Tandem genes of *Chlamydia psittaci* that encode proteins localized to the inclusion membrane

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Summary

Chlamydiae are obligate intracellular bacteria that replicate within a non-acidified vacuole, termed an inclusion. To identify chlamydial proteins that are unique to the intracellular phase of the life cycle, a lambda expression library of *Chlamydia psittaci* DNA was differentially screened with convalescent antisera from infected guinea pigs and antisera directed at formalin-fixed purified chlamydial elementary bodies (EBs). One library clone was identified that harboured two open reading frames (ORFs) with coding potential for similar-sized proteins of \( \approx 20 \) kDa. These proteins were subsequently termed IncB and IncC. Sequencing of the cloned insert revealed a strong *Escherichia coli*-like promoter sequence immediately upstream of incB and a 36 nt intergenic region between the ORFs. Sequence analysis of the region upstream of incB and incC revealed two ORFs that had strong homologies to an amino acid transporter and a sodium-dependent transporter. Immunoblotting with antisera directed at IncB or IncC demonstrated that these proteins are present in *C. psittaci*-infected HeLa cells but are absent or below the level of detection in purified EBs. Reverse transcriptase-polymerase chain reactions provided evidence that incB and incC are transcribed in an operon. Immunofluorescence microscopy demonstrated that IncB and IncC are each localized to the inclusion membrane of infected cells. No primary sequence similarity is evident between IncA, IncB or IncC, but each contains a large hydrophobic domain of similar size and character as in IncA. Analysis of the recently completed *C. trachomatis* serovar D genome database has revealed *C. trachomatis* ORFs encoding homologues to incB and incC, indicating that these genes are conserved among the chlamydiae.

Introduction

Chlamydiae are obligate intracellular pathogens causing serious health problems in both humans and animals. *Chlamydia trachomatis* is responsible for an estimated 500 million cases of trachoma worldwide (Dawson et al., 1981) and is the most common cause of sexually transmitted disease in the United States. The World Health Organization estimated 89 million new cases of genital chlamydial infections worldwide in 1995 (WHO, 1995). *C. pneumoniae* is a respiratory pathogen that has recently been implicated in atherosclerosis (Kuo et al., 1995). *C. psittaci* is primarily a pathogen of veterinary concern that causes incidental diseases of humans (Moulder, 1991).

All intracellular bacterial pathogens enter the host cell surrounded by a membrane-bound vacuole (Garcia-del-Portillo and Finlay, 1995). Survival strategies of these pathogens include escape from the vacuole (Tilney and Portnoy, 1989; High et al., 1992; Theriot, 1995), tolerance of the harsh lysosomal environment (Hackstadt and Williams, 1981; Antoine et al., 1990) and avoidance of lysosomal fusion (Horwitz, 1983; Clemens and Horwitz, 1995; Swanson and Isberg, 1996; Scidmore et al., 1996a). *Chlamydia* species, *Legionella pneumophila* and the protozoan parasite *Toxoplasma gondii* are among the intracellular pathogens contained in vacuoles that have restricted fusion with host endocytic markers. Understanding the biogenesis of these vacuoles and their interactions with the host cell are important areas for investigation because they are central to successful intracellular parasitism.

All chlamydiae display a unique biphasic life cycle with dimorphic forms that are functionally and morphologically distinct. Infection of host cells is mediated through a non-metabolic form, the elementary body (EB), whereas intracellular multiplication progresses through the reticulate body (RB), a metabolically active, non-infectious form (Moulder, 1991). This developmental cycle takes place entirely within an intracellular vacuole (the inclusion), which is a barrier that separates the chlamydia from the nutrient-rich cytoplasm and does not allow passive diffusion of nutrients and biosynthetic precursors as small as 520 Da (Heinzen and Hackstadt, 1997). Neither fluid-phase markers nor markers of early or late endosomes and lysosomes are associated with the chlamydial inclusion (Heinzen et al., 1996; Scidmore et al., 1996b; Taraska et al., 1996). These observations suggest that the inclusion interacts only minimally with the endosomal–lysosomal pathway.
Instead, the inclusion appears to interact with an exocytic pathway, as demonstrated by the specific vesicular delivery of endogenously synthesized NBD sphingomyelin (Hackstadt et al., 1995; 1996). Modification of the vesicle to intersect an exocytic pathway requires chlamydial protein synthesis, suggesting that the chlamydiae synthesize proteins that determine the vesicular interactions of the inclusion (Scidmore et al., 1996a).

To date, the only known prokaryotic protein that has been localized to a parasitophorous vacuolar membrane is IncA from C. psittaci strain guinea-pig inclusion conjunctivitis (GPIC; Rockey et al., 1995). There are at least three additional proteins that are only recognized in the context of infection (Rockey and Rosquist, 1994). In an effort to identify these proteins, we report here two additional C. psittaci-secreted proteins that are localized to the inclusion membrane. In addition, examination of sequence data from the C. trachomatis serovar D genome project has identified IncB and IncC homologues in this species.

Results

Cloning of two infection-specific proteins in C. psittaci GPIC

A lambda ZAPII library of C. psittaci (strain GPIC) genomic DNA was differentially immunoscreened with guinea-pig convalescent antisera, and antisera directed at formalinfixed C. psittaci GPIC EBs (α-EB). Eighteen clones reacted with convalescent sera but not with α-EB sera. One of these positive clones was subcloned into pBluescript SK(+) phagemid and designated pBS200-7. E. coli lysates harbouring this construct were analysed using SDS–PAGE and immunoblot analysis (Fig. 1). A 20 kDa antigen was recognized by convalescent antiserum but was not detected with α-EB sera. These results suggest that this protein(s) may be recognized only in the context of infection.

Sequence analysis of GPIC genomic clones

Sequence analysis of the 1.3 kb insert in pBS200-7 revealed two ORFs that coded for proteins each predicted to be ~20 kDa in size. These proteins were designated IncB and IncC respectively (Fig. 2).

Both IncB and IncC display remarkably similar hydrophyty plots with respect to the size and location of a hydrophobic domain that spans amino acids 100–170 within the central region of each protein (Fig. 3). The shape of the hydrophobic domains in the hydropathy plots is also very similar to a large hydrophobic domain in IncA (Rockey et al., 1995). Surprisingly, sequence analysis of IncA, B and C identifies no amino acid sequence similarity to one another. There are also no significant homologies with any other proteins in the sequence databases.

To examine ORFs surrounding incB and incC on the GPIC chromosome, a second genomic clone, pJC2, was isolated and cloned from Eco RI-digested genomic DNA using the incC sequence as a probe. Within this 5.0 kb insert of pJC2, four complete ORFs and one partial ORF were identified (Fig. 2). All five ORFs are oriented in the
same direction. The pJC2 insert contains both the IncB and IncC ORFs as well as two upstream ORFs that encode proteins of 40 and 44 kDa. An incomplete ORF downstream of incC was also identified. This partial ORF displayed no homologies in the database, but it is extremely hydrophobic as determined by hydropathy analysis (data not shown).

The predicted amino acid sequence of the 44 and 40 kDa putative proteins displayed significant similarity with a group of amino acid transporters and Na\(^+\)-dependent transporters, respectively. The predicted 44 kDa amino acid transporter protein displayed a 29% identity over a 175-amino-acid region of overlap with a glutamate transporter protein. The predicted 40 kDa Na\(^+\)-dependent

**Fig. 3.** Comparison of hydropathy profiles of the predicted amino acid sequences of IncA, IncB and IncC. Profiles were determined using the algorithm developed by Kyte and Doolittle (1982) with a window size of seven amino acids. The vertical axis displays relative hydrophilicity with negative scores indicating relative hydrophobicity. Hydrophobic regions of similar size and shape were identified and highlighted in the profiles.
transporter protein displayed a 34% identity over 116 amino acids with the hypothetical sodium-dependent transporter from *Haemophilus influenzae*. Hydropathy analysis of the predicted amino acid transporter revealed a pattern of hydrophobicity similar to those of known glutamate transporters with a characteristic long hydrophobic stretch situated in the C-terminal half of the protein (data not shown). This 44 kDa putative protein also has two predicted N-linked glycosylation sites in hydrophilic domains, which match that seen in number and location for other amino acid transporters (Utsunomiya-Tate et al., 1996). These putative transporter ORFs have a 9 nt intergenic region.

**Production of fusion proteins and immunoblot analysis**

Maltose-binding protein (MBP) fusions were constructed for all of the ORFs identified in the pJC2 insert sequence. Only the N-terminal 101 amino acids of IncB, which excludes the long hydrophobic stretch (Fig. 3), were fused to MBP because full-length expression could not be achieved. Immunoblotting of these purified antigens was used to determine which of IncB or IncC was recognized by the convalescent antisera. MBP/IncB was strongly recognized by convalescent antisera, whereas the MBP/IncC signal was faint (Fig. 4B). A parallel filter probed with anti-MBP showed that antigen concentration was similar in each sample (Fig. 4A). MBP fusions with the ORF5 product and ORFs encoding the two putative transporters were not recognized by these antisera (data not shown).

Antisera were produced against purified MBP/IncB and MBP/IncC fusion proteins in guinea-pigs. Immunoblot analysis with α-IncB and α-IncC shows that these proteins are each recognized in GPIC-infected cells, but not in uninfected HeLa cells (Fig. 5) or L2-infected HeLa cells (data not shown). Because it is recognized by convalescent antisera and is similar in size, IncB may be the 22 kDa protein described by Rockey and Rosquist (1994). IncC is detected in infected cells, but the gene is apparently not expressed or the protein is degraded in the *E. coli* (pBS200-7) clone. Neither IncB nor IncC was detected in EBs (Fig. 5).

**Fig. 4.** Immunoblot analysis of purified MBP fusion proteins probed with anti-MBP (A) and convalescent antisera (B). Only the MBP/IncB fusion is recognized by convalescent antisera. The concentration of each fusion protein is 1 μg per lane. Lane assignments: lane 1, MBP; lane 2, MBP/IncB; lane 3, MBP/IncC. Molecular weight standards are indicated in kilodaltons in the left hand margin.

**Fig. 5.** Immunoblot analysis of chlamydia-infected cells with α-IncB (A) and α-IncC (B) antisera. The lanes in A are: 1, purified GPIC EBs; 2, Uninfected HeLa cells; 3, GPIC-infected HeLa cells; 4, *E. coli* SOLR (pBS200-7). The lanes for B are: 1, purified GPIC EBs; 2, uninfected HeLa cells; 3, GPIC-infected HeLa cells; 4, *E. coli* SOLR (pBS200-7); 5, purified IncC antigen. The purified IncC antigen was obtained by proteolytic cleavage of MBP/IncC with Factor Xa (New England Biolabs) followed by electrophoresis of the 20kDa band from an SDS–PAGE gel. Molecular weight standards are indicated in kilodaltons in the left hand margin.
Subcellular localization of IncB and IncC

The intracellular distribution of IncB and IncC was investigated using immunofluorescence microscopy of C. psittaci GPIC-infected HeLa cells fixed and permeabilized 30 h post infection (PI). The 30 h time point was chosen because the inclusions are large and fully developed. Neither antigen was detected on the intracellular chlamydiae (Fig. 6), but each was detected in the inclusion membrane (Figs 7 and 8). Although both antigens were detected in the membrane, the fluorescent pattern differed, indicating that the distribution of the antigens in the inclusion membrane may be distinct. IncC resembled the circumferential staining pattern of IncA (Fig. 7; Rockey et al., 1995; 1996), whereas IncB displayed a discrete, punctate staining pattern (Fig. 8). This may suggest a concentration of the antigen in specific domains or structures in the inclusion membrane. In addition, both α-IncB and α-IncC recognized an antigen present in fibres extending across the nucleus and away from the inclusion into the cytoplasm of infected cells (data not shown) in a similar fashion to that described for IncA (Rockey et al., 1995). Inclusion membrane staining with IncC antibody was best observed using a periodate–lysine–parafomaldehyde (PLP) fixative specifically designed for staining membrane structures (Brown and Farquhar, 1989). IncB displayed a punctate staining pattern irrespective of the fixative used.

incB and incC are co-transcribed in an operon

Northern hybridization analysis was performed to identify the size of the incB and/or incC transcript and determine if this transcript is present in EBs as well as infected cells harvested 18 h PI. A 1.3 kb transcript was detected in RNA harvested from infected cells, but not from uninfected cells or purified EB (data not shown). This transcript was large enough to contain only incB and incC. An identical 1.3 kb transcript was detected when incB-, incC- and incBC-specific probes were used. This experiment supported the conclusion that incB and incC were transcribed as an operon. To confirm the Northern hybridization data, the intergenic region of the inc locus was analysed using qualitative RT-PCR (Fig. 9). Primers were selected that span the intergenic regions of nadT–incB and incB–incC (see Fig. 2). Amplification of C. psittaci genomic DNA using Pwo polymerase and these primer sets resulted in products of 403 bp and 447 bp respectively. A positive control primer set located within the incC gene yielded an amplification product of 196 bp. An RT-PCR product was observed for incB–incC, but not nadT–incB. Collectively, these data indicate that only incB and incC are transcribed in an operon.

Identification of IncB and IncC in C. trachomatis

Southern hybridization analysis using a variety of stringencies failed to show the presence of incB or incC in C. trachomatis (data not shown). However, a search of the C. trachomatis serovar D genome sequence available on the internet has revealed IncB and IncC homologues in this species. The gene arrangement shown in Fig. 2 was identical in C. trachomatis. A BLAST search of these translated sequences identified C. psittaci IncB and IncC from the database with 56% and 49% identities respectively. The hydropathy plots of these C. trachomatis homologues show a similar hydrophobic domain when compared with the C. psittaci-derived proteins (data not shown).

To determine if incBC is linked to incA on the GPIC chromosome, Southern hybridization analysis was performed on GPIC DNA double digested with several different restriction enzymes (data not shown). The sequential hybridizations of incA and then incC to the same filter containing...
genomic DNA revealed that incA and incBC are not present on the same restriction fragments. The hybridization data along with the sequence data show that incBC and incA are separated by a minimum of 8 kb on the GPIC chromosome. This separation is consistent with data in the serovar D genome sequence as well.

Discussion

We have previously shown that sera from convalescent guinea-pigs recognize unique proteins that are present in the parasitized cell but not in purified EBs, and that these sera contain antibodies specific for the inclusion membrane.

Fig. 7. Immunofluorescence microscopy of C. psittaci GPIC-infected HeLa cells at 30 h PI. The α-IncA-stained cells were fixed with methanol (A); the α-IncC-stained cells were fixed with periodate–lysine–paraformaldehyde (PLP) fixative (B) and α-IncB-stained cells fixed in methanol (C). The immunofluorescent images show staining of the inclusion membrane, but no chlamydial staining with any antisera. Bar is 10 μm.

Fig. 8. Dual immunofluorescence confocal microscopy of C. psittaci GPIC-infected HeLa cells with α-IncA and α-IncB. A is a GPIC inclusion stained with α-IncB; B is the same inclusion stained with α-IncA and C is the merged images. IncB is localized in distinct regions within the inclusion membrane. Bar is 10 μm.
of GPIC-infected HeLa cells. Rockey and Rosquist (1994) initially identified three antigens of 22, 34 and 52 kDa from C. psittaci that were only expressed in the context of infection. Using this approach, an inclusion membrane protein of chlamydial origin, termed IncA, was identified (Rockey et al., 1995). In this report, we have used similar convalescent sera to identify two additional chlamydial proteins encoded by tandem ORFs, one of which (IncB) is recognized preferentially in the context of infection. Because of its similar size and recognition by convalescent antisera, it is possible that IncB may correspond to the 22 kDa infection-specific protein described previously (Rockey and Rosquist, 1994). As was the case with IncA (Rockey et al., 1995), IncC does not appear to be one of the three proteins originally observed by Rockey and Rosquist (1994) because it is not recognized by convalescent antisera. Collectively, these results indicate that multiple proteins are present within the chlamydial inclusion membrane, some of which are antigenic and some of which are not. Alternative methodologies will probably be required to identify other proteins in the inclusion membrane.

The genes encoding IncA and IncBC are not closely linked in the chlamydial chromosome. Each possesses a hydrophobic region of similar size and character, but they show no amino acid sequence identity to each other or any protein in the database. IncA and IncC are localized to the inclusion membrane and appear to be distributed circumferentially around each of the lobes (Rockey et al., 1996). In contrast, fluorescence microscopy reveals a punctate distribution of IncB at the inclusion membrane. It is not yet clear if this distinction is accurate or a result of the fixation process. In addition, all three are localized to fibres of unknown origin that extend away from the inclusion into the infected cell. The function of these gene products remains elusive, although IncA is phosphorylated by the host cell (Rockey et al., 1997). Thus, IncA may mediate some type of communication between the inclusion and the host cell.

One perplexing issue surrounding the characterization of IncA, IncB and IncC has been our inability to identify homologues to these genes in other chlamydial species. This has led to concern that these proteins may lack relevance to chlamydial biology in general and to the human pathogens in particular. The recent availability of the completed C. trachomatis genome has facilitated the identification of C. trachomatis IncB and IncC, located at contig 3.4 in the genome sequence (http://clamydia-www.berkeley.edu:4231/). IncB in C. trachomatis appears to contain fewer amino acids relative to the corresponding protein encoded by C. psittaci, but IncC is very similar in size and structure between the two species. These sequences encode proteins with moderate identity to the C. psittaci homologues and each contains similar hydrophobic domains as IncA, IncB and IncC from C. psittaci. This result leads to exciting opportunities for analysis of the biology of the chlamydial inclusion, and supports the hypothesis that the Inc proteins may be involved in the common features surrounding chlamydial inclusion development. The genome project has also allowed us to examine regions around incB and incC to determine if these genes are linked to a C. trachomatis IncA homologue. The C. trachomatis IncA homologue is not closely linked to these genes, a result consistent with our inability to show linkage of IncA with incB/C in C. psittaci.

Recently, studies have identified homologues of a type III secretion apparatus in C. psittaci (Hsia et al., 1997) and C. trachomatis. This protein export system has been identified in a variety of bacterial pathogens (Lee, 1997) and depends on contact with the host cell to secrete bacterial-derived proteins (Barinaga, 1996). Although the Inc proteins all appear to be secreted, none of them possesses a classical type II signal sequence. Thus, there is a possibility that IncA, IncB and IncC are exported via a type III system.

Sequence analysis revealed two putative transporter genes upstream of IncB on the C. psittaci chromosome, and this arrangement is conserved in C. trachomatis. Essentially nothing is known of the ionic strength or contents of the interior of the chlamydial inclusion; the occurrence, and presumed function, of Na\(^{+}\)-dependent amino acid transporters implies the existence of a sufficiently large Na\(^{+}\) gradient to drive transport processes. This result is intriguing, but until these transporters are actually localized to either the chlamydia or the inclusion membrane one can only speculate upon possible functions or Na\(^{+}\) concentrations within the lumen of the inclusion.

The chlamydial inclusion provides the only environment known to support chlamydial replication. The origins and functions of this unusual vesicle are only now beginning to be understood (Hackstadt et al., 1997). All interactions...
with the host cell are probably mediated via activities localized at the inclusion membrane. Functions that must be provided include nutrient availability, evasion of lysosomal fusion and physical conditions to support replication as well as signal appropriate stages of chlamydial development. Characterization of functional proteins within the inclusion membrane can be expected to offer significant insights into mechanisms of chlamydial pathogenesis.

**Experimental procedures**

**C. psittaci library construction and immunoscreening**

*C. psittaci* strain GPIC DNA was extracted using a genomic DNA extraction kit (Qiagen) with one modification; dithiothreitol (5 mM) was added to the suspension buffer to assist EB lysis. DNA was partially digested with Tsp509I and ligated to EcoRI digested λ-ZAPII phage arms (Stratagene). The ligation was packaged *in vitro* with Gigapack extracts according to the manufacturer’s instructions (Stratagene). Recombinant phage were plated on *E. coli* XL-1 Blue at densities of ~10^6 PFU/150 mm (diameter) plate. After a 9 h incubation to allow development of the plaques, the plates were sequentially overlaid with nitrocellulose disks and the resulting lifts were processed for immobilblotting with convalescent antisera and antisera to fixed EBs. Of ~8000 plaques, 18 had reactivity with the convalescent sera but not sera generated against EBs. One of these was subcloned into pBluescript SK(--) phagemid by in vivo excision in the *E. coli* SOLR strain (Stratagene) and designated pBS200-7.

**DNA cloning and fusion protein production**

The plasmid pJC2 contains a 5.0 kb *EcoRI* GPIC genomic fragment cloned into the pZEro2.1 vector (Invitrogen). To construct pJC2, the *incC* ORF sequence was 32P-radiolaabeled using random priming (Gibco-BRL) and used to probe *EcoRI*-cut GPIC genomic DNA fragments separated by agarose gel electrophoresis. Fragments in the size range of the positive signal were excised from the gel and purified by Gene Clean (Bio101). The gel-purified fragments were used in a ligation along with *EcoRI*-digested pZEro2.1. Kanamycin-resistant colonies were screened by colony hybridization with radiolabelled *incC*.

MBP fusions of the five ORFs present in pJC2 were produced using the pMAL-C2 vector (New England Biolabs). The reading frame of *incC*, with the exception of the first four codons, was amplified using Pwo polymerase (Boehringer Mannheim) and pBS200-7 as the template. The upstream and downstream oligonucleotides for this amplification were 5'-AGAACCGATTTAACTCCAGGCG-3' and 5'-GCGCGGATCCGTTATTAGGTGCGCTTCTGAGTTAGG-3'. The purified MBP/IncB and MBP/IncC fusion proteins were used as antigen for the production of monospecific antibody in Hartley strain guinea-pigs according to the method of Rockey et al. (1995). The MBP/IncB fusion was also used to generate antibody in New Zealand white rabbits. Inserts in each construct were confirmed by DNA sequencing.

**DNA sequencing and sequence analysis**

The pBS200-7 and pJC2 genomic clones as well as the MBP fusions were sequenced with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems Division). Several internal primers were designed to sequence further into the cloned inserts. Sequence assembly was performed using MACVECTOR software and sequence analysis was performed with MACVECTOR software (International Biotechnologies). Hydrophilicity profiles were determined using the Kyte–Doolittle scale (Kyte and Doolittle, 1982) with a window of 7. Deduced amino acid sequences were compared with the database using the BLAST program available from the National Center for Biotechnology Information on the worldwide web. The entire nucleotide sequence of the pJC2 insert was deposited in the GenBank/EMBL Nucleotide Sequence Data Library, under accession no. AF017105.

**Electrophoresis and immunoblotting**

Polyacrylamide gel electrophoresis was conducted as described previously (Rockey and Rosquist, 1994). Immunoblotting was performed as described previously (Rockey et al., 1995).

**Immunofluorescence studies**

Chlamydiae grown in HeLa cells on sterile glass coverslips were fixed for microscopy one of two ways. Cells were either incubated in methanol for 5 min, or in the combination fixative PLP for 3 h at room temperature followed by permeabilization with 0.05% saponin (Brown and Farquhar, 1989). Immunostaining of the fixed coverslips was performed according to the method of Rockey et al. (1995) and visualized under a Nikon Microphot FXA microscope using the 63× objective and oil immersion.

**RT-PCR analysis**

RNA for RT-PCR analysis was extracted from ~2×10^14 *C. psittaci*-infected cells. A Qiagen column was used for extraction and purification according to the manufacturer’s instructions (Qiagen). RQ1 RNase-free DNase (Promega) was used to ensure removal of contaminating genomic DNA. cDNA was prepared by incubating 1.5 μg of RNA, 2.5 μM of the

reverse oligonucleotide primer, and AMV reverse transcriptase (Promega) for 1 h at 42°C in sodium pyrophosphate buffer, according to the manufacturer’s instructions. PCR reactions were carried out using 1 μl of the cDNA reaction, 1.25 μM of each oligonucleotide primer, and Pwo polymerase (Boehringer Mannheim). Each RT-PCR reaction was accompanied by a positive control reaction that used the same primer set and 10 ng of C. psittaci genomic DNA, and a negative control reaction in which 1 μl of the same RNA preparation was used as template in the PCR reaction. A control primer set, located within the incC gene, was also used as an RT-PCR control.

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