

Architecture of the Cell Envelope of *Chlamydia psittaci* 6BC

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Received 29 September 1994/Accepted 30 November 1994

The cysteine-rich envelope proteins of the elementary body form of chlamydiae are thought to be located in the outer membrane on the basis of their insolubility in the weak anionic detergent *N*-lauryl sarcosinate (Sarkosyl). We found, however, that the insolubility of the small (EnvA) and the large (EnvB) cysteine-rich proteins of *Chlamydia psittaci* 6BC in Sarkosyl is dependent on the maintenance of a supramolecular disulfide-cross-linked complex and is unlikely to be a valid indicator of outer membrane location. Consequently, we used other methods to characterize the architecture of the cell envelope of *C. psittaci* 6BC. We found that disulfide-reduced EnvA, previously shown to be a lipoprotein, segregated into the detergent phase during Triton X-114 partitioning experiments and was recovered from the membrane fraction of elementary bodies lysed by nondetergent means. In contrast, disulfide-reduced EnvB segregated to the aqueous phase in partitioning experiments and was found in the soluble fraction of elementary bodies lysed in the absence of detergents. The hydrophobic affinity probe 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)-diazirine labeled the major outer membrane protein and EnvA but did not label EnvB. Treatment of intact elementary bodies of *C. psittaci* with trypsin had no effect on the cysteine-rich proteins, although the major outer membrane protein was partially degraded. On the basis of these and other observations, we propose that EnvA is anchored to the outer membrane by its lipid moiety, with a hydrophilic peptide portion extending into the periplasm, and that EnvB is located exclusively within the periplasm. We further propose that disulfide-cross-linked polymers of EnvB are the functional equivalent of peptidoglycan, forming a disulfide-cross-linked network with the periplasmic domains of EnvA and other membrane proteins, which accounts for the osmotic stability of elementary bodies.

Members of the genus *Chlamydia* are obligate intracellular parasites. The cell envelopes of both the extracellular elementary body (EB) and the intracellular reticulate body (RB) forms resemble those of gram-negative bacteria, consisting of an outer and an inner membrane (35). The envelopes are atypical, however, in that they lack peptidoglycan (3, 15), and some envelope proteins in EBs are disulfide cross-linked (16, 19, 30). It has been proposed that the cross-linked envelope proteins form a supramolecular structure that may be responsible for the osmotic stability of EBs (16, 19, 20, 30). The predominant proteins in the cross-linked complex of EBs are the major outer membrane protein (MOMP), one or two large cysteine-rich proteins (CRPs), and a small CRP (19, 28, 32). The MOMP is also present in the logarithmically dividing RBs but is not cross-linked (16, 19, 20, 28). The CRPs, in contrast, are found in the cell envelope of intracellular chlamydiae only late in the cycle, starting at 18 to 24 h postinfection, when RB multiplication has slowed and RBs have begun to reorganize back to EBs (20, 28, 32).

Many observations suggest that the MOMP is located within the outer membrane and is surface exposed: the MOMP of EBs is susceptible to hydrolysis by trypsin (33), radioiodination (9, 21), and recognition by monoclonal antibodies (2, 10, 23). Baehr et al. (2) have suggested that the MOMP is also exposed to the periplasm, consistent with its function as a porin (5). Considerably less is known about the intracellular location of the CRPs, in part because there are no reports in the literature of successful separation of inner and outer membranes of

chlamydiae by physical means. The small 12-kDa CRP of *Chlamydia psittaci* 6BC, designated EnvA, possesses a predicted signal peptidase II-processing site and appears to be a lipoprotein with a structure similar to that of the Braun lipoprotein of *Escherichia coli* (12). The large CRP (EnvB) of *C. psittaci* 6BC is posttranslationally cleaved to a doublet consisting of approximately equimolar concentrations of 60-kDa proteins (13, 19). The primary sequence of EnvB possesses a typical signal peptidase I cleavage site (13), and EnvB is presumably processed at one or two additional downstream sites to form the mature doublet. Large CRP doublets have also been reported in lymphogranuloma venereum (1) but not trachoma (29) strains of *Chlamydia trachomatis*. The presence of potential signal peptidase recognition sequences in the large and small CRPs suggests that these cell-associated proteins are located in either the inner membrane, the outer membrane, or the periplasmic space. Neither the large nor the small CRP of *C. trachomatis* L2 appears to be surface exposed, on the basis of the failure of antibodies raised to these proteins to react with intact EBs (10). However, recently Ting and Bavoil (36) have suggested that the large CRP doublet proteins of the guinea pig inclusion conjunctivitis (GPIC) agent of *C. psittaci* may serve as surface-exposed adhesins for host cells. The insolubility of the large and small CRPs in the weak anionic detergent *N*-lauryl sarcosinate (Sarkosyl), a characteristic of integral outer membrane proteins of gram-negative bacteria (14), has led to the speculation that they are located in the outer membrane (19, 28, 32). However, it is not clear whether the insolubility of the CRPs in Sarkosyl is a function of cellular location or simply a reflection of their extensive cross-linkage to form an insoluble particulate structure. The purpose of this study, therefore, was to determine the probable cellular location of the CRPs of *C. psittaci* 6BC EBs by several methods, including reexamination of the extraction properties of the CRPs in Sarkosyl.

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MATERIALS AND METHODS

Preparation of detergent-insoluble envelope complexes. L cells (2.5×10^7) infected with 10 50% infective doses of *C. psittaci* 6BC were incubated in 25 ml of medium M199–5% fetal calf serum (GIBCO, Grand Island, N.Y.) containing 0.5 μ g of cycloheximide per ml and 100 μ Ci of [35 S]cysteine (600 Ci/mmol; NEN Research Products, Boston, Mass.) between 9 and 48 h postinfection, and EBs were harvested and purified by Renografin density centrifugation (21). Equal portions of purified EBs were incubated at 37°C for 30 min in either 2% sodium dodecyl sulfate (SDS) or 2% Sarkosyl (Sigma Chemical Co., St. Louis, Mo.) in $1 \times$ TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) in the presence or absence of 20 mM dithiothreitol (DTT), and detergent-insoluble complexes were prepared by centrifugation ($10,000 \times g$) for 30 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions and analyzed by autoradiography.

Triton X-114 phase partitioning. *C. psittaci* 6BC-infected L cells (10^7) were pulsed for 10 min at 25 h postinfection with 0.1 μ Ci of [35 S]cysteine in cysteine-free medium (MEM Select Amine; GIBCO) in the presence of cycloheximide (100 μ g/ml) and chased in medium M199–5% fetal calf serum containing cycloheximide (0.5 μ g/ml) until EBs were harvested and purified at 48 h postinfection. The purified EBs were Triton X-114 phase partitioned in the absence of reducing agents and then reextracted in the presence of 20 mM DTT and 0.64 M 2-mercaptoethanol as described previously (12). Equal portions of the aqueous phosphate-buffered saline (Dulbecco PBS; GIBCO) and the Triton X-114 phases were subjected to SDS-PAGE, and the separation of labeled proteins was examined by autoradiography.

Analysis of envelope proteins from EBs lysed in alkaline EDTA. Purified EBs were treated with 10 mM EDTA at pH 9.5, 10 mM EDTA–10 mM sodium carbonate-bicarbonate buffer at pH 10.0, or 2% SDS in the presence or absence of 20 mM DTT for 2 h at 37°C. The insoluble membrane fraction ($100,000 \times g$ centrifugation pellet) of the samples was subjected to SDS-PAGE under reducing conditions, and proteins were identified by staining with Coomassie brilliant blue and by an immunoblot of the gel. The immunoblot was reacted with a monoclonal antibody raised against EnvA (13) and a polyclonal antibody raised against the large CRP of *C. trachomatis* L2 (kindly provided by Harlan Caldwell, Rocky Mountain Laboratories, Hamilton, Mont.).

Labeling of membrane proteins with [125 I]TID. Fifteen microcuries of 3-(trifluoromethyl)-3-(*m*-[125 I]iodophenyl)diazirine ([125 I]TID; Amersham, Arlington Heights, Ill.) in a volume of 3 μ l was added with a glass capillary pipette to 100 μ l of EBs in PBS on ice in a dimly lit room. The [125 I]TID was photoactivated by exposing the mixture for 30 min to a model UVL-56 BLAK-RAY long-wave UV 366-nm lamp (UVP Inc., San Gabriel, Calif.) placed directly over a multiwell glass plate containing the samples. SDS-insoluble complexes were prepared and analyzed by SDS-PAGE and autoradiography.

Trypsin treatment of intact EBs and SDS-insoluble complexes. Purified EBs and SDS-insoluble envelope complexes prepared from purified EBs of *C. psittaci* 6BC and *C. trachomatis* L2 were suspended in $1 \times$ TE, with and without 5 mM MgCl₂, and incubated with 100 μ g of type III trypsin from bovine pancreas (Sigma) per ml at 37°C for 30 min. The reaction was stopped by the addition of trypsin inhibitor from chicken egg white (Sigma) to a final concentration of 100 μ g/ml and SDS-PAGE solubilization buffer (24). Proteins were separated by SDS-PAGE, and the large CRPs and their degradation products were detected by immunoblotting with a polyclonal antibody raised against the large CRP of *C. trachomatis* L2.

RESULTS

Solubility properties of chlamydial envelope proteins. Lysis of *C. psittaci* 6BC EBs in SDS, a method which dissolves the lipid bilayer of membranes, yielded predominantly the MOMP, EnvA (the small CRP), and EnvB (the large CRP doublet) in the insoluble centrifugation pellet (Fig. 1). The insolubility of the MOMP and the CRPs in this strong anionic detergent was dependent on interpeptide disulfide cross-links since these proteins were rendered soluble in SDS containing DTT. The MOMP and the CRPs were also insoluble in the weak anionic detergent Sarkosyl (Fig. 1), a property of integral outer membrane proteins of gram-negative bacteria (14). However, only the MOMP remained insoluble in Sarkosyl under reducing conditions. On the basis of Sarkosyl solubility, therefore, the MOMP, but not the CRPs, appears to be an integral outer membrane protein. A common procedure for determining the location of proteins in the envelope of gram-negative bacteria is the separation of inner from outer membranes on density gradients (22). For unknown reasons, we have not been successful in applying this method and variations of this method to chlamydiae (18). We therefore used alterna-

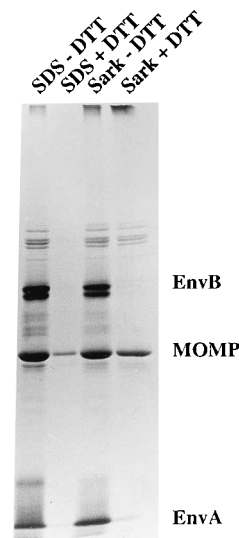


FIG. 1. Solubility of the predominant envelope proteins of *C. psittaci* in SDS and Sarkosyl. [35 S]cysteine-labeled EBs were treated with 2% SDS or 2% Sarkosyl (Sark) with and without the addition of 20 mM DTT as indicated, and the insoluble fractions were analyzed by SDS-PAGE and autoradiography under reducing conditions. Residual MOMP seen in preparations extracted with SDS and DTT and residual EnvA in preparations extracted with Sarkosyl and DTT are rendered soluble upon reextraction (data not shown).

tive methods to better characterize the cellular location of the predominant chlamydial envelope proteins.

Triton X-114 phase partitioning experiments have been used to characterize the hydrophobicity of proteins (6, 31). Lipoproteins and membrane proteins containing large hydrophobic domains segregate to the Triton X-114 phase, while water-soluble proteins partition with the aqueous PBS phase. In the absence of reducing agents, we found that the disulfide-cross-linked envelope structure of EBs failed to partition into either phase (Fig. 2) but rather was located in the insoluble pellet (not shown). When the partitioning was carried out in the presence of reducing agents, EnvB segregated exclusively to the aqueous phase, suggesting that it is unlikely to be tightly associated with a membrane. In contrast, EnvA was found entirely in the Triton X-114 phase, as previously noted (12). This observation is consistent with the lipoprotein nature of EnvA. The MOMP also segregated to the Triton X-114 phase under reducing conditions (Fig. 2), although a significant proportion was found in the insoluble pellet (data not shown). Partitioning in the Triton X-114 phase, rather than the aqueous phase, is consistent with an outer membrane location for the MOMP.

To further identify the cellular location of the predominant envelope proteins of chlamydiae, EBs were lysed by incubation in alkaline EDTA (27), and the particulate membrane fraction (containing inner and outer membranes) was separated from soluble periplasmic and cytoplasmic proteins by centrifugation. When EB lysis was carried out by this nondetergent method in the absence of reducing agent, the MOMP and both CRPs were present in the centrifugation pellet. However, when lysis was carried out in the presence of DTT to disrupt the supramolecular disulfide-cross-linked complex, only the MOMP and EnvA were found in the membrane fraction (Fig. 3).

The solubility properties of the CRPs and the MOMP of *C. psittaci* under reducing conditions are summarized in Table 1. The results are consistent with the conclusion that EnvA and the MOMP are associated with a membrane whereas mono-

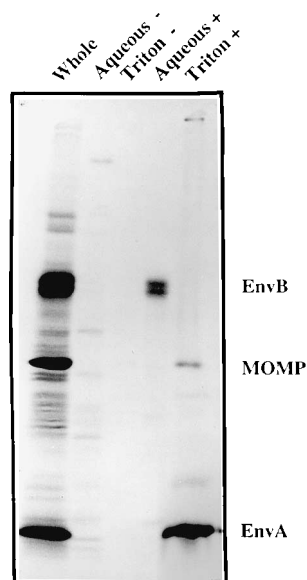


FIG. 2. Triton X-114 phase partitioning of EB envelope proteins. [^{35}S]cysteine-labeled EBs of *C. psittaci* were partitioned in Triton X-114, either in the presence or in the absence of reducing agents, and the phases were analyzed by SDS-PAGE and autoradiography. Lanes: Whole, untreated EBs; Aqueous -, aqueous phase without reducing agents; Triton -, Triton X-114 phase without reducing agents; aqueous +, aqueous phase with reducing agents; Triton +, Triton X-114 phase with reducing agents.

meric EnvB is a soluble protein not tightly associated with a membrane.

Hydrophobic affinity labeling of *C. psittaci* envelope proteins. Photoactivatable lipophilic reagents have been used to label proteins associated with the apolar core of membranes (7). A particularly useful photoactivatable hydrophobic probe is [^{125}I]TID (7, 8), which partitions to hydrophobic environments and under UV light reacts with the fatty acid chains of lipids and the side chains of amino acids. One advantage of [^{125}I]TID is that the carbene generated by photoactivation is capable of reacting with alkyl groups rather than being restricted to forming covalent adducts with nucleophiles such as NH_2 or SH groups (7). Consequently, any amino acid within a hydrophobic environment should be labeled (7). When EBs were exposed to photoactivated [^{125}I]TID, several bands in the SDS-insoluble complexes were noted following SDS-PAGE (Fig. 4). The two most mobile bands migrated to the positions expected for lipopolysaccharide and other lipids. Only two protein bands were well labeled: one comigrating with [^{35}S]cysteine-labeled MOMP and another comigrating with EnvA. No label was associated with the EnvB doublet. These results confirm that the MOMP is located in the outer membrane of EBs. They also suggest that a portion of EnvA, most likely its lipid moiety, is located within the hydrophobic environment of a membrane. The failure of [^{125}I]TID to label EnvB suggests that no part of this protein is associated with the hydrophobic core of the outer membrane.

Susceptibility of the CRPs to trypsin. Ting and Bavoi (36) have suggested that the large CRP of the GPIC agent of *C. psittaci* is an adhesin for host cells. Support for this suggestion includes the observations that the 60-kDa doublet proteins are partially degraded, with a concomitant loss of infectivity, when EBs are treated with trypsin (4). These observations imply that at least a portion of the large CRP is exposed on the surface of the GPIC agent. In contrast, we found that the large CRP of

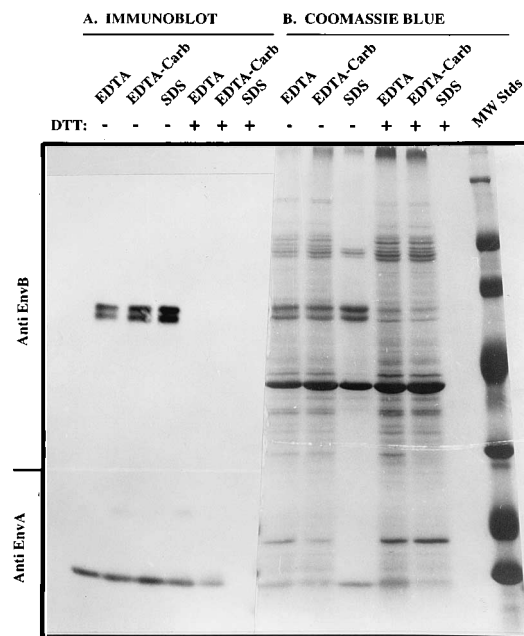


FIG. 3. Association of proteins with the membrane fraction of EBs lysed with alkaline EDTA or SDS. Purified EBs were treated with EDTA at pH 9.5 (EDTA), EDTA-sodium carbonate-bicarbonate buffer at pH 10.0 (EDTA-Carb), or 2% SDS in the absence (-) or presence (+) of DTT for 2 h at 37°C. The insoluble membrane fractions were subjected to SDS-PAGE under reducing conditions and analyzed by immunoblotting (A) and staining with Coomassie brilliant blue (B). Molecular mass markers weight standards (MW stds; 206, 111, 70, 45, 29, 18, and 15 kDa) are shown on the right.

intact EBs of *C. psittaci* 6BC was not affected by trypsin, and therefore not surface exposed, as long as an excess of Mg^{2+} was present (Fig. 5A). Trypsin also had no effect on EnvA under these conditions but did degrade about 20% of the 43-kDa MOMP to a peptide migrating with an M_r on SDS-PAGE of about 40,000 (data not shown). When Mg^{2+} ions were chelated with EDTA, the larger 60-kDa doublet protein appeared to be degraded by trypsin to several species about the size of the small doublet protein, with possible degradation of the small doublet protein as well (Fig. 5A). Under these conditions, trypsin had no obvious effect on EnvA (data not shown). Treatment of gram-negative bacteria with EDTA results in release of lipopolysaccharide (25), compromising the outer membrane permeability barrier and rendering the organisms susceptible to lysis with lysozyme (11). We presume that EDTA has a similar effect on the outer membrane of *C. psittaci* 6BC, exposing periplasmically located proteins to trypsin. SDS-insoluble envelope protein complexes were only slightly

TABLE 1. Solubility properties of the predominant envelope proteins of *C. psittaci* 6BC

Treatment ^a	Solubility		
	EnvA	EnvB	MOMP
SDS	+	+	+
Sarkosyl	+	+	-
Triton X-114 phase	+	-	+/-
Aqueous PBS phase	-	+	-
Alkaline EDTA	-	+	-

^a EBs were treated in the presence of reducing agents as described in Materials and Methods.

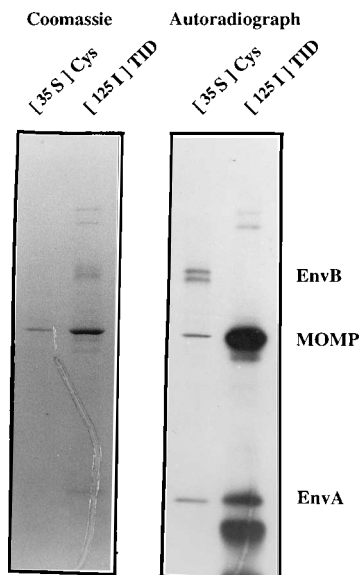


FIG. 4. [^{125}I]TID labeling of membrane proteins. EBs were intrinsically labeled with [^{35}S]cysteine or extrinsically labeled with [^{125}I]TID as described in Materials and Methods. The SDS-insoluble fractions were analyzed by SDS-PAGE followed by staining with Coomassie blue and autoradiography.

more susceptible to further degradation by trypsin, suggesting that the failure of trypsin to completely digest EnvB to small peptides (the largest predicted tryptic peptide is 3.4 kDa) depended on the conformation of the protein rather than on protection of the protein by a membrane structure (Fig. 5B). Reduction of the SDS-insoluble complex with DTT increased susceptibility to trypsin, but degradation was still incomplete (Fig. 5B). The effect of trypsin on the large CRPs in EBs of the GPIC agent (4) and *C. trachomatis* L2 (Fig. 5C) when Mg^{2+} was present was similar to the effect observed on the CRP doublet proteins of *C. psittaci* 6BC when Mg^{2+} ions were

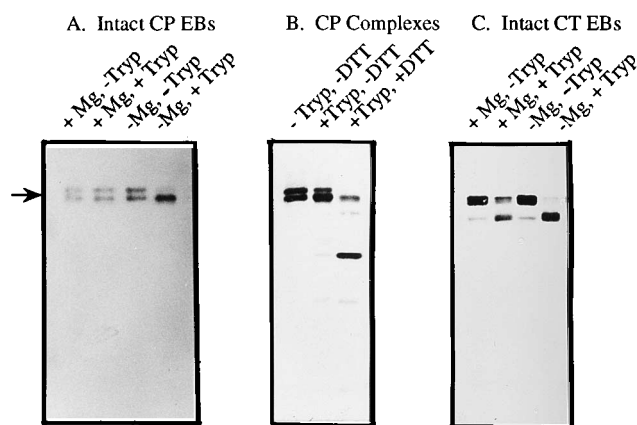


FIG. 5. Effect of trypsin on the large CRP of chlamydiae. (A) Intact *C. psittaci* 6BC (CP) EBs were suspended in $1\times$ TE buffer in either the presence or absence of 5 mM Mg^{2+} (Mg) and were incubated with and without trypsin (Tryp). (B) SDS-insoluble envelope protein complexes of *C. psittaci* EBs were suspended in $1\times$ TE buffer and incubated with and without trypsin in the presence of absence of 20 mM DTT. (C) Intact *C. trachomatis* L2 (CT) EBs were treated as described in for panel A. Proteins were separated on 11% (A and B) or 7.5 to 15% gradient (C) gels, and the large CRP peptides were analyzed by immunoblotting using a polyclonal antibody directed against the large CRP of *C. trachomatis* L2. The arrow points to the large CRP doublet proteins.

chelated; i.e., partial degradation was observed. The reason for this variability among chlamydial strains is not clear.

DISCUSSION

The original suggestion that the MOMP and the CRPs of chlamydiae are outer membrane proteins was based on the observation that they are insoluble in Sarkosyl (9, 19, 21, 28, 32). We have found, however, that only the MOMP of *C. psittaci* 6BC EBs remains insoluble in Sarkosyl when interpeptide disulfide cross-links are broken with reducing agents. The insolubility of cross-linked CRPs in Sarkosyl is not, therefore, a valid indicator of the cellular location of these proteins. Because we have not been successful in our attempts to separate inner from outer membranes by physical means (18), we further characterized the cellular location of the predominant chlamydial envelope proteins of *C. psittaci* 6BC by alternative methods.

The insolubility of reduced MOMP in Sarkosyl and in alkaline EDTA, the segregation of MOMP into the Triton X-114 phase in partitioning experiments, and the susceptibility of MOMP in intact EBs to lipophilic labeling with [^{125}I]TID and to degradation by trypsin support a variety of previous observations (2, 5, 9, 10, 21, 23, 33) that indicate that the MOMP is a surface-exposed, integral outer membrane protein. In addition, the poor solubility of the MOMP observed in Triton X-114 partitioning experiments may be a reflection of its porin structure (5), since Maher and Singer (26) demonstrated that a multisubunit channel-forming integral membrane protein (acetylcholine receptor) forms aggregates when subjected to Triton X-114 phase partitioning. The partitioning of EnvA into the Triton X-114 phase and the presence of EnvA in the centrifugation pellet when EBs are lysed by alkaline EDTA suggest that EnvA also is a membrane protein. The susceptibility of EnvA to labeling with [^{125}I]TID suggests an outer membrane rather than an inner membrane location for EnvA since it is unlikely that [^{125}I]TID can traverse the aqueous environment of the periplasm. In contrast to the MOMP, EnvA is soluble in Sarkosyl under reducing conditions and therefore is unlikely to be an integral outer membrane protein. We feel it more likely that EnvA is anchored to the inner leaflet of the outer membrane by its lipid moiety, with its hydrophilic cysteine-rich peptide portion extending into an aqueous environment. The failure of trypsin to degrade EnvA in intact EBs supports the conclusion that the peptide portion EnvA extends into the periplasm rather than into the external environment. In contrast to our observations on the MOMP and EnvA, we found no evidence that EnvB is associated with a membrane: EnvB is soluble in PBS but not Triton X-114, it is soluble in Sarkosyl, it is not found in the membrane fraction when EBs are lysed by nondetergent means in the presence of reducing agents, and it is not labeled by [^{125}I]TID. This evidence and the presence of a predicted signal peptidase I sequence in the EnvB open reading frame suggest that EnvB is translocated through the inner membrane to the periplasm, where it remains as a major structural envelope protein.

A model of the EB cell envelope of *C. psittaci* 6BC is shown in Fig. 6. EnvA and EnvB are represented as the spatial and functional analogs of murein lipoprotein and peptidoglycan, respectively, of gram-negative bacteria. MOMP, the mature form of which contains seven cysteine residues, is represented as a porin, although it likely functions as such only when it is not cross-linked (5); its precise mutimeric structure may be quite different in cross-linked EBs. The specific topological relationship among the predominant envelope proteins is not known. However, we speculate that EnvB, which contains 37

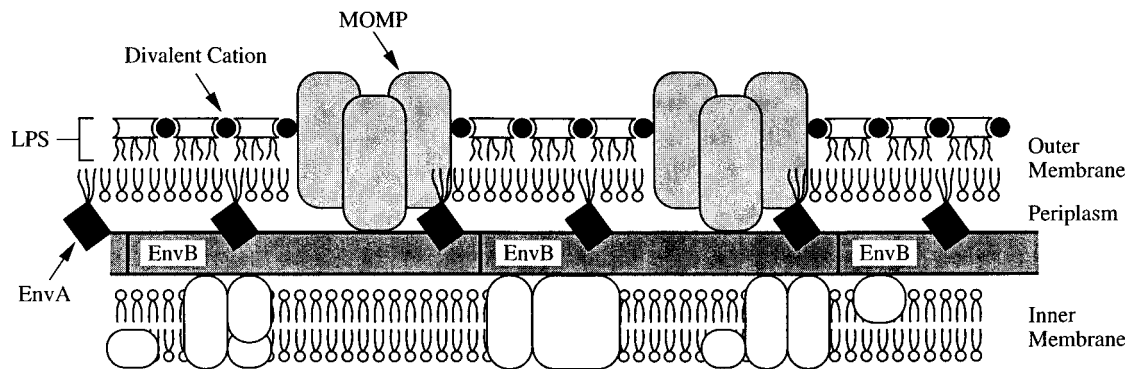


FIG. 6. Model of the cell envelope of *C. psittaci* 6BC EBs. Representation of the MOMP as a transmembrane protein in the outer membrane is based on the model of Baehr et al. (2). Its representation as a trimer is based on evidence that the cell-free MOMP possesses porin activity (5); however, a trimeric structure has not been experimentally demonstrated in either un-cross-linked RBs or disulfide-cross-linked EBs. The actual shapes of the proteins are not known, and specific inner membrane proteins have not been identified. We propose that periplasmically located, cross-linked EnvB multimers may also be disulfide bonded to the periplasmically exposed domains of other envelope proteins; however, specific intermolecular cross-links have not been identified. The MOMP/EnvA/EnvB molecular ratio has been shown to be approximately 5:2:1 (13). LPS, lipopolysaccharide.

cysteine residues, is extensively cross-linked with itself and with EnvA, which contains 14 cysteines in the proposed mature form. Implicit in this model is the suggestion that the osmotic stability of EBs is primarily due to cross-linked polymeric EnvB, although disulfide bonding between EnvB and the cytoplasmically exposed cysteine residues of other inner and outer membrane proteins may also occur. Interpeptide disulfide bonds involving EnvB, EnvA, and the MOMP have been shown to be rapidly reduced upon entry of EBs into host cells (20). Reduction of any or all of these proteins may account for the osmotic fragility of intracellular chlamydiae.

EnvB of *C. psittaci* 6BC was resistant, whereas the large CRPs *C. trachomatis* L2 were partially degraded when whole EBs were treated with trypsin, even when attempts were made to preserve the integrity of the *C. trachomatis* outer membrane by inclusion of Mg^{2+} during the treatment. Trypsin also partially degrades the large CRP in whole EBs of the GPIC agent (4). It is possible that a small proportion (for example, the N terminus of the larger doublet protein) of the large CRPs of the GPIC agent and *C. trachomatis* L2 are exposed on the surface, while none of the large CRP of *C. psittaci* 6BC is similarly exposed. Alternatively, it is possible that the outer membrane structures of *C. trachomatis* L2 and the GPIC agent are less sturdy than the structure of *C. psittaci* 6BC and are thus damaged during harvest and/or purification procedures, rendering their large CRPs susceptible to trypsin. Because it is not possible to distinguish between these alternatives at this time, the model presented in Fig. 6 applies only to *C. psittaci* 6BC, and modification of the model may be required as information on other strains becomes available. However, the large CRPs of other chlamydiae are between 71 and 85% homologous to EnvB (13). It is likely, therefore, that the large CRPs of all chlamydial strains are similar in structure, cellular location, and function.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI19570 from the National Institutes of Health.

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