The *Chlamydia trachomatis* Mip-Like Protein
Is a Lipoprotein

ANKER G. LUNDEMOSE,† DUNCAN A. ROUCH, CHARLES W. PENN, AND JOHN H. PEARCE*

Microbial Molecular Genetics and Cell Biology Group, School of Biological Sciences, University of Birmingham, Birmingham B15 2TT, United Kingdom

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The Mip-like protein of *Chlamydia trachomatis* is similar to the Mip protein of *Legionella pneumophila* and may be equally important for the initiation of intracellular infection. This article presents data which identify the chlamydial Mip-like protein as a lipoprotein. The amino acid sequence of the Mip-like protein contains a signal peptidase II recognition sequence, as is seen in procaryotic lipoproteins. Palmitic acid was incorporated into the recombinant chlamydial Mip-like protein. Globomycin, known to inhibit signal peptidase II, inhibited processing of the recombinant Mip-like protein. Labelling of chlamydial organisms with palmitic acid revealed incorporation into the native Mip-like protein.

*Chlamydia trachomatis* is an obligate intracellular bacterium mainly causing oculargonital infections in humans. *C. trachomatis* has a remarkable biphasic developmental cycle alternating between the infectious, metabolically inactive elementary body and the replicating, metabolically active reticulate body within the vacuolar inclusion membrane (15). Early events in infection are critical for the survival of the chlamydial organism and for productive infection (16, 20). This may in part depend upon evolved mechanisms of the parasite for avoidance of fusion with lysosomes and ability of the parasite to gain access to nutrients and high-energy compounds from the host cell. The molecular components responsible for these early-infection events, pivotal for productive infection, have not yet been determined. We have previously described the 27-kDa chlamydial membrane protein, the Mip-like protein, with sequence similarity to the *Legionella pneumophila* Mip protein (11, 13), a virulence factor necessary for optimal initiation of intracellular legionella infections (3, 4). The chlamydial Mip-like protein is poorly surface exposed, and specific antibodies are nonneutralizing in the absence of complement (13). The Mip-like protein has also previously been shown to have peptidyl-prolyl *cis*-trans isomerase (PPIase) activity that is inhabitable upon binding by the immunosuppressive drug FK506 or rapamycin (12). In experiments in which organisms were treated with FK506 or rapamycin prior to infection and during the early stages of infection, infectivity for McCoy cells was significantly reduced (12). These results suggest that inhibition of the isomerase of the Mip-like protein interferes with one or more early events in the infective process that determine productive intracellular infection and that the Mip-like protein may be important for optimal initiation of chlamydial infections, as with Mip of *L. pneumophila*.

The cleavage site of the signal peptidase sequence of the Mip-like protein is similar to a signal peptidase II sequence, suggesting that it is likely to be a lipoprotein (Fig. 1). Incorporation of radiolabelled palmitic acid was used to test for the presence of a lipid moiety. In this article, we demonstrate that both the recombinant and the native chlamydial Mip-like proteins are lipid modified following biosynthesis.

*Escherichia coli* recombinants pETMip 9a pLys S (expressing the Mip-like protein) and pET 9a pLys S (not carrying the *mip* gene) were cultivated in Luria broth and at an optical density of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, Mo.) (0.4 mM) was added to induce the production of recombinant protein (13). After a further 15 min of cultivation, 50 μl of [U-14C]palmitic acid (Amersham, Amersham, United Kingdom) at 50 μCi/ml in phosphate-buffered saline (PBS) with 1% Triton X-100 was added to 250 μl of culture, and cultivation continued for 45 min. To some samples of recombinant pETMip 9a pLys S, 1 μl of globomycin dissolved in ethanol at 25 mg/ml (final concentration, 100 μg/ml) was added just prior to the radioactive label. Globomycin obtained from M. Arai, Sankyo, Tokyo, Japan, was kindly provided by L. Schools, Rijksinstituut voor Volksgezondheid en Milieurubbing, Bilthoven, The Netherlands. Postlabeling, the recombinants were centrifuged, and the pellets were resuspended in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris [pH 6.8], 2.4% [wt/vol] SDS, 10% [wt/vol] glycerol, 5% [vol/vol] mercaptoethanol). The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12.5% polyacrylamide) and either stained with Coomassie blue or transferred to nitrocellulose and immunostained with Mip-specific monoclonal antibody (MAB) 112.3 (11, 13). The blot was then dried and exposed to Hyperfilm (Amersham) for 3 days. Palmitic acid was incorporated into recombinant mature Mip-like protein (Fig. 2C, lane 3). In cultures in which globomycin, known to inhibit signal peptidase II (6), had been added, significant inhibition of processing of the Mip-like protein occurred (Fig. 2A and C, lanes 2), but with some lipid incorporation evident in both the mature and the unprocessed protein (Fig. 2C, lane 2). This may have been due to the introduction of label at too short an interval following the addition of globomycin before inhibition of processing was fully established. No lipid was incorporated into proteins with the size of the Mip-like protein in the control recombinant pET 9a pLys S not carrying the *mip* gene (Fig. 2C, lane 1); labelling of other lipoproteins in the recombinants was apparent only after longer exposure of the gels (data not shown).

* Corresponding author.
† Present address: Diabetes Research, Novo Nordisk A/S, DK-2880 Bagsvaerd, Denmark.
FIG. 1. Signal peptide sequences of selected lipoproteins. MipL2, the Mip-like protein of C. trachomatis serovar L2 (13); CRPL2, outer membrane protein 3 cysteine-rich protein of C. trachomatis serovar L2 (1, 7); EcPAL, E. coli peptidoglycan-associated lipoprotein (10); EcnIPA, E. coli lipoprotein 28 (23); KpPulA, K. pneumoniae pulA gene product (pullulanase) (9); Con., consensus (21). The shaded box encloses the signal peptidase II recognition sequence; the box is further extended (no shading) for amino acids 2 to 4 in KpPulA and MipL2 to show homology. The arrow indicates the cleavage site of signal peptidase II. +1 indicates the lipid-modified cysteine, the first amino acid of the mature protein.

To label native chlamydial Mip-like protein, C. trachomatis serovar L2 was cultivated on monolayers of McCoy cells. At 22 h postinfection, the medium was replaced with a serum-free medium containing 10 μCi of [U-14C]palmitic acid per ml and 0.5 μg of cycloheximide per ml and was cultivated for a further 3 h. The monolayers were then washed once in PBS and either dissolved in SDS sample buffer or solubilized on ice in 50 mM Tris (pH 8)–150 mM NaCl–1% Nonidet P-40 for 30 min. Solubilized monolayers were centrifuged for 10 min at 10,000 × g before immunoprecipitation was carried out. For radioimmunoprecipitation of the Mip-like protein, MAB 139 or 28.2 (Mip-like protein specific [13]) was added and the mixture was incubated on ice for 1 h. Protein A-Sepharose CL-4B (Pharmacia) was then added to the samples, which were then incubated at 4°C for 1 h. Beads with immune complex were washed three times in PBS before being boiled in SDS sample buffer. Samples were separated by SDS-PAGE, and the gels were fixed, dried, and exposed to X-ray film (Amersham) for 3 weeks. In chlamidia-infected McCoy cells, [14C]palmitic acid appeared to be weakly incorporated into a protein with a size corresponding to that of the Mip-like protein (Fig. 3, lane 1). The weakness of the band detected by immunoprecipitation with Mip-specific monoclonal antibody (Fig. 3, lane 2) was expected, given the low level of Mip-like protein present in whole organisms (11) and in comparison with the prominent lipoprotein band at 14 to 15 kDa likely to represent (at least in part) the small, cysteine-rich lipoprotein that is a dominant protein in the outer membrane complex (7). Other bands evident in the infected cells (Fig. 3, lane 1) were present in uninfected cells (data not shown), and they may have arisen from posttranslational lipid modification of host cell proteins or from mitochondrion-dependent lipoprotein synthesis.

The chlamydial Mip-like protein is a lipoprotein by the following criteria. (i) Palmitic acid is incorporated into the recombinant chlamydial Mip-like protein. (ii) Globomycin, known to inhibit signal peptidase II, inhibits processing of the recombinant Mip-like protein, as shown by the incorporation experiments. (iii) The signal peptide sequence contains a signal peptidase II recognition sequence. (iv) Labeling of chlamydial organisms with palmitic acid reveals incorporation into the native Mip-like protein, as demonstrated by immunoprecipitation. (v) The Triton X-114 solubility of the Mip-like protein, despite the lack of hydrophobic domains (13), indicates the likely presence of a lipid moiety.

So far, only the small, cysteine-rich outer membrane protein of chlamydiae has been characterized as a lipoprotein (1, 7), and it is probably the chlamydial membrane structural protein with a function equivalent to that of Braun’s lipoprotein in E. coli. The chlamydial Mip-like protein is, to our knowledge, only the second lipoprotein to be identified in chlamydiae. In other bacteria, a number of lipoproteins have been identified, and they compose a diverse group of structurally and functionally distinct proteins (8).

The L. pneumophila Mip protein has no signal peptidase II site, it is not a lipoprotein, and it is surface exposed. Whether these factors indicate a function different from that in Chlamydia spp. is not known. We have previously speculated that the Mip-like protein, via properties of its N-terminal segment, is targeted to the outer membrane and that the low frequency of surface exposure arises from the release of a cell surface intermediate (13). However, the findings that the Mip-like protein is a lipoprotein and that the second amino acid of the mature protein is aspartate would suggest that it is directed to the inner membrane (22). Interestingly, the first four amino acids of the mature protein (including the asp residue; Fig. 1) are identical to the amino acids in the pullulanase protein of Klebsiella pneumoniae (9). The pullulanase is a lipoprotein secreted from the inner membrane to the exterior via a short-lived surface-exposed intermediate (5, 14, 17). The secretion is a two-step process involving insertion into the inner membrane through the lipoprotein sorting pathway and then translocation to the surface before it is released to the exterior (19); the asp residue has previously been shown to be necessary for
optimal secretion (18). The conflicting alternatives of inner versus outer membrane location for the Mip-like protein (the latter with restricted surface exposure) may thus be explained if it behaves like the pullulanase.

Whether the Mip-like protein is released from organisms is highly relevant to its mode of action. Using inhibitors of PPIase activity, we have provided evidence that the Mip-like protein is necessary for initiation of intracellular events over the first 16 h of the developmental cycle (12). We speculated that the PPIase might influence intracellular survival by modulating nutrient transport properties of the vacuolar (inclusion) membrane surrounding the newly endocytosed parasite. Indeed, mammalian PPIases have previously been suggested to regulate membrane transport channels (2). Such a proposal would require that the Mip-like protein traverse the chlamydial envelope to make contact with the inclusion membrane. Since the inclusion membrane enlarges significantly during the division of reproductive forms, release of the Mip-like protein might be required for continued membrane modification. The possibilities of secretion and release of the Mip-like protein and insertion into the inclusion membrane will be examined in future studies.

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