Molecular Characterization and Subcellular Localization of Macrophage Infectivity Potentiator, a *Chlamydia trachomatis* Lipoprotein[∇]

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Received 14 December 2006/Accepted 13 April 2007

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.

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[∇] Published ahead of print on 20 April 2007.

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 precursors 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 PS000013; www.expasy.org/prosite/) (30), DOLOP (database of bacterial lipoproteins; available at www.mrc-lmb.cam.ac.uk/genomes/dolop/) (44) with (L/V/I)(A/S/T/V/I)(G/A/S)C as the consensus pattern, and LipoP (accessible at www.cbs.dtu.dk/services/LipoP/) (33).

Homologues of proteins required for precursor export and lipoprotein biosynthesis in *E. coli* were searched for in the complete 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* LGV2 strain 434 (inactivated by a photochemical treatment affecting bacterial genomes) were either purchased from Biodec International (Milan Analytica AG, Le Milan, Switzerland) or prepared according to the method of Boleti et al. (5).

Preparation of WT rMIP and the C20A rMIP variant. The preparation of WT rMIP from *C. trachomatis* has been described previously (1). The C20A rMIP variant was prepared by in vitro site-directed mutagenesis as follows. Template DNA for the PCR was obtained from a purified WT rMIP plasmid clone (1). Bases coding for cysteine were replaced by bases coding for alanine with the QuikChange site-directed mutagenesis kit from Stratagene (Amsterdam Zuidooit, The Netherlands). For the amplification reaction, the following oligonucleotide primers containing the desired mutations (boldfaced) were used: 5'-TGCCTATCGTAGGAGCTGATAACGGAGGCGGTCGCG-3' for the 5' end and 5'-GCGAACCCTCCGTTATCAGCTCTACGATAGGCA-3' for the 3' end. Other conditions have been described previously (1).

Immunization of rabbits with WT rMIP and development of polyclonal antibodies. Two female New Zealand White rabbits (weight, 2.0 to 2.5 kg) were used by Covalab (Lyon, France) to produce antibodies to WT rMIP. Before immunization, the preimmune sera were collected for use as controls. For the first immunization, each rabbit was injected intradermally with purified endotoxin-free WT rMIP (50 µg). The injected proteins (0.5 ml) were emulsified with an equal volume of Freund's complete adjuvant. Animals were subsequently injected three times at 3-week intervals with the same immunogen emulsified with incomplete Freund's adjuvant. Injections were performed intradermally, except for the last, which was done subcutaneously. Antisera of immunized animals were collected 11 days after the third and fourth booster immunizations and examined for the presence of MIP-specific antibodies by enzyme-linked immunosorbent assay. Rabbits were sacrificed 82 days following the first injection. The immunoglobulin G (IgG) fraction of the antiserum was isolated using an immobilized protein A-agarose column.

Immunoblot analysis. Proteins from EB and from *E. coli* clones expressing either WT rMIP or C20A rMIP were separated on 12% Tris-glycine sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions, electrophoretically transferred to a nitrocellulose membrane, and probed with rabbit polyclonal anti-MIP IgG diluted 1/5,000 and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., LabForce AG, Nunningen, Switzerland) diluted 1/10,000. The membrane was developed with the enhanced chemiluminescence (ECL) immunoblot detection system before visualization with Hyperfilm (Amersham Biosciences, Buckinghamshire, United Kingdom).

In vivo radiolabeling of WT rMIP and the C20A rMIP variant with phosphatidic acid- α -dipalmitoyl-[U- 14 C]glycerol or [U- 14 C]palmitic acid. Experimental conditions were those described by Lundemose et al. (42). Briefly, cultures of recombinant *E. coli* M15(pREP4) expressing WT rMIP and the C20A rMIP variant in Luria-Bertani broth (LB) medium were induced with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Eurogentec, Geneva, Switzerland). One microcurie of phosphatidic acid- α -dipalmitoyl-[U- 14 C]glycerol (specific activity, 140 mCi/mmol; Perkin-Elmer, Schwerzenbach, Switzerland), or 1 µCi of [U- 14 C]palmitic acid (specific activity, 780 mCi/mmol; Amersham Biosciences), dissolved in toluene, dried with N₂ gas, and suspended in phosphate-buffered

saline (PBS) with 1% Triton X-100, was added to 100 µl of culture, and cultivation was continued for 1 h. To some samples, globomycin (a generous gift of Shunichi Miyakoshi, Sankyo, Tokyo, Japan) dissolved in 95% ethanol at 20 mg ml⁻¹ (final concentration, 100 µg ml⁻¹) was added 10 min before induction. The control cultures received an equivalent volume of 95% ethanol. Cells were centrifuged, resuspended in SDS sample buffer, heated for 5 min at 95°C, and subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE) with low-range Rainbow molecular weight (MW) markers that had been [14 C] labeled (2,500 to 45,000 Da) (Amersham Biosciences). The same amounts of induced WT rMIP and C20A rMIP variant upper bands were loaded onto the gel (in preliminary experiments, the corresponding volumes of cells were determined after band quantification using a densitograph and OptiQuant software). Gels were transferred to nitrocellulose membranes and visualized by autoradiography before being probed with the anti-penta-His monoclonal antibody (Mab) (QIAGEN AG, Basel, Switzerland).

N-terminal amino acid sequencing. Purified WT rMIP was separated on 18% SDS-PAGE gels (Novex; Invitrogen, Basel, Switzerland) under reducing conditions and transferred to polyvinylidene difluoride membranes (Immobilon-PSQ; Millipore, Volketswil, Switzerland) using a semidry transfer cell (Novex, Invitrogen) in 10 mM 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS) buffer, pH 11, containing 10% (vol/vol) methanol. The blot was stained with Ponceau Red S and dried, and bands of interest were excised. Samples were then subjected to four cycles of Edman degradation (Atheris Laboratories, Geneva, Switzerland).

Purification of native MIP by immunoprecipitation. A total of 2×10^{10} EB were harvested by centrifugation, resuspended in 1 ml of a solution containing 50 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 extraction at 4°C, insoluble material was removed by centrifugation at $20,000 \times g$ for 5 min at 4°C. A Seize X protein A immunoprecipitation kit (Pierce, Perbio Science, Lausanne, Switzerland) was then used according to the manufacturer's instructions to purify native MIP. Briefly, purified IgG antibodies raised in rabbits against WT rMIP 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 rMIP 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 released by a base treatment. Strong-acid hydrolysis was performed in 6 M 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 (Separtys 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 *N,O*-bis(trimethylsilyl)trifluoroacetamide, to identify possible hydroxylated fatty acids. All the analytes were then analyzed with 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; Masslab, Manchester, 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. Electron impact 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.

Mild-alkaline hydrolysis of [U- 14 C]palmitic acid-labeled WT rMIP. *E. coli* 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- 14 C]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).

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sp|P26623|MIP_CHLTR      ---MKNILSWMLM-FAVALPIVGCD--NGGGSQ-----TSATEKSMVEDS 39
sp|Q9PJK1|MIP_CHLMU      ---MKNILSWMLM-FAVALPIVGCD--NGGGSQ-----TSAMGKDMVEDS 39
sp|Q9Z7P3|MIP_CHLPN      ---MNRWNVLVATVALALSVASCDVRSKDKDKDQGSLEYKDNKDNDI 47
tr|Q824R2|Q824R2        ---MKKRWHLMITMVVSFSIASCDSSSQRGNQ----IAQVEEKAANDS 43
tr|Q6ME92|Q6ME92_PARUW   MFKQTKLLALASFGMFICGVLHANDANIATNQTN---KSVQASTETPKS 47
                          ..      . : . : . : . : . : . : . : . : . :

sp|P26623|MIP_CHLTR      ALTDNQKLSRTFGHLLSRQLSRTEDFSLDLVEVIKGMQSEIDGQSAPLTD 89
sp|Q9PJK1|MIP_CHLMU      VLTDNQKLSRTFGHLLARQLSSTEDFTLDLTEVIKGMQSEIEGKSAPLTD 89
sp|Q9Z7P3|MIP_CHLPN      ELSDNQKLSRTFGHLLARQLRKSEDMFFDIAEVAKGLQAEVLKQSAPLTE 97
tr|Q824R2|Q824R2        QLSVNQKLSRTFGHLLARQLRKSEIMMDIAEVAKGLQGELECKSAPLTE 93
tr|Q6ME92|Q6ME92_PARUW   DQIDMQKLSAEAFGHFIGNLQAP-GLQFDLEAIKGIREGAAGKPAPLSE 96
                          *: *: *: *: *: *: . . . : *: : *: : . : *: *:

sp|P26623|MIP_CHLTR      TEYEKQMAEVQKASFEAKCSENLASAEKFLKENKEKAGVIELEPNKLQYR 139
sp|Q9PJK1|MIP_CHLMU      SEYETQMALVQKASFEKKCSENLASAEKFLKENKDEGVIELEPNKLQYR 139
sp|Q9Z7P3|MIP_CHLPN      TEYEKQMAEVQKLVFEKKSKENLSLAEKFLKENSKNAGVVEVQPSKLQYK 147
tr|Q824R2|Q824R2        SEYEKQMAEIQKLVFEKKAKENLSLAERFLQENKNSGVVEVQADKLQYR 143
tr|Q6ME92|Q6ME92_PARUW   KEYEEMMAAVQERAFKEMSTKNLKNANDFMIKNSQSTDVTEIVPGKLQYS 146
                          .*** ** *: *: . : ** *: *: : *... . * *: . : ****

sp|P26623|MIP_CHLTR      VVKEGTGRVLSGKPTALLHYTGSGFIDGKVFDSSEKNKEPILLPLTKVIPG 189
sp|Q9PJK1|MIP_CHLMU      IVKEGTGRVLTGKPNALLHYTGSGFINGKVFDTSEKNKDPILLPLTKVISG 189
sp|Q9Z7P3|MIP_CHLPN      IIEGAGKAISGKPSALLHYKGSFINGQVFSSEGNNEPILLPLGQTIPG 197
tr|Q824R2|Q824R2        IVKEGTGRAISGKPSALLHYKGTTFIDGQVFSSEANKEPILLPLAQTIPTG 193
tr|Q6ME92|Q6ME92_PARUW   VEKEGTGPVVEPHSSPKIHYTGKYQDGTVFGTSEEMDGPITIPLDQTIPG 196
                          : *: *: . : . : . : *: *: : * *: *: * . * : *: . : * . *

sp|P26623|MIP_CHLTR      FSQGMQGMKEGEVRVLYIHPDLAYGTAGQLPPNSLLIFEVKLIEANDDNV 239
sp|Q9PJK1|MIP_CHLMU      FSQGMQGMKEGEVRVLYIHPDLAYGTSGQLPPNSLLIFEVKLIEANDDNV 239
sp|Q9Z7P3|MIP_CHLPN      FALGMQGMKEGETRVLYIHPDLAYGTAGQLPPNSLLIFEINLIQASADEV 247
tr|Q824R2|Q824R2        FALGMQGMKEGETRILYIHPDLAYGTSGQLPPNSLLIFEINLIETDDSV 243
tr|Q6ME92|Q6ME92_PARUW   FSKGIVGMKEGEKRRLYVHPDLGYGTGTGQLPPNELLIFDIEVIKANSDDA 246
                          *: *: *: *: * *: *: *: *: *: *: *: *: *: *: *: *: *:

sp|P26623|MIP_CHLTR      SVTE----- 243
sp|Q9PJK1|MIP_CHLMU      SVAE----- 243
sp|Q9Z7P3|MIP_CHLPN      AAVPQEGNQGE----- 258
tr|Q824R2|Q824R2        AAQNTENENTAS----- 255
tr|Q6ME92|Q6ME92_PARUW   KTKTGKSADDAEEDLFIDEEYELEPAQKNAKTPEASNTSAKN 290

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FIG. 1. Sequence alignment of *Chlamydia trachomatis* (accession number P26623), *Chlamydia muridarum* (Q9PJK1), *Chlamydomphila pneumoniae* (Q9ZP3), *Chlamydomphila 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.

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⁻¹ 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 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, 10⁹ 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, 10⁹ EB were washed three times with ice-cold PBS (pH 8.0) and resuspended in PBS. The biotinylation reaction was performed with 2 mM sulfo-succinimidyl-6-(biotinamido)hexanoate (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 by three washes with 10 mM Tris and inactivated biotin was removed by two washes with PBS (pH 8.0). Surface-biotinylated EB were subsequently lysed with 1% Triton X-100. Biotin-labeled proteins were separated from unlabeled proteins by affinity purification using streptavidin-agarose beads (Amersham Biosciences). The beads were first preincubated in 10 mM Tris and 3% bovine serum albumin in order to prevent nonspecific protein binding. After capture of biotinylated proteins, elution by boiling in Laemmli sample buffer, SDS-PAGE analysis, and membrane transfer, MIP was probed 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 in ascitic fluid 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).

Surface immunoprecipitation of native MIP. To investigate possible surface exposure of *C. trachomatis* MIP, surface immunoprecipitation of MIP was performed according to the work of Shang et al. (58). Briefly, 0.5 × 10¹⁰ intact EB were mixed with rabbit polyclonal anti-MIP IgG or biotinylated MAb 124.4 against soluble S1 ribosomal protein (39) and gently shaken for 2 h at 30°C. Agglutinated EB were pelleted at 2,000 × *g* for 15 min, resuspended in 5 mM MgCl₂ in PBS, pelleted again at 2,000 × *g* for 15 min, and then resuspended in 450 µl of a solution containing 10 mM Tris HCl (pH 8.0), 1 mM EDTA, and a protease inhibitor cocktail (Complete, EDTA-free; Roche). To this suspension

TABLE 1. Prediction of *Chlamydia* MIPs (FKBP-type PPIases) as putative lipoproteins according to different bioinformatic tools

Chlamydial species (strain[s])	Gene (ordered locus name)	Primary accession no. (Swiss-Prot/TrEMBL)	Result by the following computer prediction program:			N-terminal amino acid sequence ^a	Predicted mass of prolipoprotein (kDa)
			PROSITE	DOLOP	LipoP		
<i>Chlamydia trachomatis</i> (D/UW-3/Cx)	CT541	P26623	—	+	+	MKNILSWMLMFAVALPIVGC	26.7
<i>Chlamydia muridarum</i> (MoPn/Nigg)	TC0828	Q9PJK1	—	—	+	MKNILSWMLMFAVALPILGC	26.8
<i>Chlamydomonas caviae</i> (GPIC)	CCA00078	Q824R2	+	+	+	MKKRWHLMITMVVSFSIASC	28.2
<i>Chlamydomonas pneumoniae</i> (AR39, CWL029, J138, TW-183)	CPn0661, CP0086, CpB0687	Q9Z7P3	+	+	+	MNRRWNLVLATVALALSVC	28.3
" <i>Ca. Protochlamydia amoebophila</i> " (UWE25)	pc0383	Q6ME92	+	—	+	MFKQTLLALASFGMFIGC	31.7

^a Lipobox motifs are boldfaced.

50 μ l 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 $\times$ g for 20 min. Sodium deoxycholate (final concentration, 0.2%), SDS (0.1%), and 20 μ l 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 and were finally resuspended in sample buffer. Samples were then processed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-MIP IgG or anti-S1 ribosomal protein MAb 124.4 and HRP-IgG conjugates (Amersham Biosciences). Labeled proteins were visualized by chemiluminescence. Immunoprecipitation control experiments were performed in parallel under the same conditions as those described above except that either no antibody or preimmune serum was mixed with intact EB. As an additional control, S1 ribosomal protein was also immunoprecipitated from EB lysates.

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), *Chlamydomonas pneumoniae* (strains AR39, CWL029, J138, and TW-183) (35, 52, 59), *Chlamydia muridarum* (strain MoPn/Nigg) (52), *Chlamydomonas caviae* (strain GPIC) (53), and "*Candidatus* 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, showing a high degree of sequence conservation (Fig. 1). Pairwise alignments of MIP sequences from pathogenic chlamydiae (*C. trachomatis*, *C. muridarum*, *Chlamydomonas pneumoniae*, and *Chlamydomonas caviae*) resulted in 58 to 88% identity, with 88% for the two *Chlamydia* MIP sequences and 71% for the two *Chlamydomonas* MIP sequences. The lowest scores (36 to 40%) were obtained when pairwise alignments were performed between MIPs from pathogenic chlamydiae and the MIP from the environmental chlamydial species "*Ca. Protochlamydia amoebophila*" (28; also data not shown). Even though lower levels of homology were observed between these strains, they show that the *mip* gene was already present in the ancient core gene set of chlamydiae, because "*Ca. Protochlamydia amoebophila*" is a *Chlamydia*-related symbiont of free-living amoebae, representing an early-diverging (about 700 million years ago) evolutionary sister group of present-day pathogenic chlamydiae (28).

Prediction of MIP sequences as putative lipoprotein precursors according to different bioinformatic analyses. To examine whether the NH₂-terminal regions of the selected MIP sequences corresponded to the typical prokaryotic 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 *C. 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, *C. 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 *C. trachomatis* 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-

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

Gene name	Ordered locus name	Primary accession no. (Swiss-Prot/TrEMBL)	Definition (Swiss-Prot/TrEMBL)
<i>ffh</i>	CT025	O84028	Signal recognition particle GTPase
<i>secA</i>	CT701	O84707	Preprotein translocase SecA subunit
<i>secD/secE</i>	CT448	O84454	Protein export
<i>secE</i>	CT321	O84323	Preprotein translocase subunit
<i>secY</i>	CT510	P28539	Preprotein translocase SecY subunit
<i>lnt</i>	CT534	O84539	Apolipoprotein N-acyltransferase (EC 2.3.1.)
<i>lspA</i>	CT408	O84413	Lipoprotein signal peptidase (EC 3.4.23.36) (signal peptidase II)
<i>lgt</i>	CT252	O84254	Prolipoprotein diacylglycerol transferase (EC 2.4.99.)

^a Strain D/UW-3/Cx was used.

FIG. 2. Immunodetection of MIP in *C. trachomatis* EB and in cultures of *E. coli* M15(pREP4) overexpressing WT rMIP-His₆ and the C20A rMIP-His₆ variant. Proteins were separated by 12% Tris-glycine SDS-PAGE, blotted onto a membrane, and detected with rabbit polyclonal anti-MIP IgG and HRP-conjugated goat anti-rabbit IgG. Molecular mass markers (in kilodaltons) are indicated on the left. In cultures of rMIP, two forms of the protein were detected. The solid arrow indicates the position of a protein of about 32 kDa, consistent in MW with the precursor-like forms and present in both WT rMIP-His₆ and the C20A rMIP-His₆ variant. The open arrow indicates the position of a protein of about 27.6 kDa, consistent in MW with the mature-like form and present only in WT rMIP-His₆. In *C. trachomatis* EB, the MW of the mature form is about 1 kDa lower than that in WT rMIP due to the absence of the His₆ tag in the COOH-terminal position.

mine if cysteine²⁰ is the lipobox cysteine of MIP, a C20A rMIP variant was prepared after site-directed mutagenesis where cysteine²⁰ was changed to alanine (17). The two different recombinant proteins were prepared with a six-histidine (His₆) tag in the COOH-terminal position. To assess whether WT rMIP-His₆ and C20A rMIP-His₆ were comparable in MW to native MIP, immunoblot analysis was performed using MIP-specific polyclonal antibodies. One band of about 26.6 kDa was detected in *C. trachomatis* EB, whereas two bands of about 27.6 and 32 kDa were detected in WT rMIP-His₆ and only one band of about 32 kDa was detected in C20A rMIP-His₆ (Fig. 2). The band of about 27.6 kDa was consistent in MW with the mature-like form, obtained after signal peptide release, since the MW was similar to the molecular size (26.6 kDa) of the only band observed in *C. trachomatis* EB. The 1-kDa difference observed corresponded to the weight of the His₆ tag present in the COOH-terminal position of WT rMIP. The 32-kDa band, absent in *C. trachomatis* EB, was consistent in MW with precursor-like forms of MIP, although this requires further confirmation.

To confirm the presence of the predicted lipid modification, *in vivo* labeling experiments with *E. coli* clones expressing WT and C20A rMIP were initially performed in the presence of phosphatidic acid-L- α -dipalmitoyl-[U-¹⁴C]glycerol, which is known as a substrate for the enzyme diacylglycerol transferase (56). This precursor was used instead of [U-¹⁴C]glycerol to avoid nonspecific labeling by glycerol catabolite products. When this esterified glycerol donor was used, a main radioactive band of 32 kDa and a faint band of 27.6 kDa were detected in the total-cell extract of the WT rMIP clone whereas no radioactive band was detected in the C20A rMIP clone (Fig.

FIG. 3. Immunodetection and diacylglycerol radiolabeling of rMIP in cultures of *E. coli* M15(pREP4) overexpressing WT rMIP-His₆ and the C20A rMIP-His₆ variant. Cells were grown in LB medium. Expression of WT rMIP and C20A rMIP was induced by the addition of 0.4 mM IPTG, and MIPs were further labeled with phosphatidic acid-L- α -dipalmitoyl-[U-¹⁴C]glycerol. Proteins were separated by SDS-PAGE, His₆-tagged proteins were detected with the anti-penta-His MAb, and labeled proteins were visualized by autoradiography. Solid arrows indicate the position of a protein of about 32 kDa, corresponding to the precursor forms and present in both WT rMIP-His₆ and the C20A rMIP-His₆ variant. Open arrows indicate the position of a protein of about 27.6 kDa, corresponding to the mature form and present only in WT rMIP His₆.

3). These results indicated that a substantial amount of U-¹⁴C-esterified glycerol was used in WT rMIP lipidation and allowed identification of the radioactive 32-kDa band as the diacylglycerol-modified prolipoprotein and of the radioactive 27.6-kDa band as the diacylglycerol-modified lipoprotein obtained after peptide signal release.

To further assess the presence of fatty acids in WT rMIP and their dependence on the lipobox cysteine²⁰, *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 total-cell extract of the WT rMIP clone, whereas no radioactive band 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 prolipoprotein 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 cysteine²⁰ eliminates processing by lipoprotein signal peptidase II. To confirm that the upper band was the precursor form with the signal peptide still present, microse-

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 μ g 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.

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 C_{12:0} to C_{22:0} fatty acids, with three major fatty acids (accounting for more than 92% of total fatty acids) corresponding to n-C_{18:0}, n-C_{16:0}, and n-C_{14:0}. Branched (i.e., isosaturated and anteisaturated) fatty acids were found with odd carbon numbers (C₁₅ and C₁₇) in very small amounts (Table 3). The presence of characteristic fatty acids i-C_{15:0} and ai-C_{15:0} in *C. trachomatis* has been reported previously (3, 19). No unsaturated or hydroxylated fatty acids were identified. The same fatty acids, in the same proportions, were predominant in acid and base hydrolysates of native and WT rMIP proteins, indicating that the *E. coli* vector system is capable of providing a reliable source of rMIP that has a fatty acid composition very similar to that of the native MIP.

To determine whether amide-linked fatty acids were present, the mass ratios of total fatty acids to ester-linked fatty acids were estimated. They were found to be equal to 1 for WT rMIP and 1.12 for native MIP. These results are consistent with the absence of amide-linked fatty acids in WT rMIP and the presence of a small proportion in native MIP. To further investigate this possibility, the presence of amide-linked fatty

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

Fatty acid released ^a	Release of the indicated fatty acid (% of total fatty acid release) from:			
	Native MIP		WT rMIP	
	After base hydrolysis	After acid hydrolysis	After base hydrolysis	After acid hydrolysis
n-C _{12:0}	0.74	1.84	0.97	1.27
n-C _{13:0}	0.44	0.47	0.13	0.92
n-C _{14:0}	6.78	7.73	7.39	6.91
i-C _{15:0}	0.4	0.32	0.18	0.44
ai-C _{15:0}	0.48	0.47	0.24	0.37
n-C _{15:0}	1.43	1.37	0.88	1.16
n-C _{16:0}	37.44	39.25	36.82	34.24
i-C _{17:0}	0.37	0.26	0.25	0.44
ai-C _{17:0}	0.59	0.53	0.59	0.73
n-C _{17:0}	1.87	1.55	1.76	1.54
n-C _{18:0}	48.07	45.24	49.86	51.06
n-C _{19:0}	0.29	0.2	0.11	0.2
n-C _{20:0}	0.95	0.67	0.71	0.6
n-C _{22:0}	0.15	0.11	0.1	0.12

^a n, 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).

acids in WT rMIP was examined by mild-alkaline methanolysis, 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 methanolysis released a significantly larger amount of [U-¹⁴C]palmitic acid radioactivity from the upper band than from the lower band (92% $\pm$ 2% versus 82% $\pm$ 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. 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 μ g 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.

FIG. 6. Subcellular localization of native MIP. COMC was prepared after EB treatment in the weak anionic detergent sodium lauryl sarcosinate (Sarkosyl). Proteins from Sarkosyl-soluble and -insoluble (COMC) fractions of intact EB were separated on 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. Equivalent starting amounts of EB were present in Sarkosyl-soluble and -insoluble fractions. MIP was detected with rabbit polyclonal anti-MIP IgG, MOMP with an anti-MOMP MAb (clone 165), and soluble S1 ribosomal protein with anti-S1 MAb 124.4 (39).

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 under these experimental conditions, sulfo-NHS-LC-biotin did not label the intracellular protein hsp60 in intact EB. As a control, the experiment was performed on an EB lysate where hsp60 could be visualized as a biotin-labeled protein (Fig. 7B).

FIG. 7. Immunoblot analysis of streptavidin-bound fractions from nonbiotinylated, surface-biotinylated, and lysate-biotinylated EB. Intact EB were either left untreated or biotinylated with sulfo-NHS-LC-biotin (Pierce) for 1 min on ice and then lysed. Intact EB were also lysed before being biotinylated. All biotinylated molecules were captured with streptavidin-agarose. Streptavidin-bound fractions were analyzed by SDS-PAGE and immunoblotting. MIP was probed with polyclonal anti-MIP IgG (A) and hsp60 with an anti-hsp60 MAb (B).

In addition, it should be noted that the MIP band obtained from the total-EB lysate displayed a slightly higher MW than the band obtained from surface-biotinylated EB, indicating 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 either 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 Prosit 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-

FIG. 8. Surface immunoprecipitation of native MIP. Intact EB were incubated with either no antibody, preimmune serum, polyclonal anti-MIP IgG, or biotinylated MAb 124.4 against soluble S1 ribosomal protein (39) and lysed. MIP was immunoprecipitated in the presence of protein G-Sepharose, and the S1 antigen-biotinylated antibody complex was captured with streptavidin-agarose. Samples were then processed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-MIP IgG or a streptavidin-HRP conjugate. (A and B) Labeled proteins were visualized by chemiluminescence. (B) A lysate of EB was also prepared, mixed with biotinylated MAb 124.4 against soluble S1 ribosomal protein, and then processed as described above.

ported 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 in WT rMIP labeling was observed between the two precursors: when phosphatidic acid-L- α -dipalmitoyl-[U-¹⁴C] glycerol was used as an esterified glycerol donor, both the mature and precursor forms of WT rMIP were radioactive, whereas when [U-¹⁴C]palmitic acid was used as a fatty acid precursor, only the mature form was radioactive. The accumulation of the precursor form (i.e., of diacylglycerol-modified prolipoprotein) observed when phosphatidic acid-L- α -dipalmitoyl-[U-¹⁴C]glycerol was used as a precursor implies that signal peptidase II was a rate-limiting step. One explanation could be the wrong configuration of the dipalmitoyl glycerol moiety transferred from phosphatidic acid to the prolipoprotein. Such a scenario is possible, since an asymmetric C atom is 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 methanolysis 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 methanolysis, a treatment that releases ester-linked but not amide-linked fatty acids (47). Indeed, about 10% of [U-¹⁴C]palmitic acid radioactivity remained linked to WT rMIP after alkaline methanolysis, 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 pathogenesis 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.

ACKNOWLEDGMENTS

This work was supported by grants from the Novartis, Albert-Boeni, and de Reuter foundations as well as by grant 3200B0-107883 from the Fonds National Suisse de la Recherche Scientifique (to S.B.).

We are grateful to John Pearce for supplying MAbs against MIP and S1, Ian Maclean for antibodies against hsp60, Shunichi Miyakoshi for globomycin, Olivier Dorchie for silver staining, Carine Poussin and Frédérique Lisacek for help in bioinformatics, Paul Bradfield for assistance in editing the manuscript, and Lorenza Eder-Colli, Danielle Burger, Agathe Subtil, Alice Dautry-Varsat, Gisou van der Groot, and Jacques Deshusses for helpful advice.

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