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Processing and Surface Presentation of the *Mycoplasma hyorhinis* Variant Lipoprotein VlpC

CATHERINE M. CLEAVINGER, MARY F. KIM, AND KIM S. WISE*

Department of Molecular Microbiology and Immunology, University of Missouri—Columbia, Columbia, Missouri 65212

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The variant surface lipoprotein VlpC of *Mycoplasma hyorhinis* was shown to be processed by cleavage of a characteristic prokaryotic prolipoprotein signal peptide. In addition, a *vlpC::phoA* fusion protein expressed and translocated in *Escherichia coli* was recognized by surface-binding monoclonal antibodies, which identified the characteristic region II of Vlps, containing divergent external sequences proximal to the membrane, as an exposed portion of these surface proteins subject to immune recognition and selection.

Organisms of the genus *Mycoplasma* are distinct among prokaryotes in that they possess a single limiting membrane in the absence of a cell wall (1). This structure must provide all critical functions related to morphology, nutrient transport, signal transduction, environmental adaptation, and surface colonization in the host. How mycoplasma membrane surface structure emerged during the reductive evolution and chromosomal diminution of these organisms is not clear. However, accumulating evidence suggests that lipoproteins may have played a particularly versatile role in this process. Amphiphilic membrane proteins that are lipid modified, or encoded by sequences characteristic of prokaryotic lipoproteins, have been reported as potential components of transport systems and as abundant membrane proteins involved in antigenic variation or adherence in mycoplasmas (reviewed in references 19 and 20). The varied occurrence of mycoplasmal lipoproteins emphasizes the need to determine how these components are processed in the organism in order to understand their assorted functions. Unfortunately, as with some other prokaryotes (18), classic approaches to defining lipoprotein processing (24) have not been readily applicable to mycoplasmas. For example, incorporation of [^{3}H]glycerol into lipid moieties is precluded when serum-containing medium is required, and an inability to specifically inhibit signal peptide processing with globomycin has been noted (19).

Fortunately, an alternative strategy to directly assess mycoplasma lipoprotein processing emerged during studies of the variable lipoprotein (Vlp) system of *Mycoplasma hyorhinis* (14), an etiologic agent causing polyarthritis in swine (15). Several Vlps have been identified (12, 14) and constitute a family of prokaryotic surface lipoproteins that have been characterized both biochemically and at the gene level (13, 14, 25). They are encoded by separate, divergent *vlp* genes and have been shown, in vitro, to undergo high-frequency phase variation. Vlps also display size variation involving extensive sets of repetitive structures at their C termini that are subject to length variation by intragenic recombination (25). Biochemical analysis has established the overall topology of Vlps, which are oriented with their hydrophilic sequence external to the single plasma membrane of the organism (13, 14, 25). They are lipid modified and amphiphilic, although it is not known if the lipid moiety alone or in conjunction with an uncleaved signal sequence determines their amphiphilicity. Despite their overall sequence differences, each Vlp contains three regions that are structurally similar (Fig. 1): region I, containing a conserved prolipoprotein signal peptide ending in the tetrapeptide sequence A-I-S-C; region II, characterized by general sequence divergence, limited composition of uncharged amino acids, and interspersed blocks of homologous amino acid sequences; and region III, composed of distinctive C-terminal repetitive units bearing the charge motif $(- + -)_n$. Monoclonal antibodies (MAbs) to surface epitopes that are unique to VlpC, unique to VlpB, or common to VlpB and VlpC have been described (3, 4, 11, 14) and define critical regions subject to immune recognition. The precise locations of these epitopes have not been determined.

Here we provide direct evidence for cleavage of the signal peptide from VlpC, a representative variable surface lipoprotein. Also, in the course of this study, previously described surface-exposed epitopes were localized to a highly variable region of Vlps, proximal to the plasma membrane.

Vlp signal peptide cleavage in *M. hyorhinis*. Since the highly conserved signal sequence of Vlps (25) is similar to functionally characterized prolipoprotein signal peptides in other bacteria (7, 24), they are predicted to be translocated and cleaved according to the typical prolipoprotein processing pathway (7, 24). In light of difficulties with classic approaches to assessing lipoprotein modification and processing in mycoplasmas, an alternative approach was required. An unusual distribution of amino acids in the VlpC sequence provided the opportunity to directly assess Vlp signal processing in *M. hyorhinis*. The VlpC coding region contains five leucine residues, all of which occur within the signal peptide, and one cysteine residue, which is the predicted N-terminal amino acid of mature VlpC if cleavage of the signal sequence occurs (25). Therefore, by metabolically labeling organisms with either L-[^{14}C]leucine or L-[^{35}S]cysteine the precursor and processed forms of VlpC can be clearly identified and differentiated. Labeling of Cys in the protein is unambiguous, since no methionine residues are present in the VlpC sequence (25) and selective labeling of VlpC with Cys but not Met has been demonstrated (14).

To monitor signal peptide processing, *M. hyorhinis* organisms were metabolically labeled as previously described (14) with either 20 μCi of L-[^{14}C]leucine (311 mCi/mmol; NEN Research Products, Boston, Mass.) per ml or 80 μCi of L-[^{35}S]cysteine (1,097 Ci/mmol; NEN) per ml. Triton X-114

* Corresponding author. Phone: (314) 882-8138. Fax: (314) 882-4287. Electronic mail address: wise@medsci.mbp.missouri.edu.

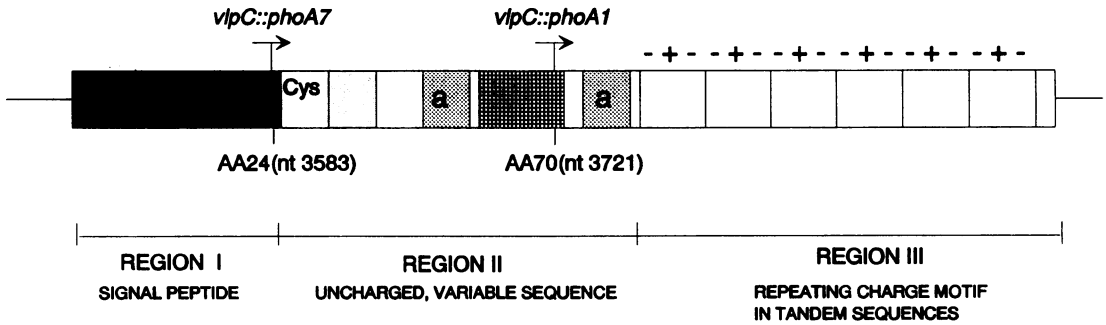

FIG. 1. Schematic representation of pre-VlpC. The coding sequence of VlpC (and other defined Vlps) is divided into three structurally distinct regions with the indicated features. Regions II and III are hydrophilic and extend outward from the plasma membrane (13, 14, 25). Shaded boxes designate blocks of sequence similarity present in other Vlps. Boxes designated "a" indicate portions shared only by VlpB and VlpC (25). The approximate locations of two *vlpC::phoA* fusions are shown above the pre-VlpC open reading frame, with corresponding amino acid (AA) and nucleotide (nt) positions (according to reference 25) indicated below each fusion.

(TX-114)-phase proteins, representing phase-fractionated organisms from 0.5 ml of broth culture (2, 14), were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blot transfer for immunostaining and autoradiography (14). Labeling with L-[^{35}S]cysteine revealed a single discrete TX-114-phase protein of 37 kDa (Fig. 2A, top, lane 1) which was identified as VlpC by immunostaining the same blot with MAb to VlpC (F192C17 [3, 4]) (Fig. 2A, bottom, lane 1). Labeling with L-[^{14}C]leucine showed no autoradiographic band corresponding to the VlpC immunoblot even though many other proteins were labeled with this amino acid (Fig. 2A, bottom, lane 2, compare top and bottom). To confirm the differential metabolic labeling of VlpC further, radioimmunoprecipitation of TX-114-phase material with MAb to VlpC (F192C17) was performed (Fig. 2A, lanes 3 and 4). Authentic VlpC immunoprecipitated from TX-114-phase proteins of *M. hyorhinis* was labeled with L-[^{35}S]cysteine (Fig. 2A, lane 3, compare top and bottom) but failed to label with L-[^{14}C]leucine (Fig. 2A, lane 4, compare top and bottom). The inability to label immunologically isolated and defined VlpC with L-[^{14}C]leucine, under conditions in which this label was readily incorporated into other mycoplasma proteins (Fig. 2A, lane 2, top), offers strong evidence that the VlpC signal is cleaved in *M. hyorhinis*. The precise position of cleavage was not established by this experiment, but the site can be localized to a region of 4 amino acids between Leu and Cys residues in the sequence.....LIAISC....., which contains the previously identified (25) prokaryotic prolipoprotein cleavage motif (underlined) at the C terminus of the signal peptide.

Identification of unprocessed VlpC in *E. coli*. To determine the ability of Vlp lipoprotein-like signal sequences to be processed by the classic prolipoprotein pathway, we examined the expression, in *E. coli*, of recombinant VlpC (rVlpC). The *vlpC* gene was cloned from an isogenic sibling (i.e., one with a similar *vlpC* coding sequence) of the *M. hyorhinis* variant labeled in the preceding experiment. Originally planned to analyze inhibition of signal processing with globomycin, this experiment instead established the lack of VlpC signal cleavage in *E. coli* and concomitantly provided an important control showing the ability to metabolically label the VlpC precursor with L-[^{14}C]leucine. HB101(pH2; pGP1-2) (25) was used to overexpress rVlpC by using the T7 expression system (17) with modifications as described by Jung et al. (8). rVlpC expression

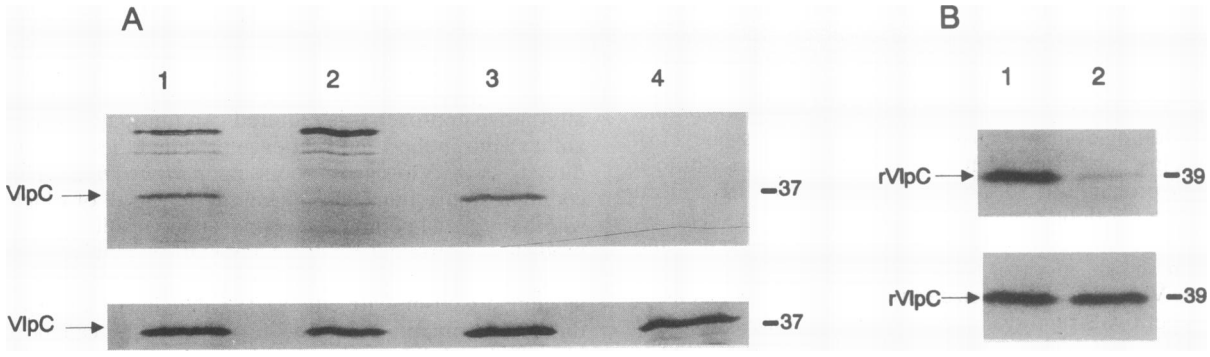

FIG. 2. Processing of authentic VlpC and rVlpC. (A) Metabolic labeling (top) and immunoblot (bottom) of VlpC in *M. hyorhinis*. TX-114-phase protein from *M. hyorhinis* was metabolically labeled with L-[^{35}S]cysteine (lanes 1 and 3) or L-[^{14}C]leucine (lanes 2 and 4). These samples (lanes 1 and 2) or proteins immunoprecipitated from these samples (lanes 3 and 4) with MAb to VlpC (F192C17) were run on SDS-PAGE gels and transferred to nitrocellulose. Blots were autoradiographed (top) and immunostained (bottom) with MAb to VlpC (F192C17). (B) Metabolic labeling (top) and immunostaining (bottom) of rVlpC. Protein extract from HB101 (pH2; pGP1-2) cells metabolically labeled with either L-[^{35}S]cysteine (lane 1) or L-[^{14}C]leucine (lane 2) were run on SDS-PAGE gels prior to transfer to nitrocellulose. An autoradiograph (top) was obtained following immunoblot analysis (bottom) with MAb to VlpC (F192C17). VlpC-specific bands on the autoradiographs and the immunoblots are superimposable. VlpC products are indicated by arrows, with molecular masses (in kilodaltons), determined by comparison with size markers run on the same gel, indicated to the right.

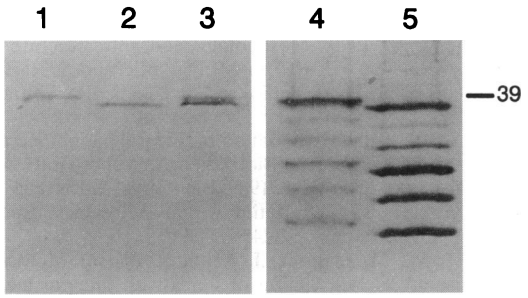

FIG. 3. Size difference between recombinant VlpC and authentic VlpC. Immunoblot analysis of intact or partial tryptic fragments of rVlpC and mycoplasma-derived VlpC is shown. Overexpressed protein from HB101 (pH2; pGP1-2) (lane 1), TX-114-phase proteins from *M. hyorhinis* (lane 2), and a combination of both (lane 3) were run on SDS-PAGE gels, transferred to nitrocellulose, and subjected to immunoblot analysis with MAb to VlpC (F192C17). Partial tryptic digests of samples in lanes 1 and 2 are shown in lanes 4 and 5, respectively. The relative molecular mass of rVlpC is indicated in kilodaltons as in Fig. 2.

was selectively induced in the presence of rifampin in medium containing 5 μCi of L-[^{14}C]leucine per ml or 30 μCi of L-[^{35}S]cysteine per ml, and cells were subjected to SDS-PAGE, immunoblotting, and autoradiographic analysis as previously described (14). MAb to VlpC (F192C17) identified a single, discrete VlpC product that was labeled with both L-[^{35}S]cysteine (Fig. 2B, lane 1, compare top and bottom) and L-[^{14}C]leucine (Fig. 2B, lane 2, compare top and bottom). This result provided a positive control showing that VlpC can be labeled with L-[^{14}C]leucine and concomitantly showed that the protein is apparently not processed in *E. coli*. This was supported by the inability to identify any processed form of VlpC in *E. coli* by using either radiolabeled compound or the sensitive MAb immunoblot.

To further compare rVlpC expressed in *E. coli* with authentic (mycoplasmal) VlpC, the relative sizes of the products were assessed. TX-114-phase proteins (2, 14) from the same *M. hyorhinis* clonal variant described above (containing the same *vlpC* structural gene as the recombinant construct) and the gel-eluted (21, 22) rVlpC expressed in *E. coli* were subjected to SDS-PAGE and immunoblotted (Fig. 3). The recombinant product migrated slightly more slowly than the authentic VlpC product (Fig. 3, lanes 1 to 3), with a difference in size consistent with that predicted by the presence of a signal peptide. This result also shows the ability to resolve and identify any processed products, which were not seen in *E. coli*. The size difference between the rVlpC product in *E. coli* and the authentic Vlp product from the mycoplasma was accentuated by partial digestion of each with L-1-tosylamine-2-phenylethyl-chloromethyl ketone. This created a set of products truncated by removal of C-terminal repetitive structures (13, 14) (Fig. 1). As shown in Fig. 3 (lanes 4 and 5), six partial tryptic products were derived from each protein as predicted by the *vlpC* sequence (25). This is characteristic of the VlpC C-terminal sequence and confirmed that the recombinant and authentic products were similar in their region III structures. However, the periodic ladders clearly differed by 2 to 4 kDa, again corresponding to the size difference predicted by the presence versus the absence of a signal peptide. These observations, in conjunction with the selective labeling of the recombinant rVlpC with Leu, indicate that the VlpC signal peptide is processed in *M. hyorhinis* but not in *E. coli*. The lack of rVlpC processing is not fully understood at this time. Although prolipoprotein processing of some heterologous recombinant lipoproteins, including those from spirochetes (16) and gram-positive species (6), has been shown to occur in *E. coli*, recombinant lipoproteins from other organisms have not been cleaved in this host (18). It has been argued that this lack of cleavage may be due to a slight difference in signal peptidase II specificity (18).

Identification of VlpC surface epitopes. To understand the location of surface accessible epitopes on Vlp proteins, alkaline phosphatase fusions to rVlpC were generated with the transposon Tn*phoA*. For the purpose of mutagenesis, a 669-bp *Hin*dIII fragment bearing the VlpC coding sequence but not the complete *vlp* promoter sequence (25) was cloned into the corresponding site of pGEM7ZCAT in an orientation allowing for the expression of *vlpC* from pLac. This plasmid was designated pMH7C-H2. The vector pGEM7ZCAT, a chloramphenicol-resistant derivative of pGEM7Zf(+) (Promega Biotec), was constructed for an unrelated study and was generated by replacing an 830-bp *Sca*I-*Nae*I fragment with a 950-bp end-filled *Bst*BI Cm^r cassette from pBR325 (10). *E. coli* CC118 (9) was transformed with pMH7C-H2 and infected with λTn*phoA* as previously described by Gutierrez et al. (5), and enzymatically active fusions, representing approximately 1.5% of the total number of transductants, were selected by the ability to utilize the chromogenic phosphatase substrate 5-chloro-4-bromo-3-indolyl phosphate. The junction of each active fusion was determined by sequence analysis with a *phoA*-specific oligonucleotide (5′-GCCCGGTTTTCCAGAA CAGG-3′), and two *vlpC*::*phoA* fusions were selected for further study. The junction of *vlpC*::*phoA1* is located just after amino acid 70 of pre-VlpC near the C-terminal boundary of region II (Fig. 1). The corresponding 57-kDa fusion product (VlpC-AP1) was identified by SDS-PAGE and immunoblotting (14) with a MAb to alkaline phosphatase (Chemicon International, Inc., Temecula, Calif.). The junction of *vlpC*::*phoA7* is located just after amino acid 24 of the VlpC signal peptide (Fig. 1) and was found to produce a 53-kDa fusion protein (VlpC-AP7). This fusion product also demonstrated the membrane translocation capability of the predicted signal peptide, even though the fusion is located just upstream of (5 residues preceding) the proposed signal peptidase II lipoprotein cleavage site (7).

It was anticipated that VlpC surface epitopes located upstream of the fusion junction would be present on the fusion protein. Importantly, with the fusion constructs shown, the role of region II as a target of immune recognition could be tested with MAbs previously shown to bind distinct, surface-exposed epitopes. Cells expressing fusion proteins were harvested from mid-logarithmic-phase cultures and analyzed by SDS-PAGE. As predicted from its lack of mature VlpC sequences, VlpC-AP7 contained no VlpC surface epitopes (data not shown). In contrast, VlpC-AP1 contained one of two VlpC-specific epitopes, defined with MAb F20C17F (Fig. 4), clearly localizing this epitope to region II (Fig. 1). Since this MAb mediates complement-dependent mycoplasma killing (11), this region of VlpC could further be implicated as a potential target of a mycoplasmacidal immune response in vivo. An epitope shared by VlpB and VlpC and defined with surface binding MAb 4C1A (14) was also present on VlpC-AP1 and thus was similarly localized to region II. Amino acid sequences shared between VlpB and VlpC (also located in region II) may contribute to this epitope structure (Fig. 1). Localization of two classes of surface epitopes to region II marks this domain as a surface-exposed portion of VlpC accessible to antibody binding. This may provide a possible basis underlying the observed variation in this region, which could result from

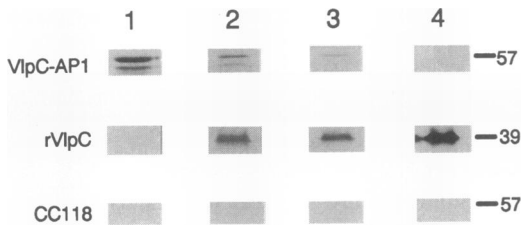

FIG. 4. Mapping of VlpC surface epitopes. Immunostaining of VlpC-AP1, rVlpC, and CC118 is shown. Extracts from CC118 or CC118 (*vlpC::phoA1*) cells or gel-purified rVlpC were separated by SDS-PAGE in the same gel and electrophoretically transferred to nitrocellulose. VlpC-AP1 was detected with MAb to AP (column 1), and parallel samples were then screened for the presence of VlpC-specific surface epitopes by immunoblotting with MAb F20C17F to VlpC (column 2), MAb 4C1A to VlpB/C (column 3), or MAb F192C17 to VlpC (column 4). Relevant portions of each blot corresponding to the position of VlpC-AP1 or rVlpC or the equivalent position in channels containing control CC118 preparations are shown. Approximate molecular masses, in kilodaltons, are indicated to the right as in Fig. 2.

selective pressure imposed by the host immune system (23). An additional VlpC-specific epitope defined with MAb F192C17 was not present in the VlpC-AP1 construct, suggesting that it is located either in the distal end of region II or in region III or that it corresponds to a conformational motif not preserved in the fusion protein. It is not yet known how variation in the number of region III repeats expressed in Vlp size variant isolates might affect the surface accessibility of region II epitopes; however, now that region II epitopes have been defined these relationships can be further investigated.

Two important conclusions arise from this study. First, mycoplasmas have retained a mechanism (including a possible signal peptidase II homolog) to cleave prolipoproteins. Since mature Vlps lack a signal peptide and contain only a continuous hydrophilic sequence that has been shown to extend externally from the membrane (13, 14, 25), they appear to be anchored to the single membrane of *M. hyorhinis* solely by fatty acyl chains, which is typical of prokaryotic lipoproteins. Acyl chains appear to be the feature that dictates their amphiphilicity in detergent fractionation. The absence of any transmembrane sequence (now including the signal sequence) also rules out certain biological functions for mature Vlps, such as signal transduction by polypeptide sequences spanning the plasma membrane.

Second, the unusual maintenance of divergence in Vlp sequences throughout two structurally distinct regions of the protein may now be better understood. Together regions II and III (Fig. 1) constitute the entire external hydrophilic Vlp protein sequence. Our results show that region II, which is more proximal to the membrane, contains epitopes recognized by surface-binding antibodies and therefore may be subject to diversification by immune selection. These features are consistent with the previously proposed model of Vlp topology (20, 23).

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