Type III secretion genes identify a putative virulence locus of Chlamydia

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Summary

Four genes of Chlamydia psittaci strain guinea pig inclusion conjunctivitis (GPIC), whose predicted products are highly homologous to structural and regulatory components of a contact-dependent or type III secretion apparatus, were isolated. Related to genes present in several animal and plant bacterial pathogens, these genes may represent a section of a previously undetected chromosomal virulence locus analogous to several recently described virulence-associated type III secretion loci. The existence of contact-dependent secretion in Chlamydia strongly suggests that these bacteria use pathogenic mechanisms that are similar to those of other intracellular bacterial pathogens. Unlike other intracellular bacteria, however, chlamydiae are metabolically inactive extracellularly and only become capable of global protein synthesis several hours after infection. This implies that chlamydial contact-dependent secretion is only active from within, uniquely after the bacteria have been internalized by eukaryotic cells. The possible role(s) of this pathway in chlamydial pathogenesis are discussed.

Introduction

Chlamydial sexually transmitted, respiratory and ocular diseases are major health problems worldwide (Schachter, 1988; Grayston and Thom, 1991). Central to the pathogenesis of all chlamydiae are the ability to enter into efficiently, and subsequently survive and multiply within, mucosal epithelial cells. The actual molecular mechanisms underlying these processes, however, have remained elusive, in large part because genes dedicated to chlamydial virulence have not been isolated to date.

Previous studies that have sought to elucidate the molecular basis of the interaction between the infectious elementary body and the susceptible epithelial cell have suggested that abundant surface-associated proteins and/or heparan sulphate-like glycosaminoglycans promote the efficient internalization of the chlamydial elementary body by the eukaryotic cell (Su et al., 1990; Zhang and Stephens, 1992; Schmied et al., 1991; Ting et al., 1995; Kuo et al., 1996). Within 15 min after infection of cultured HeLa cells by Chlamydia trachomatis serovar L2, several host proteins are found to be tyrosine phosphorylated (Birkeland et al., 1994). During the course of infection, Chlamydia-containing vacuoles redistribute from the cellular to the nuclear periphery in a F-actin-dependent mechanism (Majeed and Kihlstrom, 1991) which is regulated by intracellular Ca2+. Later, elementary bodies differentiate to reticulate bodies, which multiply, and eventually (40 h after infection) the intracellular chlamydial vacuole(s) expand(s) to occupy nearly all intracellular space not already claimed by the nucleus or cellular organelles. From a few hours after infection onwards, the parasitophorous vacuole membrane is thought to expand by the acquisition of host membrane components (Hackstadt et al., 1996; van Ooij et al., 1997) and proteins synthesized by Chlamydia (Rockey et al., 1995; Taraska et al., 1996; Bannantine et al., 1997). Among these, Rockey et al. (1997) have recently shown that a protein made in reticulate bodies is translocated to the outer surface of the Chlamydia vacuole where it may be phosphorylated by a host enzyme. These observations suggest that chlamydiae, like other intracellular pathogens, subvert host intracellular trafficking pathways in order to fulfill their own pathogenic needs.

We report here the characterization of four chlamydial genes that putatively express structural and regulatory components of a contact-dependent secretion apparatus. As this pathway often lies at the heart of the interaction between eukaryotic cells and infecting bacteria, further investigation of contact-dependent secretion in Chlamydia promises to contribute important information concerning the molecular mechanisms governing the obligate intracellular pathogenesis of chlamydiae.

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Results and discussion

Four genes of Chlamydia encode homologues of contact-dependent (type III) secretion genes

We have isolated a fragment of the Chlamydia psittaci strain guinea pig inclusion conjunctivitis (GPIC) genome, which includes seven major contiguous, non-overlapping open reading frames, the first four of which (ORFs A–D) represent putative genes of a previously undetected contact-dependent or type III secretion apparatus (Salmond and Reeves, 1993; Mecsas and Strauss, 1996; Lee, 1997) (Fig. 1). ORFs E and F, located immediately downstream, encode, respectively, a 58 537-Da homologue (28% identity, 50% similarity) of the product of malQ (malM) of Streptococcus pneumoniae (Lacks et al., 1982) and a 66 207-Da homologue of a hypothetical 55.3-kDa polypeptide mapping in the rfaH–rfe intergenic region of E. coli (Daniels et al., 1992). In E. coli, malQ encodes a cytoplasmic 4-α-glucanotransferase (amylomaltase) that catalyses maltodextrin polymer formation from maltose with concomitant release of free glucose (Wiesmeyer and Cohn, 1960). ORFG encodes a hypothetical 39 675-Da polypeptide with no significant homology to any entry in the databases. Hence, ORFs E to G are most probably not functionally related to the contact-dependent secretion locus and are not dealt with further in this report.

Contact-dependent secreted substrates in bacterial intracellular pathogens, such as Yersinia, Salmonella and Shigella, are secreted proteins uniquely dedicated to the establishment and sustainment of the parasitic relationship between the pathogen and the host eukaryotic cell, and are usually genetically linked to secretory genes. Thus, the four putative contact-dependent secretion genes described here may identify the first chromosomal locus from Chlamydia, which is dedicated to virulence. We have provisionally designated ORFA and ORFB, respectively, cds1 and cds2 (for contact-dependent secretion), to refer to the more highly conserved membrane-associated components of the secretion apparatus. For gene products that are less conserved because they encode proteins with species specificity, we have adopted the system currently used for Yersinia. Hence, ORFC is named copN (for Chlamydia outer protein homologue of YopN) and ORFD is named scc1 (for specific Chlamydia chaperone) (Fig. 1).

Full-length cds1 and cds2 encode predicted polypeptides of 40 473 and 78 027 Da respectively. Homology searches have revealed that the Cds1 sequence is highly similar to that encoded by yscU of yersiniae (Allaoui et al., 1994; Bergman et al., 1994) (32% identity, 58% similarity), spaS (spa40) of Shigella flexneri (Sasakawa et al., 1993) (31% identity, 58% similarity), spaS (Groisman and Ochman, 1993) and ssaU (Hensel et al., 1997) of Salmonella typhimurium (30% and 32% identity, 54% and 55% similarity respectively). Significant Cds1 homologies were also detected with the products of flhB of Bacillus subtilis (Carpenter et al., 1993) and S. typhimurium (Minamino et al., 1994), and hrpN of Ralstonia solanacearum (Gough et al., 1993).

The Cds2 amino acid sequence is most homologous to that encoded by lcrD of yersiniae (Plano et al., 1991) (47% identity, 72% similarity), with high homologies also detected with the products of flhA of B. subtilis (Carpenter and Ordal, 1993) and S. typhimurium (Minamino et al., 1994), invA (Galan et al., 1992) and ssaV (Hensel et al., 1997) of S. typhimurium, mxiA of S. flexneri (Andrews and Maurelli, 1992), hrpO of R. solanacearum (Gough et al., 1993), sepA of EPEC (Jarvis et al., 1993), flbF of Caulobacter crescentus (Webb et al., 1991) and of virulence-associated genes from several bacterial plant pathogens. Homology of the Cds2 sequence to selected sequences from animal pathogens is shown in Fig. 2.

Southern hybridization analysis using a cds probe identified conserved sequences in purified genomic DNA from C. trachomatis serovar L2 grown in Amiens, France, and C. pecorum strain IB1 grown in Nouzilly, France (Fig. 3). Moreover, the presence of cds2 sequence in C. trachomatis serovar D was confirmed by preliminary data from the Chlamydia Genome Sequencing Project currently underway in California (R. Stephens, personal communication). Finally, cds2 sequence was also polymerase chain reaction (PCR) amplified using cds2-specific oligonucleotide primers and purified genomic DNA from C. trachomatis L2 and from C. pneumoniae TWAR grown in Seattle, USA, as templates (not shown). The fact that the C. psittaci strain used here and chlamydiae of the three other species originated from five different geographical locations indicates that the cloned DNA is of chlamydial origin and is not that of a bacterial contaminant of tissue culture.

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Thus, although phenotypic differences exist in the expression of virulence between different Chlamydia species (e.g. species and tissue tropism, survival in macrophages), all species appear to possess a conserved mechanism of secretion, which in other bacteria is dedicated to the expression of virulence. This is consistent with other intracellular pathogens, which, although expressing distinct virulence traits, possess highly conserved contact-dependent secretion genes (Groisman and Ochman, 1993).

The product of copN is a homologue of Yersinia YopN (LcrE), a substrate of the secretion pathway involved in its down-regulation.

The predicted product of copN is a polypeptide of 398 amino acid residues for a calculated molecular weight of 43 093 Da. The CopN sequence is most homologous to that of the yopN (lcrE) gene product of yersiniae (Viitanen et al., 1990; Forsberg et al., 1991) (23–24% identity; 49–50% similarity) (Fig. 4). Further analysis also reveals Q

Fig. 2. Amino acid sequences of SepA (EPEC) (Jarvis et al., 1995), SsaV (S. typhimurium) (Hensel et al., 1997), MxiA (S. flexneri) (Andrews and Maurelli, 1992), InvA (S. typhimurium) (Galan et al., 1992), LcrD (Y. pestis) (Plano et al., 1991) and Cds2 (C. psittaci GPIC) (this work) are compared. Dots indicate a gap in a given alignment. Identities in at least four of the sequences are shaded. The sequence of a peptide against which a monospecific rabbit antiserum was prepared is underlined.

Fig. 3. Southern blot analysis of cds conserved sequence in Chlamydia spp. Purified Pst I-restricted genomic DNA from C. pecorum IB1 (lane A) and C. trachomatis serovar L2 (lane B) was hybridized to a probe corresponding to the first 817 bp of the sequence shown in Fig. 1.
homology of CopN to the products of invE of S. typhimurium (Ginocchio et al., 1992), mxiC of S. flexneri (Andrews and Maurelli, 1992; Allaoui et al., 1993) and hrpJ of Erwinia amylovora (Bogdanove et al., 1996) (Fig. 3). In contrast to the Cds1–2 homologies, the homology of CopN with YopN, InvE, MxiC and HrpJ is generally lower and must allow for sizeable gaps in sequence corresponding to significant size differences between these polypeptides.

In yersiniae, the YopN protein is itself a substrate of contact-dependent secretion, which, unlike other Yops, remains associated with the outer surface of the outer membrane (Forsberg et al., 1991). Upon contact with the target cell, YopN is thought to participate in the transmission of a signal leading to derepressed yop expression and in directing the assembly of the secretion apparatus in the contact zone (Rosqvist et al., 1994). Thus, while YopN is itself a contact-dependent secreted protein, a direct role in virulence has not been reported since the essential function of YopN is apparently to control the polarized delivery of Yops through the secretory pathway (Boland et al., 1996; Rosqvist et al., 1994; Forsberg et al., 1994). It is therefore possible, in analogy with Yersinia, that CopN function may be to upregulate Cop expression and secretory activity upon contact between intracellular reticulate bodies and the plasma membrane-derived parasitophorous Chlamydia vacuole membrane.

The product of scc1 is a homologue of specific Yersinia chaperones (Syc)

The predicted product of scc1 is a polypeptide of 146 amino acid residues for a calculated molecular weight of 16 176 Da and isoelectric point of 5.2. The Scc1 sequence is homologous to that of the YscE chaperone protein of Y. enterolitica (Viitanen et al., 1990) and CopN (C. psittaci GPIC) (this work) are compared. Dots indicate a gap in a given alignment. Identities in at least three of the sequences are shaded. Additional identities between CopN and YopN are boxed.

Fig. 4. Amino acid sequences of HrpJ (E. amylovora) (Bogdanove et al., 1996), MxiC (S. flexneri) (Andrews and Maurelli, 1992; Allaoui et al., 1993), InvE (S. typhimurium) (Ginocchio et al., 1992), YopN (Y. enterolitica) (Viitanen et al., 1990) and CopN (C. psittaci GPIC) (this work) are compared. Dots indicate a gap in a given alignment. Identities in at least three of the sequences are shaded. Additional identities between CopN and YopN are boxed.
recognized a protein doublet of apparent molecular weight 69 kDa present in GPIC lysates (Fig. 5), in slight discrepancy with the calculated molecular weight (78 kDa). Moreover, the doublet band was only detected in samples that had not been boiled before SDS–PAGE. Identical properties (abnormal migration and irreversible heat-induced aggregation) have been observed with LcrD of Y. pestis (Plano et al., 1991). We conclude that the observed 69-kDa doublet band most probably represents an expressed form of the cds2 product during the GPIC intracellular life cycle.

To demonstrate the functionality of the chlamydial genes further, we undertook complementation experiments using a lcrD mutant of Y. pestis that is deficient in the low calcium response. Although the Cds2 sequence is most closely related to that of LcrD, neither the full-length cds2 nor a cds2–lcrD hybrid gene (corresponding to the fusion [Met-1–LcrD–Ile-18]–[Ile-28–Cds2–Leu-318]–[Ala-310–LcrD–Leu-699] under lcrD promoter control) could restore a low-calcium response to host yersiniae (not shown). These results are in contrast to the observed functional replacement of InvA by a LcrD–InvA hybrid in experiments that assessed restoration of invasiveness to an invA mutant of S. typhimurium (Ginocchio and Galan, 1995). An important structural property of Cds2, which may be relevant to this apparent functional difference, is its uniquely basic pI (9.17 calculated pl for Cds2 compared with 4.94–5.96 for LcrD, MxiA, InvA, SepA and SsaV). Since 9 of 20 predicted charge differences between Cds2 and LcrD are located in segments proposed to face the cytoplasm (Plano et al., 1991), the observed lack of functional complementation may reflect different structural requirements for assembly of the secretion apparatus in Yersinia and Chlamydia or different substrate specificities.

The cds locus of C. psittaci is phylogenetically unique

In yersiniae, yscU is located upstream of lcrD, is separated from it by several genes also involved in the secretory pathway and belongs to a cluster that is transcribed in the opposite orientation to that of lcrD (Allaoui et al., 1994). In SPI1 and SPI2 of salmonellae and in shigellae, the corresponding yscU homologues, spaS, ssaU and spa40, are located several genes downstream of and in the same transcriptional orientation as the lcrD homologues invA, ssaV and mxiA (Venkatesan et al., 1992; Groisman and Ochman, 1993; Sasakawa et al., 1993; Hensel et al., 1997). Yet another variation on the theme is seen in Chlamydia, in which the yscU homologue, cds1, lies immediately upstream of the lcrD homologue, cds2 (Fig. 1), separated from it by only three nucleotides. Thus, while the putative chlamydial genes display high homology with their counterparts in enteric pathogens, particularly yersiniae, the general topography of the chlamydial cds locus appears to be unique.

Donnenberg et al. (1997) first noted that some contact-dependent secretion loci have a lower G + C content, e.g. 38.4% G + C for the locus of enterocyte effacement (LEE) of EPEC vs. 50–51% for E. coli genomic DNA. In contrast, the calculated G + C for cds1–scc1 is 40%, close to the value for chlamydial genomic DNA (41–44%) (Moulder et al., 1984), and close to the value for the LEE of EPEC. The observed G + C homogeneity within Chlamydia combined with a lack of distinguishable repeat sequences in the ssc1–orfE intergenic sequence does not currently support, although it does not exclude, the possibility that the chlamydial cds locus is included in a classical pathogenicity island, as defined by its recent acquisition by horizontal transfer. Recent data from the Chlamydia Genome Sequencing Project led by Dr R. Stephens (http://chlamydia-www.berkeley.edu:4231/) have also revealed a putative gene encoding a homologue (54% identity) of YscN of yersiniae. In Yersinia, yscN encodes a cytoplasmic membrane-bound ATPase, thought to be required for energizing Ysc–Lcr contact-dependent secretion (Woeslyn et al., 1994) and lies at the proximal end of the yscN–yscU eight-gene operon (Bergman et al., 1994). The organization of the yscN–yscU cluster is conserved in Shigella, Salmonella SPI1 and SPI2 (Groisman and Ochman, 1993; Bergman et al., 1994; Hensel et al., 1997), but differs in EPEC where several genes, including sepA, encoding a homologue of LcrD of Yersinia, and sepC, encoding a PuI-like outer membrane protein homologous to YscC of Yersinia, intervene between the yscN and yscU homologues (sepB and sepF respectively) (Donnenberg et al., 1997). In C. trachomatis, several additional ORFs encoding hypothetical products with weak homology to secretory pathway components YscT of Yersinia (ORF D031) and InvG of Salmonella (ORF D028) and
to potential secreted proteins (e.g. products of ORFs D029 and D035) are closely linked to the yscN homologue (ORF D033). However, further away on either side are several hypothetical genes that are apparently unrelated to virulence, such as rrf (ORF D025) and gyrB (ORF D037). If one assumes that the cds locus is conserved in *C. trachomatis*, this would imply that the yscN and hypothetical yscU homologues of *C. trachomatis* not only are not part of the same operon as in EPEC, but are also genetically unlinked. Notwithstanding possible strain differences within the *Chlamydia* genus and the fragmented information on which this analysis is currently based, this may suggest that contact-dependent secretion is genetically evolved in *Chlamydia*, perhaps as an insurance mechanism against loss of essential function by a single deletion event. Moreover, this apparent ‘dispersion’ of contact-dependent secretion genes distinguishes *Chlamydia* from facultative intracellular pathogens and constitutes a departure from classical pathogenicity islands.

Possible role(s) of contact-dependent secretion in *Chlamydia*

Since the genes that we have identified so far are involved in secretion, further sequence analysis of linked genes is necessary before the virulence properties of the cds locus can be assessed. Although flagellar subunits are also translocated via a type III secretion pathway, chlamydiae themselves are non-motile, and phylogenetic analysis groups Cds2 with its homologues in animal pathogens, the closest relationship being with LcrD (data not shown). Therefore, since chlamydiae are bacteria whose pathogenesis (and biology) is strictly dependent on its interaction with eukaryotic cells, the most likely activities of contact-dependent secreted gene products in *Chlamydia* concern the establishment and sustenance of the host–parasite relationship.

In the context of a virulence-associated contact-dependent secretion in *Chlamydia*, it is tempting to speculate about the possible role(s) of its secreted protein substrates (Cops) in chlamydial biology. Chlamydiae are characterized by a unique intracellular developmental cycle, such that the extracellular, infectious form of the organism, the aptly named elementary body, is metabolically inactive, while the intracellular form, the reticulate body, is a ‘normally’ growing bacterium. Elementary body metabolic dormancy therefore predicts that contact-dependent secretion will be a property of the reticulate body, i.e. will occur only after chlamydiae have been internalized and have differentiated. This sharply contrasts chlamydiae with other pathogens in which contact-dependent secretion is triggered from without, i.e. upon extracellular contact between bacteria and eukaryotic cell. While contact-dependent secretion is necessarily a property of intracellular chlamydiae, it is nonetheless possible that secreted virulence factors affect elementary body infectivity. Under this scenario, some Cops secreted intracellularly by the reticulate body would remain stably anchored at the chlamydial surface during late reticulate body differentiation and would contribute to initial pathogenic processes, either during entry, in a manner similar to the *Shigella* IpaBCD invasins (Menard et al., 1993), or immediately after, upon interacting with endosomal components. Consistent with the latter is the observation that certain host proteins are specifically tyrosine phosphorylated as early as 15 min after infection (Birkeland et al., 1994), i.e. several hours before elementary body maturation.

A second, non-exclusive possibility compares chlamydial contact-dependent secretion to that in *Yersinia*. Contact of yersiniae with the target cell membrane triggers expression and coupled translocation of virulence factors (Yops) across the juxtaposed plasma membrane (Rosqvist et al., 1994; Boland et al., 1996; Pettersson et al., 1996) through a channel spanning the two bacterial membranes and the plasma membrane of the target cell. Once injected, Yops are thought to interact with and/or modify host molecules, often subverting signal transduction pathways, ultimately leading to pathogenesis. Growing chlamydiae similarly are often observed to contact the plasma membrane-derived vacuole membrane in a relationship thought to promote the acquisition of host nutrients and nucleotides (Moulder, 1991). Alternatively, this juxtaposition may also facilitate the contact-induced polarized translocation of virulence factors through the secretion pathway.

Possible substrates of contact-dependent secretion in *C. psittaci* GPIC are two proteins, termed Inc, recently identified by Rockey et al. (1995) as antigens specific to growing chlamydiae, which are translocated into the parasitophorous vacuole membrane (Bannantine et al., 1997). The currently better characterized IncA lacks a leader sequence, is serine–threonine phosphorylated during infection and portions of it face the outer (cytosolic) side of the *Chlamydia* vacuole membrane (Rockey et al., 1997). In other intracellular bacterial pathogens, contact-dependent secreted virulence factors are often enzymes (e.g. protein phosphatases), whose main role is to modify host proteins involved in signal transduction cascades. The disruption of these pathways usually leads to the subversion of essential host trafficking mechanisms in order to satisfy the pathogen’s own parasitic needs. A number of recent studies have indicated that chlamydiae do indeed exploit host intracellular trafficking. Soon after infection, the host cytoskeleton is involved in the redistribution of internalized chlamydiae from the cellular to the nuclear periphery (Majeed et al., 1993; Schramm and Wyrick, 1995), and calcium-dependent annexins are found colocalized with
the *Chlamydia vacuole* (Majeed *et al*., 1994). Dependent on the multiplicity of infection and on time elapsed after infection, development of the *Chlamydia vacuole* may involve fusogenicity with both early and late endosomes (van Ooij *et al*., 1997), and with Golgi-derived exosomes from which sphingolipids are redirected to the parasitophorous vacuole membrane and to membranes of growing reticulate bodies (Hackstadt *et al*., 1996). Globally, these observations are indicative of a high level of meddling by *Chlamydia* with host intracellular trafficking pathways and provide ample biological justification for the existence in *Chlamydia* of a contact-dependent secretion pathway, which, similarly to that of other intracellular pathogens, is dedicated to intercepting and modulating host signalling pathways.

**Experimental procedures**

**Strains and DNA preparation**

The strain of *C. psittaci* GPIC used in this study was obtained from the laboratory of Roger Rank, University of Arkansas for Medical Sciences, Little Rock, AR, USA. Purified (Batteiger and Rank, 1987) GPIC elementary bodies grown on HeLa 229 cells (ATCC CCL 2.1) were used to purify genomic DNA as described previously (Palmer and Falkow, 1986).

Cloning, nucleotide sequence analysis and computer analysis of sequence data

A weakly hybridizing clone, pYP601, was isolated from a BamHI GPIC genomic library in pBluescript pSK+. Using the degenerate oligonucleotide probe 5'-GA(A,G)TT(C,T)TT(C,-T)(A,T)(C,G)NTT(C,T)T-3' based on a conserved segment of the DsbA periplasmic thioreductase of *Escherichia coli* (Bardwell *et al*., 1991) (data not shown). The insert in pYP601 is a BamHI fragment of 7.5 kb. The upstream fragment, which includes the complete *cst1* sequence, was isolated by chromosome walking. For each strand, nucleotide sequence analysis was performed on overlapping segments, either by subcloning small fragments into Bluescript pSK–, subsequently sequencing using T3 and T7 primers, or by subcloning larger fragments into pMOB, subsequently generating nested -6 insertions and sequencing using transposon-specific primers as described previously (Strathmann *et al*., 1991). A combination of manual (Sanger *et al*., 1977) and automated (Applied Biosystems) sequencing methods were used for the analysis of the complete region. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number U88070.

Open reading frames were identified using MAP, homologies of the predicted translation products to sequences of the SWISSPROT (release 31.0) were identified using FASTA alignments were generated using PILEUP (gap creation and extension penalties of 3.00 and 0.10 respectively) and percentage similarity and identity were obtained using GAP of the University of Wisconsin Genetics Computer Group (UWCGG) package at the Human Genome Mapping Project Resource Centre, Hinxton, Cambridge, UK.

**Other methods**

Southern blot analysis was performed as described previously (Sambrook *et al*., 1989). SDS–PAGE and immunoblotting were as described by Laemmli (1970) and Towbin *et al.* (1979) respectively.

Polyclonal antiserum monospecific for Cds2 was obtained by immunization of one New Zealand white rabbit with a Cds2-specific peptide (Fig. 2) synthesized using the multiple antigen peptide system (BioSynthesis, Lewisville, TX, USA).

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