strong-acid hydrolysis, whereas only esterified fatty acids were liberated by acid hydrolysis. Strong-acid hydrolysis was performed by placing 0.15 ml aliquots of MIP and HCl at 100°C overnight under nitrogen and was followed by fatty acid saponification in KOH. Alkaline hydrolysis was performed in 5% KOH in aqueous methanol at 100°C overnight under nitrogen. After acidification, fatty acids were subsequently recovered by repeated extraction with methylene chloride and purified using anion-exchange solid-phase extraction cartridges (Sep-Pak AG, Grellingen, Switzerland), highly selective for acidic compounds. Then fatty acids were methyl esterified with 14% (vol/vol) boron trifluoride in methanol (Fluka AG, Buchs, Switzerland) and recovered by repeated extraction with methylene chloride. At last, hydroxyl groups were further derivatized to trimethylsilyl ethers by the addition of O,N-bis(trimethylsilyl) trifluoroacetamide, to identify possible hydroxylated fatty acids. All the analytes were then analyzed by a DB-5 fused silica capillary column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm) (Agilent J & W Scientific, Basel, Switzerland). Analytical gas-liquid chromatography was carried out on a Hewlett-Packard 5890 gas-liquid chromatograph interfaced with a quadrupole mass spectrometer (Trio-2 VG; Micromass, West Sussex, United Kingdom). For sample analysis, the column temperature was held at 80°C for 1 min before it was raised to 300°C at a rate of 3°C min⁻¹. Impact ion mass spectra were obtained at 70 eV, and individual fatty acid methyl esters were identified by their fragmentation pattern and ion masses. Fatty acid methyl ester levels are given as percentages of the total peak area recovered on the chromatogram.

In vivo radiolabeling of WT MIP and the C20A rMIP variant with phosphatidic acid–L-[14C]glycerol or [U-14C]palmitic acid. WT MIP, E. coli cells expressing WT MIP were grown in LB medium in the presence of 0.4 mM IPTG and 100 μg/ml globomycin and were labeled with [U-14C]palmitic acid. Bacteria were pelleted, washed, treated at 37°C for 2 h with 0.1 N NaOH in 90% methanol, and neutralized. Proteins were next separated by 12% SDS-PAGE and silver stained with a Silver Stain Plus kit (Bio-Rad, Reinach, Switzerland).
Gels were dried and subjected to autoradiography. Digital images were processed using a densitograph and OptiQuant software.

Preparation of COMC. COMC was prepared as described previously (49). Briefly, C. trachomatis L2 EB were suspended (5 mg/ml of EB protein) in PBS, pH 7.4, containing 2% (wt/vol) Sarkosyl (Sigma, Fluka AG) and 1 mM EDTA. This suspension was incubated at 37°C for 1 h with mixing every 5 min and was then centrifuged at 100,000×g for 1 h. The insoluble pellet was resuspended in the same buffer with 10 mM dithiothreitol, incubated for 1 h at 37°C with mixing every 5 min, and centrifuged as described above. The pellet was washed twice in PBS to remove excess detergent. This Sarkosyl-insoluble material consisted of COMC (10). Proteins from Sarkosyl-soluble and -insoluble fractions were subjected to 12% SDS-PAGE analysis and transferred to a nitrocellulose membrane. MIP was detected with polyclonal anti-MIP IgG diluted 1/5,000, major outer membrane protein (MOMP) with an anti-MOMP MAb (clone 165; Biodesign International) diluted 1/5,000, and soluble S1 with anti-S1 ribosomal protein MAb 124.4 (39) diluted 1/1,000. Membranes were next probed with HRP-conjugated goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) diluted 1/10,000 and developed with the ECL immunoblot detection system before visualization with Hyperfilm (Amersham Biosciences). Digital images were processed using a densitograph and OptiQuant software.

EB surface biotinylation. For total-protein biotinylation, 10^9 EB were solubilized in lysis buffer containing 1% Triton X-100 in PBS and EDTA-free protease inhibitor cocktail (Complete, Roche). Solubilization was carried out by overnight rotation at 4°C. For surface biotinylation, 10^9 EB were washed three times with ice-cold PBS (pH 8.0) and resuspended in PBS. The biotinylation reaction was performed with 2 mM sulfosuccinimidyl-6-(biotinamido)hexanate (sulfo-NHS-LC-biotin; Pierce) for 1 min on ice. For EB lysate, the residual sulfo-NHS-LC-biotin was quenched with 10 mM Tris and inactivated biotin was eliminated by buffer exchange using a desalting column. For surface-biotinylated EB, quenching was performed with 2 mM sulfosuccinimidyl-6-(biotinamido)hexanate (sulfo-NHS-LC-biotin; Pierce) for 1 min on ice. For surface-biotinylated EB, quenching was performed with 2 mM sulfo-NHS-LC-biotin for 1 min on ice. For EB lysate, the residual sulfo-NHS-LC-biotin was quenched with 10 mM Tris and inactivated biotin was eliminated by buffer exchange using a desalting column. For surface-biotinylated EB, quenching was performed with 2 mM sulfo-NHS-LC-biotin for 1 min on ice. For EB lysate, the residual sulfo-NHS-LC-biotin was quenched with 10 mM Tris and inactivated biotin was eliminated by buffer exchange using a desalting column. For surface-biotinylated EB, quenching was performed with 2 mM sulfo-NHS-LC-biotin for 1 min on ice.

Surface immunoprecipitation of native MIP. To investigate possible surface exposure of C. trachomatis, surface immunoprecipitation of MIP was performed according to the work of Shang et al. (58). Briefly, 0.5 × 10^10 intact EB were mixed with rabbit polyclonal anti-MIP IgG and HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), and Hsp60 was probed with a MAb against hsp60, kindly provided by Ian Maclean (University of Manitoba, Winnipeg, Manitoba, Canada), and HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Labeled proteins were detected with the ECL immunoblot detection system before visualization with Hyperfilm (Amersham Biosciences).

FIG. 1. Sequence alignment of Chlamydia trachomatis (accession number P26623), Chlamydia muridarum (Q9PJK1), Chlamydophila pneumoniae (Q9ZP3), Chlamydophila caviae (Q824R2), and Ca. Protochlamydia amoebophila (Q6ME92) MIPs as performed by the CLUSTAL multiple alignment program. Asterisks indicate identities, and colons (conserved substitutions) and periods (semiconserved substitutions) indicate similarities, in the lines below the sequences.
50 μl of 10% protein-grade Triton X-100 (Calbiochem, Juro Supply, GmbH, Lucerne, Switzerland) was added, and the mixture was agitated overnight at 4°C. The insoluble material was removed by centrifugation at 16,000 x g for 20 min. Sodium deoxycholate (final concentration, 0.2%), SDS (0.1%), and 20 μl of a slurry of protein G-Sepharose or streptavidin-agarose beads (Amersham Biosciences) were added to the supernatant. These mixtures were gently agitated for 1 h at 4°C. The protein G-Sepharose- or streptavidin-agarose-antibody-antigen complexes were washed three times in 10 mM Tris HCl (pH 7.5)–500 mM NaCl–1% Triton X-100–0.2% deoxycholate–1 mM EDTA and once in 10 mM Tris HCl for 20 min. g

Results

MIP is conserved among five different chlamydial species.

To determine the degree of homology of MIP within five different chlamydial species, amino acid sequences were aligned. The five MIP-like protein sequences, deduced from the complete genomic sequences of five different species—Chlamydia trachomatis (strain D/UW-3/Cx) (63), Chlamydothila pneumoniae (strains AR39, CWL029, J138, TW-183) (35, 52, 59), and “Ca. Protochlamydia amoebophila” (strain UWE25) (28)—were available in the Swiss-Prot/TrEMBL database. In a 243- to 88% identity, with 88% for the two species “Ca. Chlamydia trachomatis” (strain GPIC) (53), and “Ca. Protochlamydia amoebophila” (strain UWE25) (28)—were available in the Swiss-Prot/TrEMBL database. In a 243- to 290-residue overlap, 78 residues were identical in all five MIP sequences. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae homologues appeared to be highly conserved sequences within the five different chlamydial species.

Table 1. Prediction of Chlamydia MIPs (FKBP-type PPases) as putative lipoproteins according to different bioinformatic tools

<table>
<thead>
<tr>
<th>Chlamydia species (strain[s])</th>
<th>Gene (ordered locus name)</th>
<th>Primary accession no. (Swiss-Prot/TrEMBL)</th>
<th>Result by the following computer prediction program:</th>
<th>N-terminal amino acid sequence*</th>
<th>Predicted mass of prolipoprotein (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia trachomatis (D/UW-3/Cx)</td>
<td>CT541</td>
<td>P26623</td>
<td>PROSITE DOLOP LipP</td>
<td>MKNL5SWMFLMVAVLPGC</td>
<td>26.7</td>
</tr>
<tr>
<td>Chlamydia pneumoniae (MoPnNigg)</td>
<td>TCO826</td>
<td>Q9PJ1K</td>
<td>PROSITE DOLOP LipP</td>
<td>MKNL5SWMFLMVAVLPGC</td>
<td>26.8</td>
</tr>
<tr>
<td>Chlamydothila pneumoniae (AR39, CWL029, J138, TW-183)</td>
<td>CCa0078</td>
<td>Q82AR2</td>
<td>PROSITE DOLOP LipP</td>
<td>MKRRWHLMTMVMVVPSASC</td>
<td>28.2</td>
</tr>
<tr>
<td>“Ca. Protochlamydia amoebophila” (UWE25)</td>
<td>pC0338</td>
<td>Q6MEF2</td>
<td>PROSITE DOLOP LipP</td>
<td>MFKQTKLLASFGMFIC</td>
<td>31.7</td>
</tr>
</tbody>
</table>

* Lipobox motifs are boldfaced.

Table 2. Gene names, accession numbers, and definitions in Swiss-Prot/TrEMBL of Chlamydia trachomatis* proteins homologous to those required for secretory machinery and lipoprotein biosynthesis in Escherichia coli

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Ordered locus name</th>
<th>Primary accession no. (Swiss-Prot/TrEMBL)</th>
<th>Definition (Swiss-Prot/TrEMBL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ffh</td>
<td>CT025</td>
<td>O84028</td>
<td>Signal recognition particle</td>
</tr>
<tr>
<td>secA</td>
<td>CT701</td>
<td>O84707</td>
<td>GTPase</td>
</tr>
<tr>
<td>secDsF</td>
<td>CT448</td>
<td>O84454</td>
<td>Protein export</td>
</tr>
<tr>
<td>secY</td>
<td>CT321</td>
<td>O84323</td>
<td>Preprotein translocase subunit</td>
</tr>
<tr>
<td>secF</td>
<td>CT510</td>
<td>P28539</td>
<td>Preprotein translocase subunit</td>
</tr>
<tr>
<td>int</td>
<td>CT534</td>
<td>O84539</td>
<td>Apolipoprotein N-acyltransferase (EC 2.3.1.1)</td>
</tr>
<tr>
<td>lbpA</td>
<td>CT408</td>
<td>O84413</td>
<td>Lipoprotein signal peptidase (EC 3.4.23.36) (signal peptidase II)</td>
</tr>
<tr>
<td>lgt</td>
<td>CT252</td>
<td>O84254</td>
<td>Preprotein diacylglycerol transferase (EC 2.4.99)</td>
</tr>
</tbody>
</table>

* Strain D/UW-3/Cx was used.

Prediction of MIP sequences as putative lipoprotein precursors according to different bioinformatic analyses. To examine whether the N-H2-terminal regions of the selected MIP sequences corresponded to the typical protonkyotic lipoprotein precursor patterns, three different computer prediction programs were used. All these MIP proteins were predicted to be possible lipoproteins by one or several prediction methods, with clear identification of their lipobox sequences. The cysteine in position 20 was predicted to be the lipobox cysteine of Chlamydia trachomatis MIP (Table 1).

Presence of homologues of proteins required for precursor export and lipoprotein biosynthesis in E. coli. Based on a previous analysis originally performed on E. coli and now commonly used a model (26), homologues of proteins required for precursor export and lipoprotein biosynthesis were found in the complete proteomes of five different chlamydial species. As an example, the Chlamydia trachomatis protein homologues are presented in Table 2. Even when proteins were identified as putative or probable, they appeared as highly conserved sequences within the five different chlamydial species. For example, the Chlamydia pneumoniae protein homologues appeared to be 45 to 65% similar and 28 to 45% identical to E. coli proteins (data not shown).

MIP has classical lipoprotein features. By analogy to other bacterial lipoproteins and according to established prediction methods, fatty acids were thought to be attached to a glycerol molecule linked by a thioether bond to cysteine20. To deter-
mine if cysteine is the lipobox cysteine of MIP, a C20A rMIP variant was prepared after site-directed mutagenesis where cysteine was changed to alanine (17). The two different recombinant proteins were prepared with a six-histidine (His) tag in the COOH-terminal position. To assess whether WT rMIP-His6 and C20A rMIP-His6 were comparable in MW to their dependence on the lipobox cysteine (20), E. coli clones expressing WT and C20A rMIP were radioactively labeled in vivo using [U-14C]palmitic acid as an acyl donor. Only one radioactive band of 27.6 kDa was detected in the C20A rMIP clone (Fig. 4). These results showed that the radioactive 27.6-kDa band represents an acyl-modified lipoprotein which may be either diacylated or triacylated according to analogies made with other bacterial lipoproteins.

To confirm that the precursor form of WT rMIP is processed by signal peptidase II, [U-14C]palmitic acid radiolabeling was performed in the presence of globomycin, a signal peptidase II inhibitor (31). Under these conditions, [U-14C]palmitic acid incorporation was observed in both the upper and lower bands (Fig. 4), as previously reported for C. trachomatis MIP (42). The radiolabeled upper band corresponds to an accumulation of the diacylglycerol-modified lipoprotein due to inhibition of peptide signal cleavage by globomycin. These results demonstrate that in E. coli, WT rMIP is processed by signal peptidase II. As expected, globomycin had no detectable effect on the size of C20A rMIP on the immunoblot, showing that replacement of cysteine (20) eliminates processing by lipoprotein signal peptidase II. To confirm that the upper band was the precursor form with the signal peptide still present, microse-
quencing was performed on the 32-kDa band of WT rMIP, which demonstrated an intact NH₂-terminal sequence corresponding to the uncleaved MIP precursor (data not shown). All these results confirm that E. coli enzymes recognize the MIP lipoprotein motif and add a lipid tail to the recombinant protein.

To further analyze the lipid component of MIP, fatty acids were identified in purified native MIP as well as WT rMIP by gas chromatography-mass spectrometry analysis. Native MIP was purified by immunoprecipitation and WT rMIP by nickel chelate affinity chromatography (1). The types and distributions of fatty acids present in the native and WT rMIP proteins were very similar, comprising C₁₂ to C₂₂ fatty acids, with three major fatty acids (accounting for more than 92% of total fatty acids) corresponding to n-C₁₈:0, n-C₁₆:0, and n-C₁₄:0.

The presence of characteristic fatty acids was very similar, comprising C₁₂ to C₂₂ fatty acids, with three major fatty acids (accounting for more than 92% of total fatty acids) corresponding to n-C₁₈:0, n-C₁₆:0, and n-C₁₄:0.

Table 3. Types and distributions of fatty acids identified from purified native and WT rMIP proteins

<table>
<thead>
<tr>
<th>Fatty acid released*</th>
<th>Native MIP</th>
<th>WT rMIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After base hydrolysis</td>
<td>After acid hydrolysis</td>
</tr>
<tr>
<td>n-C₁₂:0</td>
<td>0.74</td>
<td>1.84</td>
</tr>
<tr>
<td>n-C₁₃:0</td>
<td>0.44</td>
<td>0.47</td>
</tr>
<tr>
<td>n-C₁₄:0</td>
<td>6.78</td>
<td>7.73</td>
</tr>
<tr>
<td>i-C₁₅:0</td>
<td>0.4</td>
<td>0.32</td>
</tr>
<tr>
<td>ai-C₁₅:0</td>
<td>0.48</td>
<td>0.47</td>
</tr>
<tr>
<td>n-C₁₆:0</td>
<td>1.43</td>
<td>1.37</td>
</tr>
<tr>
<td>i-C₁₇:0</td>
<td>0.37</td>
<td>0.26</td>
</tr>
<tr>
<td>ai-C₁₇:0</td>
<td>0.59</td>
<td>0.53</td>
</tr>
<tr>
<td>n-C₁₈:0</td>
<td>1.87</td>
<td>1.55</td>
</tr>
<tr>
<td>n-C₁₉:0</td>
<td>48.07</td>
<td>45.24</td>
</tr>
<tr>
<td>n-C₂₀:0</td>
<td>0.29</td>
<td>0.2</td>
</tr>
<tr>
<td>n-C₂₀:0</td>
<td>0.95</td>
<td>0.67</td>
</tr>
<tr>
<td>C₁₂:0</td>
<td>0.15</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* a, linear (straight chain); i, methyl branched chain with iso structure (methyl group at the penultimate carbon atom); ai, methyl branched chain with anteiso structure (methyl group on the third carbon from the end).

To investigate this possibility, the presence of amide-linked fatty acids in WT rMIP was examined by mild-alkaline methanalysis, a treatment that releases ester-linked but not amide-linked fatty acids (47). Treatment was performed on WT rMIP labeled with [U-¹⁴C]palmitic acid in the presence of globomycin, which blocked lipoprotein maturation, in order to obtain both precursor and mature forms of MIP. The precursor form was used as a negative control for amide-linked fatty acid, because the presence of signal peptide prevented the addition of amide-linked fatty acids. After SDS-PAGE separation, gel silver staining showed that no protein was lost during treatment whereas autoradiography demonstrated a notable decrease in radioactivity (Fig. 5). Analysis of results obtained after image digitalization revealed that alkaline methanalysis released a significantly larger amount of [U-¹⁴C]palmitic acid radioactivity from the upper band than from the lower band (92% ± 2% versus 82% ± 5% [mean of four experiments]; P = 0.01). These results indicate that if some amide-linked fatty acids are present, NH₂ acylation is not completed.

MIP is present in C. trachomatis outer membrane complex. Since C. trachomatis MIP was reported to be a membrane protein located in both EB and RB (38), the presence of MIP

FIG. 4. Immunodetection and palmitic acid radiolabeling of rMIP in cultures of E. coli M15(pREP4) expressing WT rMIP-His₆ and the C20A rMIP-His₆ variant. Cells were grown in LB medium either in the presence (+) or in the absence (−) of 0.4 mM IPTG, vehicle (ethanol), and 100 μg ml⁻¹ globomycin. Proteins were subjected to SDS-PAGE, His₆-tagged proteins were detected with the anti-penta-His MAb, and labeled proteins were visualized by autoradiography. Solid arrows indicate the prolipoprotein forms, and open arrows indicate the mature form of WT rMIP.

Table 3. Types and distributions of fatty acids identified from purified native and WT rMIP proteins

- n-C₁₂:0
- n-C₁₃:0
- n-C₁₄:0
- i-C₁₅:0
- ai-C₁₅:0
- n-C₁₆:0
- i-C₁₇:0
- ai-C₁₇:0
- n-C₁₈:0
- n-C₁₉:0
- n-C₂₀:0
- n-C₂₂:0

FIG. 5. Mild alkaline hydrolysis of [U-¹⁴C]palmitic acid-labeled WT rMIP. E. coli M15(pREP4) cells expressing WT rMIP were grown in LB medium in the presence of 0.4 mM IPTG and 100 μg ml⁻¹ globomycin and were labeled with [U-¹⁴C]palmitic acid. Bacteria were pelleted, washed, treated with 0.1 N NaOH in methanol for 2 h, and neutralized. Proteins were separated by 12% SDS-PAGE and silver stained, and the gel was dried and subjected to autoradiography. Results from one representative experiment are shown.
in the outer membranes of intact EB was investigated by treatment with the weak anionic detergent sodium lauryl sarcosinate (Sarkosyl). After immunoblot analysis using polyclonal anti-MIP IgG and MAbs against MOMP and S1, image digitalization showed that 60% of native MIP was present in the Sarkosyl-soluble and 40% in the Sarkosyl-insoluble fraction. In contrast, 85% of MOMP was found in the Sarkosyl-insoluble fraction, whereas 100% of the soluble S1 ribosomal protein was found in the Sarkosyl-soluble fraction (Fig. 6). Two bands were obtained for MOMP, as already reported from similar experiments (10, 37, 66, 67). These results suggest that native MIP might have a dual localization, in both the inner (Sarkosyl-soluble) and outer (Sarkosyl-insoluble) membranes (20), as shown for other bacterial lipoproteins (4, 9, 23, 27, 58, 61, 64).

To eliminate the possibility that native MIP could be part of a Sarkosyl-insoluble supramolecular complex by disulfide cross-linking (18), treatment was carried out in the presence of dithiothreitol as a reducing agent.

**MIP is exposed at the surfaces of *C. trachomatis* EB.** The presence of native MIP in COMC did not indicate whether MIP is in the inner or the outer leaflet of the outer membrane. Its possible exposure at the surface was determined by two independent approaches involving exposure of intact EB to a biotinylation reagent and to a MIP-specific polyclonal antibody. EB surface proteins were biotinylated for 1 min using sulfo-NHS-LC-biotin (Pierce), a water-soluble, membrane-impermeant reagent. An EB lysate was prepared, and surface biotin-labeled proteins were separated from unlabeled proteins by affinity purification using streptavidin-agarose beads and subjected to SDS-PAGE analysis and membrane transfer.

A MIP-specific polyclonal antibody detected native MIP in this fraction, whereas an anti-hsp60 MAb did not reveal the cytosolic protein hsp60 (2) (Fig. 7A and B). This demonstrated that probably more biotinylated sites (i.e., lysine residues) were accessible to the biotinylation reagent in the total-EB lysate than in intact EB. These results support the idea that MIP is partly exposed at the surface and argue against the possibility that sulfo-NHS-LC-biotin gained access to intracellular proteins under our experimental conditions.

To further assess MIP surface exposure, a surface immunoprecipitation technique was used (58). Intact EB were incubated with polyclonal IgG specific for MIP or with a biotinylated MAb to soluble S1 ribosomal protein, followed by gentle washes to remove unbound antibodies. The MIP antigen-antibody complex was isolated using protein G-Sepharose beads, and the S1 antigen-biotinylated MAb complex was isolated using streptavidin-agarose beads. After solubilization, complexes were analyzed by electrophoresis and immunoblotting. As shown in Fig. 8, a large amount of MIP was found in the surface-immunoprecipitated material, showing that MIP is accessible to antibody on the surfaces of intact EB (Fig. 8A), whereas soluble S1 ribosomal protein was detected only in the EB lysate (Fig. 8B). These results indicate that native MIP is surface exposed, and they complement the results obtained after EB surface biotinylation.

**DISCUSSION**

The present work describes, for the first time, the lipid component of a *Chlamydia* lipoprotein and demonstrates that the structure is similar to that of the *E. coli* murein lipoprotein, the best-characterized bacterial lipoprotein (25). This conclusion is supported by several lines of evidence from bioinformatic analysis and radiolabeling experiments. In silico analysis of the MIP amino acid sequence revealed the presence of a probable signal peptide according to the DOLOP and LipoP prediction programs but not according to the strict predictive rule of Prosite pattern PS00013 (30). The predicted lipobox was IVGC, with cysteine20 being the lipobox cysteine. The first experimental evidence came from Lundemose et al., who re-
reported the incorporation of radiolabeled palmitic acid into native MIP (41). Further experimental evidence has been provided in this study and was mainly obtained with an E. coli clone expressing WT rMIP, because of technical difficulties in working with chlamydiae.

The first step of MIP synthesis, i.e., the addition of a diacylglycerol moiety to lipobox cysteine20, was demonstrated by the incorporation of radiolabeled esterified glycerol and radiolabeled palmitic acid into WT rMIP, whereas no label was incorporated into the C20A rMIP variant. However, a difference was shown to be present at the 2 position of the esterified glycerol. The commercial preparation has the R configuration, whereas the natural substrate has the S configuration (65). The next step, involving the cleavage of the signal peptide, was demonstrated by the inhibition of WT rMIP processing in the presence of globomycin, a specific inhibitor of signal peptidase II. The subsequent step, involving the aminoacylation of the cysteine residue by an amide-linked fatty acid, was investigated both by gas chromatography-mass spectrometry analysis of native and WT rMIP and by mild-alkaline methanalysis of WT rMIP. Both procedures demonstrated that aminoacylation was not completed. Gas chromatography-mass spectrometry analysis of WT rMIP found no amide-linked fatty acid, whereas a small amount was found by alkaline methanalysis, a treatment that releases ester-linked but not amide-linked fatty acids (47).

Indeed, about 10% of [U-13C]palmitic acid radioactivity remained linked to WT rMIP after alkaline methanalysis, whereas 33% should remain in the case of total acylation, with one amide-linked and two ester-linked acyl chains. Even in native MIP, the NH2-terminal cysteine residue did not appear to be completely modified with amide-linked fatty acids, because a mass ratio of total fatty acids to ester-linked fatty acids of 1.12 was obtained; in the case of total acylation, this ratio should be 1.5. The present ratio of 1.12 indicated that only 24% of cysteine20 in native MIP had amide-linked acyl chains. Taken together, this would imply that even if native MIP appears to have a slightly higher proportion of amide-linked acyl chains, the two forms of MIP are similar and are products of incomplete posttranslational modifications, as has been reported previously for other bacterial lipoproteins (6, 7, 14, 15).

Overall, these results confirm that E. coli processes WT rMIP using a pathway identical to that for its own lipoproteins, except that posttranslational modifications can become rate-limiting steps, as evidenced by the fact that E. coli expressed WT rMIP both as a precursor and as the mature form, as previously reported (6, 13, 15, 36, 70). In C. trachomatis, the lipoprotein biosynthetic pathway is not known. The fact that protein homologues of all essential components of the Sec general secretion pathway in E. coli were found in chlamydiae supports the notion that prolipoprotein translocation across the inner membrane involves Sec translocase (16). Since homologues of enzymes required for lipoprotein biosynthesis in E. coli were also found in chlamydiae, subsequent lipid modification most likely involves the same pathway as that identified in E. coli.

The final point of investigation in the present study was the subcellular localization of native MIP. Native MIP was found to be present in both inner and outer membranes of EB and was shown to be present at the surfaces of EB by biotinylation and immunoprecipitation experiments. These results are consistent with previous reports demonstrating that at least a portion of MIP is exposed on the chlamydial surface (38, 41, 45, 55), and they suggest that the rules and mechanisms regulating MIP sorting and transport to the EB outer surface have yet to be fully characterized. The “+2 rule” for lipoprotein sorting in the E. coli cell envelope (72) cannot be applied to MIP. Indeed, the second amino acid of mature MIP, immediately after cysteine20, is aspartic acid, a residue considered to be specific for inner membrane retention (71). However, other lipoproteins have also been reported to have sorting signals different from those dictating inner or outer membrane localization in E. coli lipoproteins (50, 54, 57, 73).

One important question relating specifically to MIP cellular location is the exact function in vivo of this protein in C.
trachomatis MIP. If a subset of MIP remains attached to the inner membrane, it could act as a PPIase and be involved in the folding of periplasmic or outer membrane proteins after their translocation across the cytoplasmic membrane. If MIP is located at the surfaces of EB, it might play a key role in pathogenicity and host immunity, as has been shown for other lipoproteins implicated in several important bacterial infections, including infections with Mycobacterium tuberculosis (8), Treponema pallidum (68), Listeria monocytogenes (21), Haemophilus influenzae (60), Campylobacter jejuni (32), and Borrelia burgdorferi (51). As a surface PPIase, MIP might also be involved in initiating productive chlamydial infection (40). However, the exact contribution of the PPIase activity to the functional properties of MIP is still unclear, since no bacterial or host substrates for MIP have been identified so far.

In conclusion, the present work shows that MIP is a classical lipoprotein, partly exposed at the surfaces of EB. However, as with other lipoproteins (73), the sequence of molecular events leading from inner membrane expression to exposure at the surface remains unknown, as does the nature of the MIP function. These are important issues that deserve further investigation.

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