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MINIREVIEW

Disulfide Cross-Linked Envelope Proteins: the Functional Equivalent of Peptidoglycan in Chlamydiae?

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INTRODUCTION

Chlamydiae are eubacteria that are currently separated into four species: *Chlamydia psittaci* and *Chlamydia pecorum*, which are important animal pathogens, and *Chlamydia trachomatis* and *Chlamydia pneumoniae*, which are significant agents of sexually transmitted, ocular, respiratory, and other infections in humans. Chlamydiae possess several intriguing features that are unique among eubacteria. One such feature is their unique developmental cycle, which takes place entirely within a cytoplasmic inclusion vacuole in a eucaryotic host cell (recently reviewed in references 7 and 33). The cycle is initiated when an elementary body (EB) is taken into a host cell by receptor-mediated endocytosis. Extracellular EBs are very small cocci (about 0.3 μm in diameter) that are metabolically inert and osmotically stable. Within minutes after an EB enters a host cell, its dormancy is broken and it commences a lengthy reorganization process which converts it into the reticulate body (RB) form. The RBs are about 1 μm in diameter, divide by binary fission, and are osmotically fragile. RBs divide logarithmically from about 6 h to about 20 h postinfection. The cycle becomes asynchronous by 20 h, with some RBs continuing to divide while others begin to reorganize into the infectious EB form. Generally, the host continues to support chlamydial growth until 30 to 72 h postinfection before it lyses and a mixture of several hundred RBs, EBs, and intermediate forms is released. At least one reason for the restriction of chlamydiae to an intracellular environment for growth is their apparent inability to generate ATP by respiratory or fermentative metabolism. To compensate for this deficiency, RBs scavenge host-supplied NTPs by translocation mechanisms.

Another unusual feature of chlamydiae is their cell envelope structure, a subject recently reviewed by Raulston (40). The envelope is gram negative in that it includes an inner membrane and a lipopolysaccharide-containing outer membrane. Unique features include 18 to 22 regularly-spaced dome-shaped surface projections, which are observed by scanning electron microscopy on only one hemisphere of both EBs and RBs (17, 29). Transmission electron microscopic studies suggest that fibrillar projections extend through these domes and possibly through the vacuolar membrane within which RBs divide (27, 28, 39). Neither the function nor the chemical composition of these structures is known. Perhaps the most striking feature of the chlamydial envelope is its apparent lack of peptidoglycan (PG), although chlamydiae possess penicillin-binding proteins (3) and are sensitive to drugs that inhibit PG synthesis, such as penicillin G and D-cycloserine. The conclusion that chlamydiae lack PG is based largely on the failure to detect muramic acid in chlamydiae (3, 16). Supporting observations include the failure to detect a PG layer by electron microscopy and the failure of antibodies directed against PG to react with chlamydiae (24). When infected cells are incubated with suitable concentrations of penicillin or D-cycloserine, cell division is inhibited, abnormal RB forms accumulate in the inclusion vacuole, and the development of infectious EBs does not occur. The abnormal forms are many times the size of normal RBs and contain internal membranous structures resembling miniature chlamydiae (30, 36). Generation of normal RBs and reorganization of RBs to EBs occurs upon the removal of penicillin or D-cycloserine. Morphologically similar abnormal forms can be induced in infected cells by treatment with interferon-γ and starvation of amino acids (6, 10).

TWO PARADOXES

The sensitivity of a bacterium lacking PG to penicillin and D-cycloserine is a paradox and the focus of a recent review by James W. Moulder (36). Moulder carefully built up the rationale behind several resolutions to the paradox: chlamydiae contain subdetectable levels of PG that nonetheless play a critical role in cell division and reorganization; chlamydiae possess a unique, penicillin-sensitive PG that has escaped detection because it contains a carboxylated sugar other than muramic acid; and one or more of the chlamydial penicillin-binding proteins has a unique function in chlamydial cell division and reorganization, unrelated to transpeptidation and transglycosylation. With equal care, Moulder then proceeded to argue that all of these explanations, while possible, were improbable. Thus, the anomaly of the sensitivity of chlamydiae to penicillin remains unresolved. A second paradox stemming from the lack of PG in chlamydiae, the osmotic stability of EBs, is the focus of this review.

EB ENVELOPE PROTEINS FORM A DISULFIDE CROSS-LINKED SUPRAMOLECULAR COMPLEX

Hatch et al. (23) were the first to report that the major outer membrane protein (MOMP) of *C. psittaci* EBs is insoluble in sodium dodecyl sulfate in the absence of mercaptoethanol and suggested that disulfide bonds might account for maintenance of the structural integrity of the organism. However, it was Newhall and Jones (38) who brought the concept of disulfide cross-linkage of proteins as a substitute for PG to the forefront of the minds of chlamydial researchers by elegantly demonstrating that the MOMP has interpeptide cross-links to itself in EB preparations of *C. trachomatis*. Shortly thereafter, Hatch et al. (21) reported that EBs of *C. psittaci* possess a small (M_r, 12,000) cysteine-rich protein (CRP) and a large (M_r, 60,000)

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CRP doublet that are absent in dividing RBs. Recently, Melgosa et al. (34) identified a 100-kDa disulfide cross-linked envelope protein in *C. pneumoniae*, and I have observed several high-molecular-weight cross-linked envelope proteins in *C. trachomatis* L2 and *C. psittaci* 6BC which have not been further characterized (20). Several investigators (22, 37, 42) have since found that the large and small CRPs are first made late in the developmental cycle, coincident with the slowing of RB cell division and reorganization of RBs back into EBs. Hatch et al. (21) demonstrated that the CRPs and the MOMP are so extensively disulfide cross-linked in EBs that they fail to enter into the stacking gel during sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions, whereas unreduced MOMP in logarithmically dividing RBs runs in the gels as a monomer. The actual arrangement of interpeptide cross-links among chlamydial envelope proteins remains unknown to this day. It is clear, however, that many chlamydial envelope proteins are disulfide cross-linked in some manner so as to form, in aggregate, a supramolecular structure in EBs that is not found in osmotically fragile RBs.

The genes encoding the CRPs of many chlamydial strains have been cloned and sequenced. Allen and Stephens (1) demonstrated that the large CRP doublet is generated by the posttranslational processing of a single primary gene product, and Lambden et al. (26) determined that the small and large CRPs are encoded by a bicistronic operon that is only expressed late in the developmental cycle. DNA sequencing also revealed that the CRPs are, indeed, cysteine-rich. For example, the mature form of the small CRP of *C. psittaci* 6BC (68 amino acids) contains 14 cysteine residues (20.5% of the total amino acid content), and the mature form of the larger pair of the CRP doublet (517 amino acids) contains 37 cysteine residues (7.2% of the total amino acid content) (13). Some investigators also refer to the MOMP as cysteine-rich; however, only 7 cysteines are found in the 380-amino-acid mature protein of *C. psittaci* 6BC (1.8% of the total amino acid content) (13).

Perhaps the best evidence that disulfide bonds play a role in maintaining the structural integrity of EBs is the observation of Hackstadt et al. (18) that EBs are lysed when incubated for 30 min in the presence of dithiotreitol. There is indirect evidence that the supermolecular cross-linked structure may also contribute to the metabolic dormancy of EBs. Bavoil et al. (5) found that the octylglucoside-insoluble fraction of *C. trachomatis*, consisting largely of the MOMP, has porin activity, but only if disulfide bonds are reduced and the resulting sulfhydryl groups are alkylated before the assay is performed. However, simple reduction of the MOMP and other envelope proteins with dithiothreitol does not seem to be sufficient for activation of NTP translocation in EBs, an activity normally associated with RBs (22).

The mechanism by which chlamydial envelope proteins become cross-linked has not been delineated. Hatch et al. (22) found that the CRPs and the MOMP remain largely in the reduced state as long as *C. psittaci* remains intracellular but are spontaneously cross-linked when the host cell is lysed with a nonionic detergent. In contrast, Newhall (37) reported the formation of cross-links in *C. trachomatis* late in the cycle before host-cell lysis and suggested an enzymatic mechanism for disulfide bond formation. Membrane-associated and periplasmic protein disulfide isomerases, similar to those recently found in gram-negative bacteria (4), may mediate the cross-linking process. The large CRP itself may serve this function, since a potential sulfhydryl-oxidoreductase active site is located between amino acids 424 and 427 (following the numbering scheme of Everett and Hatch [13]) in *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*. The mechanism of reduction of disulfide bonds among envelope proteins early in the developmental cycle, including the identity of the reductant, also is not understood. Reduction has been shown, however, to occur very rapidly, perhaps simultaneously with uptake of the organisms, and requires de novo chlamydial protein synthesis (22).

ARCHITECTURE OF THE CHLAMYDIAL ENVELOPE

For several reasons it is difficult to experimentally determine the location of chlamydial proteins within the envelope structure. First and foremost, only small quantities of chlamydiae are easily obtained for cell fractionation experiments. For example, about only 50 to 200 mg of chlamydial protein can be recovered from 10 liters of infected tissue culture cells. A second complication is that chlamydiae possess a developmental cycle that includes osmotically fragile RBs and a continuum of RB-EB transition forms of unknown stability. Although EBs are considerably more dense than RBs, no preparation of EBs purified by density gradient centrifugation is likely to be completely free of RBs and intermediate forms. Even highly enriched preparations of EBs may contain organisms with damaged outer membranes. This is particularly true of *C. trachomatis* EBs that are not exposed, or are only briefly exposed, to a non-host environment during their natural infection cycle. Therefore, methods designed to detect surface exposure of a protein must be interpreted with caution if weak signals or partial effects are observed. Third, the traditional method of separating inner and outer membranes on the basis of differences in density has not, for reasons that are unclear, been accomplished with chlamydiae (14).

Despite the problems described above, the MOMP has been shown to be an integral outer membrane protein (OMP) by multiple criteria: possession of a conventional signal peptide (44), the predicted membrane-spanning properties expected of an OMP (2), and unambiguous labeling when intact EBs are exposed to various surface-acting reagents, including lactoperoxidase-generated iodine radicals (9, 23) and fluorescent and gold-tagged antibodies (11, 25).

Determining the cellular location of the CRPs has proved more difficult. On the basis of immunogold labeling studies, neither the large doublet nor the small CRP appear to be located on the surface of EBs (11, 48). They are, nonetheless, generally referred to as OMPs on the basis of their insolubility in the weak anionic detergent sodium lauryl sarcosinate (Sarkosyl)—a reagent that has been used to distinguish the integral OMPs of *Escherichia coli* from other membrane proteins (15). However, Everett and Hatch (14) have recently shown that the insolubility of the CRPs of *C. psittaci* 6BC in Sarkosyl is not an inherent property of these proteins but rather is a function of their engagement in the supramolecular envelope complex. That is, the CRPs are rendered soluble in Sarkosyl under reducing conditions, whereas the MOMP, a true integral OMP, remains in the particulate fraction. The small CRP does appear to be associated with the outer membrane, however. Structural analysis suggests that the small CRP is a typical bacterial murein lipoprotein, with an N-terminal cysteine modified by amide linkage with a fatty acid residue and by thioether linkage with glycerol-fatty acid (12). Although the peptide portion of the small CRP is highly hydrophilic, the mature lipoprotein is soluble in Triton X-114 under reducing conditions and is labeled with the lipophilic reagent 3′-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) (14). From these observations, and by analogy to the Braun lipoprotein of *E. coli*, the cysteine-rich lipoprotein is most probably anchored to the inner leaflet of the outer membrane by its lipid moiety, with the peptide portion extending into the periplasm.

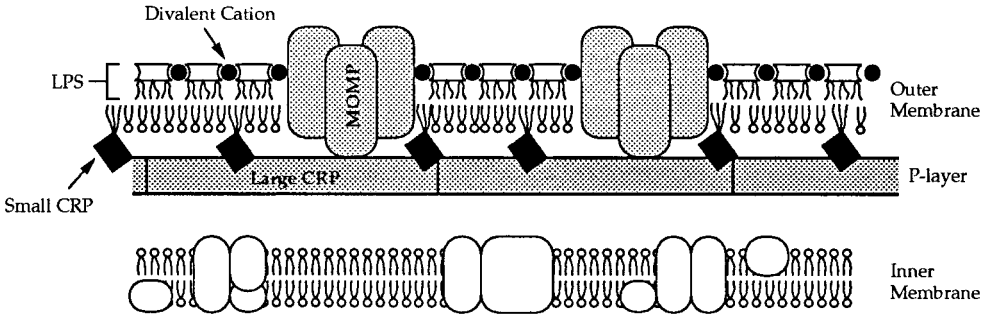

FIG. 1. Model of the envelope of chlamydial EBs. The model was adapted from the model presented by Everett and Hatch (14). Only the MOMP, the large CRP, and the small CRP are shown in the outer membrane and the periplasm; many other proteins are probably located in these areas. The actual shapes of the proteins and the existence of specific interpeptide cross-links have not been established. The P layer, which may consist entirely of cross-linked large CRP, has been observed by electron microscopy (29, 31, 35). Specific inner membrane proteins have not been identified.

The primary amino acid sequence of the large CRP doublet provides only limited clues as to its cellular location. N-terminal sequencing of the larger of the doublet proteins of *C. trachomatis* L2 indicates that posttranslational processing occurs at a predicted signal peptidase I site (1), suggesting a membrane or a periplasmic location. Several observations by Everett and Hatch (14) favor the periplasm as the location of the large CRP doublet of *C. psittaci* 6BC. First, the doublet appears to be hydrophilic, being extracted from intact EBs under reducing conditions in detergent-free aqueous buffers containing EDTA but not by Triton X-114. Second, no portion of the large CRP doublet of *C. psittaci* 6BC appears to be embedded in a lipid bilayer, since it is not labeled with [^{125}I]TID. Recent unpublished work in my laboratory confirms these results for *C. trachomatis* L2. It should be noted that [^{125}I]TID is a relatively nonspecific lipophilic reagent, labeling the side chains of all amino acids (and fatty acids) exposed to a hydrophobic environment. Third, the large CRP doublet proteins of intact *C. psittaci* 6BC EBs are susceptible to digestion with trypsin only after incubation in 10 mM Tris–1 mM EDTA (14), which presumably strips the chlamydial outer membrane of lipopolysaccharide or otherwise compromises the integrity of the outer membrane, thereby giving trypsin access to the periplasm in much the same way that Tris-EDTA treatment of other gram-negative bacteria allows lysozyme to degrade PG. The earlier observation by Everett and Hatch (14) that trypsin treatment of EBs of *C. trachomatis* L2 caused partial degradation of the large CRP doublet even in the absence of Tris-EDTA (treatment with Tris-EDTA greatly augmented the effect of trypsin) may be explained by the treatment of the *C. trachomatis* EBs with 0.05% Nonidet P-40 (5 min, on ice) to eliminate osmotically fragile RBs and intermediate forms. We have recently found that Nonidet P-40 alters the surface properties of *C. trachomatis* L2, rendering the otherwise inert large CRP susceptible to trypsin. On the other hand, the effects of trypsin on *C. trachomatis* L2 and *C. psittaci* observed in my laboratory differ from those observed by Patrick Bavoil's research group on the GPIC strain of *C. psittaci* (46, 47). These investigators found that the larger of the CRP doublet proteins was degraded to peptides of approximately the same size as the short doublet protein when EBs (not treated with detergent and not incubated in Tris-EDTA) were incubated with trypsin, suggesting that a small portion of the larger doublet protein is surface exposed. Further analysis may reconcile the differences in these two sets of studies.

A modified version of the model proposed by Everett and Hatch (14) of the architecture of the chlamydial EB envelope is shown in Fig. 1. The model includes only the MOMP and the large and small CRPs and does not reflect the relative sizes, shapes, and quantities of these proteins. A number of additional envelope proteins (34, 41, 43, 45) and a surface-associated glycosaminoglycan (49) have been recently characterized but have been excluded for simplicity. The MOMP is shown as a trimer because of its potential role as a porin (5, 32); however, its multimeric structure when cross-linked in EBs is not known.

The most conspicuous feature of this working model is the placement of the large CRP in the periplasm, based largely on the solubility properties of the protein and its failure to be labeled with [^{125}I]TID. The ability of trypsin to degrade partially the large CRP in EBs of *C. psittaci* GPIC and, at least under some conditions, the large CRP of *C. trachomatis* L2 and *C. psittaci* 6BC leaves open the possibility that a small portion of the protein is exposed on the surface of chlamydiae. Indeed, Ting and Bavoil and Ting et al. (46, 47) recently suggested that the N terminus of the larger of the two CRP doublet proteins in *C. psittaci* GPIC may be surface exposed and may play a role in the adhesion of this chlamydial strain to host cells. If some portion of the large CRP does extend through the outer membrane, its passage most likely is mediated through a hydrophilic protein channel since the protein is extracted with aqueous buffers and is not labeled with [^{125}I]TID. A recent electron microscopic study carried out by Jane Raulston of the University of North Carolina supports a periplasmic location for most of the large CRP. Figure 2 shows one of her electron micrographs in which a thin section of *C. psittaci* 6BC EBs has been treated with anti-large CRP polyclonal rabbit serum followed by reaction with immunogold-labeled anti-rabbit serum. Particles in this and other sections were most frequently located in the periplasm in close association with the inner leaflet of the outer membrane.

The presence of the large CRP in the periplasm raises the intriguing hypothesis that it, perhaps in conjunction with other cysteine-containing proteins, is the functional equivalent of PG in chlamydial EBs. Drawing the analogy between the large CRP and PG even closer, the CRP, in addition to being cross-linked to itself, may also be disulfide cross-linked to the small cysteine-rich lipoprotein—just as the Braun lipoprotein is cross-linked by peptide bonds to the PG in *E. coli* (19). Unfortunately, the cross-linkage of EB envelope proteins is so extensive and complex that it has defied analysis to date, and the existence of interpeptide disulfide bonds between specific proteins is speculative. There is, however, physical evidence that a periplasmic protein layer in close association with the outer membrane exists in chlamydiae. Matsumoto and Manire (31) and Miyashita et al. (35), who examined EB cell wall

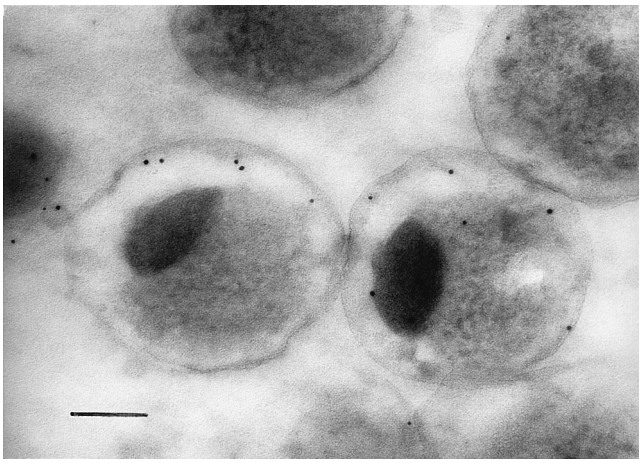

FIG. 2. Immunogold labeling of thin sections of *C. psittaci* 6BC EBs. Thin sections were treated with monospecific rabbit antisera raised against the large CRP followed by reaction with gold-tagged goat anti-rabbit antibodies. Electron-dense gold particles are most frequently noted on the inner surface of the outer membrane. The inner membrane is associated with the cytoplasm, which shrank in the fixation process. Electron-dense nucleoids, typical of EBs, can be seen in the cytoplasm. The electron micrographs were kindly supplied by Jane Raulston, University of North Carolina, Chapel Hill. The bar is equal to 100 nm.

preparations (sodium dodecyl sulfate-insoluble extracts) shadow cast with platinum palladium alloy, have observed a regular array of hexagonally packed subunits on the inner surface of the outer membrane. This array, which I will term the periplasmic or P layer, appears to be morphologically similar to the surface or the S layer found on some gram-negative bacteria (8). Consistent with the late-stage-specific large CRP being part of this P layer, the P layer was not found in RBs (31).

The proposed disulfide cross-linked P layer in EBs provides a satisfying solution to one of the paradoxes raised at the beginning of this review: EBs are osmotically stable but lack PG. By extension, the osmotic fragility of RBs can be explained by the lack of disulfide cross-linked envelope proteins. Haunting questions remain, however. Is there a direct relationship between the penicillin sensitivity of chlamydiae and formation of the disulfide cross-linked P layer? What is the evolutionary basis for the supramolecular cross-linked structure? Did it evolve in chlamydiae independent of similar structures in other bacteria? Which came first, the loss of PG, or the emergence of cross-linked envelope proteins? Regarding the evolution of the P layer, I offer this final speculation. Perhaps the large CRP once formed an external S layer which was useful in some primitive extracellular environment. As chlamydiae adapted to an intracellular environment, they may have lost the ability to translocate the protein through the outer membrane, retaining it (or most of it) in the periplasm. Then, perhaps roughly in parallel with the gradual loss of highly cross-linked PG, the large CRP and other envelope proteins may have evolved an ever-more-extensive network of disulfide cross-linked proteins which compensated for the weakening PG structure. While its evolution remains a mystery, the chlamydial cell envelope appears, at present, to be a unique adaptation of chlamydiae which allows them to divide intracellularly (when cross-links are reduced) and to survive extracellularly (when proteins are cross-linked).

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