AN ABSTRACT OF THE THESIS OF

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Chlamydiae are obligate intracellular bacteria that infect a variety of eukaryotic hosts and affect normal host processes. Within host cells, their developmental cycle takes place inside non-acidified vacuoles termed inclusions. An inclusion membrane composed primarily of secreted chlamydial synthesized proteins called Incs encloses the inclusion. At this location, Incs have a direct access to the host cytosol and are hypothesized to mediate important host- chlamydial interactions. One such Inc is IncA, a protein that participates in fusion of chlamydial inclusions. Host proteins that are localized to the inclusion are also hypothesized to participate in these interactions. Such interactions are thought to facilitate chlamydial intracellular development and survival. As a genetic system to manipulate chlamydiae does not currently exist, characterizations of such proteins rely on the use of contemporary molecular techniques. In this work, these techniques were utilized to (1) identify and characterize host phosphoproteins whose abundance and subcellular localization were altered as a result of chlamydial infection and (2) analyze the transcription of the *incA*

mRNA in clinical isolates that have *incA* sequence polymorphisms, lack detectable IncA, and have chlamydial inclusions that are non-fusogenic. Adducin, Raf-1 and PKB (protein kinase B) are previously unreported host proteins whose abundance and subcellular localization are altered in *Chlamydia*- infected cells. Adducin is a cytoskeleton- associated, actin- capping protein whose phospho- protein abundance is depleted in the Triton X-100 soluble (TS) fractions of infected cells, specifically at mid-to-late time points post- infection. However, adducin abundance is unchanged in the total protein lysates, suggesting that the subcellular localization of the phosphorylated protein is affected by chlamydial infection. A fraction of adducin is also localized at the margin of the chlamydial inclusion and localization is independent of intact microtubules or actin. Raf-1 is a signaling protein whose abundance is also depleted in the TS fractions of infected cells. While phospho- PKB abundance is comparable in the total protein lysates between *C. trachomatis*- infected and mock- infected cells, the phospho- PKB is depleted in the total protein lysates of *C. caviae*- infected cells. This result suggests that *C. caviae* infection affects PKB phosphorylation events. The transcription analysis of *incA* demonstrated that *incA* is transcribed in cells infected by the wild type or non- fuser strains, initiating at a common transcriptional start site. However, the abundance of *incA* transcripts in cells infected by a non- fuser strain is reduced relative to those infected by the wild type strains.

Analysis of Chlamydial and Host Proteins Associated With Infection

by
Hencelyn G. Chu

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Hencelyn G. Chu, Author

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DEDICATION

Without David and Cassie, this work would not have been completed. And I am grateful for them – for enduring many challenges with me these past few years and supporting me through it all. This thesis is dedicated to them.

Analysis of Chlamydial and Host Proteins Associated With Infection

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1.1 INTRODUCTION

Chlamydiaceae are a group of obligate intracellular bacterial pathogens that infect a broad spectrum of host organisms, from amoebas to humans. Chlamydial development and replication occur inside host cells in a non- acidified vacuole termed the inclusion. In order for *Chlamydiae* to establish an intracellular niche inside eukaryotic hosts, it has evolved or acquired capabilities to evade the immune system, enter host cells, replicate inside a suitable environment, and escape to re-initiate its intracellular development again in other host cells. Critical to the successful chlamydial infection are unique, largely uncharacterized intracellular host- pathogen interactions.

1.2. CHLAMYDIAE AND ASSOCIATED DISEASES

Chlamydiaceae are divided into two genera: *Chlamydia* and *Chlamydophila*. The genus *Chlamydia* includes *C. trachomatis*, *C. muridarum*, and *C. suis* species. *C. trachomatis* is predominantly an etiologic agent for human pathogens while *C. muridarum* and *C. suis* are mouse and swine pathogens, respectively. The *Chlamydophila* genus is a larger and more diverse group with six species, including significant human and animal pathogens such as *C. pneumoniae*, *C. psittaci*, and *C. caviae*. Based on 16S rRNA sequences, the *Chlamydia* and *Chlamydophila* species are closely related (120, 164). However, based on 16S rRNA sequence relatedness, the chlamydial taxonomy was reorganized to include three new families, *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliacea* (42, 134). While these organisms

are termed “environmental chlamydiae”, some evidence suggests that they may also be associated with clinical disease in humans and animals (89).

There is considerable variation within the species *C. trachomatis*, and this has led to classifications within the species based on biological differences (biovars) or serological differences (serovars). There are two important biovars of *C. trachomatis*: the trachoma biovar and the lymphogranuloma biovar. Biovars are based on differences observed both clinically and experimentally. The trachoma and lymphogranuloma venereum (LGV) biovars are largely human pathogens distinguished by different clinical presentations. LGV biovar infections (caused by serovars L1, L2, L2a, and L3) originate at a mucosal site, but serious disease is associated with systemic dissemination and proliferation in cells of the lymph nodes (139). Infections by strains of the trachoma biovar are confined mucosal epithelia, such as the urogenital tract or the conjunctiva (136). The trachoma biovars include serovariants A to K as well as Ba, Da, and Ia. They are associated with trachoma, an infection of conjunctival epithelia that, through persistence and re- infection, causes a chronic disease that leads to blindness. In developing countries, trachoma has an estimated annual incidence of 80 million cases with 6 million individuals blinded as a result of this disease (169). Experimentally, the LGV and trachoma biovars are also differentiated by their ability to infect tissue culture cells (84, 86, 88). Based on serological identification, serovars or serological variants of trachoma and LGV have been identified and designated with letters and/or numbers.

Sexually transmitted *C. trachomatis* infection results in a variety of secondary anomalies including mucopurulent cervicitis, urethritis, salpingitis, lymphogranuloma

venereum, endometritis, opthalmia neonatorum, and infant pneumonia (136). An estimated 92 million adults worldwide contract sexually transmitted chlamydial infection each year and are associated with 60% of tubal infertility cases and 40% of ectopic pregnancy cases (117). Recent work by Nusbaum *et al* suggests that previous infection of *C. trachomatis* increases the likelihood of HIV and/or HPV transmission, demonstrating that the mucopurulent discharge associated with chlamydial and gonorrheal infections increase the risk of HIV transmission at least three- to five-fold (112). Therefore, in the United States, *Chlamydia* infection control that uses contemporary molecular diagnostic approaches programs are currently being developed (29, 138).

While chlamydial infections are easily resolved with antibiotic treatment, there is evidence that tetracycline resistance exists in *C. suis*, chlamydial strains found in swine. Research in our laboratory suggests that this may have resulted from horizontal transfer of resistance genes from other bacteria (40). Available veterinary vaccines protect against chlamydial disease, not infection, and there is currently no vaccine for human chlamydial disease (91).

1.3. STRUCTURAL COMPONENTS OF CHLAMYDIAE

Characteristic of all chlamydiae is a biphasic developmental cycle marked by two distinct forms, namely the elementary bodies (EB) and the reticulate bodies (RB). The EB are infectious and metabolically inactive forms (102). Because of their ability to attach to and invade cells, their primary responsibility is to propagate infection. To initiate the infection, EB are internalized into host via membrane- derived vesicles

termed inclusions (102). Between 1 and 4 hours following contact, EB undergo differentiation to become RB, the metabolically active and non-infectious forms (102).

The RB stage is characterized by bacterial cell division via binary fission and active metabolic activity when most chlamydial transcripts are expressed and translated into proteins (18, 108). Intracellular development proceeds as the RB de-differentiate back into EB in preparation to leave the host cell and reinitiate development in other host cells.

1.3.1. THE ELEMENTARY BODY (EB)

The EB is a small (~0.3 μm), circular, electron dense form of *Chlamydia*, and highly adapted to extracellular survival (24, 97). Its size, metabolic inactivity, and resistance to physical disruption (i.e. moderate sonication) is comparable to "spore-like" forms of other bacteria. Its outer membrane complex, however, lacks detectable peptidoglycan (102). Although a complete set of genes encoding peptidoglycan biosynthetic components is present in the chlamydial genome, many investigators have yet to demonstrate that *Chlamydiae* produce peptidoglycan (11, 51). Several investigators hypothesize that peptidoglycan may be degraded during the RB to EB transition by amidases found in the chlamydial genome (30). Nevertheless, the undetectable peptidoglycan does not explain why chlamydial growth is inhibited by compounds such as penicillin and β-lactam derivatives (103).

The chlamydial EB envelope is composed of three layers, the outer membrane, the periplasmic layer, and the inner membrane. Components of the outer membrane include major outer membrane protein outer membrane (MOMP), lipopolysaccharide

(LPS), and polymorphic outer membrane proteins (POMP). Other proteins associated with the outer membrane and the periplasmic space include the cysteine-rich outer membrane complex A and B (OmcA and OmcB) (5, 76, 155).

The MOMP is the most dominant constituent of the outer membrane and is hypothesized to be an adhesin or a porin (5, 76, 155). Sequencing of MOMP has demonstrated that it has four distinct segments giving rise to different epitopes that are specific to serotype, serogroup, species, or subspecies. Su *et al* demonstrated that these regions are also associated with adherence and their structural variants may enable chlamydiae to interact with different host receptor structures, offering an explanation to differential tissue tropism exhibited by chlamydial serovars (159). Except for in *C. pneumoniae*, it is surface exposed and highly immunogenic in all chlamydial strains; they are poor vaccine candidates in that protection is serovar- specific and short-lived (66, 158, 159). However, recently, Wolf *et al* utilized partial solubilization of C. pneumoniae MOMP and demonstrated that its conformational epitopes, while structurally different from *C. trachomatis* MOMP, is a species-specific neutralizing-specific antibody and is surface exposed (183). MOMP is "susceptible" to reducing agents that render the EB capable of transient metabolic activity and RNA synthesis (65, 105). This supports the hypothesis that MOMP functions as a chlamydial porin. While MOMP is expressed throughout the developmental cycle, Omp2 (*C. psittaci* EnvA) and Omp3 (*C. psittaci* EnvB) are classified as late chlamydial gene products (65, 178).

The outer membrane also contains chlamydial LPS, a truncated form of typical LPS that is conserved among all chlamydial species (23, 111). Antibodies that attach to chlamydial LPS do not inhibit chlamydial infection.

The POMPs, encoded by *Pmps*, reside in the outer membrane and are surface-exposed in all chlamydial species, except *C. pecorum* (6, 58, 81, 92, 93, 153). In *C. psittaci* isolated from sheep, at least six genes, termed the *pomp90* and *pomp98* families, are present; nine genes, *pmpA* to *pmpH*, are present in *C. trachomatis*; and in *C. pneumoniae*, 21 genes, *pmp1* to *pmp21*, have been identified. Not only are these genes important components of the outer membrane, they also have been characterized as novel members of the bacterial type V autotransporter family of proteins that is used by *Neisseria*, *Haemophilus*, *Bordatella*, *Helicobacter*, *Shigella*, and *Escherichia coli* for protein secretion (70). Similar to the structure of other bacterial autotransporters, they have a signal sequence at the amino-terminal end, a passenger domain, and a carboxy-terminal translocating unit that assembles to form a β- barrel pore. Chlamydial secreted proteins utilize a sec- dependent secretion system, access the inner membrane, and cross the periplasm into the outer membrane via the β- barrel pore through which the passenger domain passes through to be translocated to the bacterial surface. Also, Werhl *et al* performed extensive work in characterizing *C. pneumoniae* PmpD translocation and proposed that it is an important adhesin that activates inflammation processes (179). They discovered that a portion of the PmpD passenger domain is post-translationally modified and translocates to the bacterial surface. Antibodies to PmpD block chlamydial infectivity. Additionally, incubation of recombinant PmpD with human monocytes increased secretion of IL-8, a

proinflammatory cytokine that is upregulated in human and mouse macrophages infected with *C. pneumoniae*. These data suggest perhaps that viable *C. pneumoniae* may not be needed to induce production of this cytokine but can be accomplished by acellular components.

The EB cytoplasm contains DNA, DNA binding proteins, and RNA (165). At least two DNA binding proteins, Hc1 and Hc2, are detected in the EB (19, 59, 118). Not only are these proteins eukaryotic histone homologs, but also possess sequence identity with other DNA binding proteins such as AlgP of *Pseudomonas aeruginosa* and HrdB *Streptomyces coelicolor* (Rockey/Matsumoto). AlgP is a bacterial histone-like element that is also transcriptional activator of mucoidy, a virulence factor (37). HrdB is a sigma-like transcriptional activator for *Streptomyces coelicolor* (148).

Ultrastructural analysis of the EB via electron microscopy revealed surface projections that were regularly spaced approximately 50 nm apart and hexagonnally arranged (52, 99, 107). Several investigators suggest that these are typical of type III secretion system (TTSS) structures and may provide a pathway and mechanism for chlamydial proteins to be secreted into the inclusion membrane (15, 77).

1.3.2. THE RETICULATE BODY (RB)

Distinguishing an RB from an EB via electron microscopy is not a difficult task; identifying structural components that differentiate these two structures is much more complex. Basically, RB is characterized by the lack of proteins associated with EB and while some RB- specific proteins have been identified, they are not abundant. RB-specific proteins include cyclic AMP (cAMP)- binding activity (80), early

expressed Inc proteins (146), p52 (129), and *C. trachomatis* TroA and p242 (8). It is also likely that the proteins required for anabolic processes are also present in RB. Additionally, inclusion membrane proteins (Incs) are typically associated with the RB developmental stage and not EB.

1.3.4. GENERAL PROPERTIES OF THE INCLUSION

The chlamydial inclusion is composed of two main regions: the luminal space and the inclusion membrane (IM). Depending on the developmental stage, the lumen is occupied predominantly by EB or RB as well as chlamydial synthesized molecules within the developmental forms. However, chlamydial dependence on its host for optimal development may require transport of host-derived molecules to the inclusion. Heinzen *et al* determined that molecules which are 520 Da or less are likely to enter the inclusion by passive diffusion (60, 68). These include small ions such as Na^+, Ca^+, K^+ and H^+. Grieshaber *et al* determined that the concentration of these ions in the inclusion are very similar to the concentration in the host cell cytosol (54). Molecules larger than 520 Da will require some type of active transport that requires IM transporters. However, while the genome reveals many metabolite transporters, no membrane transporters are localized to the IM and host derived molecules likely access the inclusion by other means (60, 68). Sphingomyelin, a source of nutrients and lipids, likely access the inclusion by fusion events between the exocytic vesicles carrying sphingomyelin and chlamydial inclusion.

Historically, *Chlamydiae* are known as “energy parasites”, meaning that they rely solely on the host for ATP and other energy intermediates. However, genome

sequencing has recently identified chlamydial genes that encode enzymes involved in ATP and NADPH biosynthesis (153). Functional analysis of some ATP intermediates such as pyruvate kinase, phosphoglycerate kinase, glyceraldehyde-3 phosphate dehydrogenase, and glucose-6-phosphate dehydrogenase) determined that genes encoding these products are indeed functional (78). *Chlamydiae* also have two ADP/ ATP translocases that enable ADP exchange for host ATP (170). Transcriptional analyses of these genes suggest that the genes encoding the translocases are expressed early during the developmental cycle and the energy intermediates are expressed during the middle to late developmental stages of chlamydial development (147). This suggests that *Chlamydiae* require host ATP during early development, utilize their translocases to import host ATP in exchange for ADP, and have the ability to independently produce ATP and NADPH.

Finally, several experiments were conducted to determine the pH of the inclusion lumen by different methods. Independent investigators have determined that the pH inside the chlamydial inclusion are between 6 and 7, approximating that of the host cytosol (54, 142). This lends support to the findings that chlamydiae reside in a vacuole that is non-acidified (69, 141).

1.3.5. GENERAL PROPERTIES OF THE INCLUSION MEMBRANE (IM)

The chlamydial inclusion membrane is the primary barrier between the host and chlamydial compartments with several important functions. First, the inclusion membrane offers chlamydiae shelter from the host immune response by inhibiting direct bacterial contact with major histocompatibility complex (MHC) I and II antigen

processing pathways. Second, it is a selective barrier that facilitates acquisition of nutrients and other molecules required for growth. Finally it is a compartment for facilitating export of chlamydial proteins to the cytosol and for positioning chlamydial Incs optimally so that they can interact with host proteins.

1.4. PROTEINS IN THE INCLUSION MEMBRANE (IM)

Chlamydial Incs are defined as proteins that localize to the inclusion membrane and are mediators of chlamydial interaction with its host. During the pregenomic era, the discovery of Incs was a result of serological detection and molecular cloning assays. Initially, Rockey and Rosquist *et al* identified infection-specific proteins in *C. caviae* GPIC- infected tissue culture cells (129). These proteins are absent in the infectious EB. By using sera from guinea pigs recovering from challenges with *C. caviae* GPIC, they were able to identify protein antigens p22, p34, and p52. An expansion of this work utilized screening of an expression library of *C. psittaci* DNA with convalescent antisera from infected animals as well as hyperimmune antisera obtained from animals infected by formalin- killed purified chlamydiae. The convalescent antisera identified a 39 kDa protein that was present in infected cell protein lysates, the RB, and was also localized in the inclusion membrane of infected HeLa cells. Eventually, this 39 kDa protein is renamed IncA (inclusion membrane protein A), and utilized extensively to label the inclusion membrane proteins (128). By using similar screening methods, other Incs, namely IncB and IncC, were identified in C. psittaci- infected cells (10).

C. psittaci IncA was further characterized in different assays to determine that it is exposed on the cytoplasmic side of the developing inclusion and it is a serine/ threonine phosphoprotein that is likely phosphorylated by host cell kinases (60). By using a vaccinia-virus recombinant expressing IncA, it was determined that this phosphorylation event can occur independent of a chlamydial background. Subsequently, Scidmore *et al* used a membrane fractionation approach, in which antisera were raised against a total membrane fraction that was purified from HeLa cells infected by *C. trachomatis* (146), to identify three additional chlamydial specific genes (IncD- IncG). Similar to the *C. caviae* Incs, these were also tested by immunofluorescence to demonstrate their localization to the IM. Further analyses confirm that these Incs are transcribed as an operon early in the developmental cycle, suggesting that they have an important role in remodeling the nascent chlamydial inclusion at early time points post infection.

Once the first *Chlamydia* genome was sequenced and annotated, the sequence prediction and identification of other chlamydial Incs became possible. First, it was determined that *C. trachomatis* has a gene (CT119) that encoded a protein similar to the *C. caviae* IncA. From the completed genome sequences of *C. trachomatis* and *C. pneumoniae*, Rockey *et al* utilized bioinformatics and molecular approaches to identify a unique motif shared by most Incs, a protein domain with a bi-lobed hydrophobic region of 50-60 amino acids (7). While the functional role of this motif has not been extensively characterized, it is hypothesized to serve a role in anchoring the Inc proteins in the IM. To identify proteins from the completed genome sequences that have this bi-lobed hydrophobic domain, predicted amino acid sequences from

each chlamydial open reading frame were screened for the hydrophobic motif. Rockey *et al* identified 45 ORFs in *C. trachomatis* and over 65 in *C. pneumoniae*, representing 3.1% and 5.4%, respectively, of the ORFs in the entire genome. In the recently completed *C. caviae* genome, approximately 50 putative *inc* genes, or 4.7% of the entire genome, have been identified. To determine if the hydrophobic domains are associated with the putative Inc localization in the IM, antisera against six of the candidate *C. trachomatis* Inc proteins, including CT223, CT229, CT288, CT233 (*incC*), CT442, and CT484, were produced. Of these six genes, only CT484 did not localize in the IM (7). Toh *et al* expanded this work not only by examining the amino acid sequences of candidate Incs but also included the Inc protein structure (172). By using *in silico* approaches, they were able to examine ORFs in genomes of other bacterial organisms, particularly *Mycobacterium tuberculosis*, and conclude that Inc proteins are unique to *Chlamydiae*. This suggests that Incs may be essential proteins for the chlamydial obligate intracellular lifestyle and/or virulence. In addition, Incs were discovered recently in the genome sequences of *Parachlamydia spp*., bacterial species that inhabit amoeba and are also closely related to *Chlamydiae* (75), suggesting that the interactions of these species with their hosts may be similar to those of pathogenic human and animal chlamydial species.

While a majority of IM proteins are Incs with the characteristic hydrophobic domain, not all proteins that localize in the IM have this domain. This includes CopN, a component of the chlamydial type III secretion (T3S) machinery and the $CD8^+$ T cell target Cap1 (45, 48).

An interesting paradox involving the study of Incs is the discovery that, amidst a general reductive evolutionary strategy, Incs have undergone expansion in various chlamydial strains. Evidence of this expansion is apparent in *inc* genes that are (a) unique to a particular chlamydial species, (b) common within the genus, and (c) represented within each sequenced chlamydial genome but demonstrate expansion at an accelerated rate. Examples of Incs that are unique to a chlamydial species are genes found in *C. trachomatis*, including CT115- CT119 and CT223-CT229 (7, 146), genes found in *C. caviae* such as CC793, CC794, CC797, CC799 and CC800, genes found in *C. muridarum* such as TC495 and TC496, and *C.pneumoniae* genes that include CP0010, CP1054, and other sequences (35, 176). Such genes are hypothesized to function in species-specific roles that may contribute to successful intracellular development and/or virulence. Examples of other *inc* genes that are conserved within the genus include *incB*, *incC*, and, although has not been shown to reside in the IM, CT484, and their homologs. These candidate Incs are perhaps involved in host-cell pathogen interactions that enable chlamydiae to tolerate common challenges associated with intracellular living.

An example of an Inc that appears to have undergone expansion at an accelerated rate is CT058 and its homologs. Not only does this gene have homologs in all examined chlamydial species, but the genes flanking this gene are also conserved (123). Bannantine *et al* also determined that CT058 is an Inc that has undergone gene duplication and has limited similarity with *C. pneumoniae* proteins (7). An example of a candidate *inc* gene expansion within species by gene duplication

is demonstrated by the CP1054 family of *C.pneumoniae*, which is absent in other species but expanded at a high level in *C. pneumoniae* (176).

1.4.1. TRANSCRIPTION OF *inc* GENES

Chlamydiae temporally regulate the transcription of the *inc* genes. Prior to global transcription or microarray analyses, Shaw *et al* utilized reverse-transcriptase polymerase chain reaction (RT-PCR) to categorize gene expression according to three temporal classes, such as early, mid-cycle, or late gene expression (147). This work was expanded by Belland *et al* when their group performed microarray analyses of *C. trachomatis* D/UW-3 at different time points during the developmental cycle (18). Their data revealed that approximately a third of the 28 genes upregulated at 1h pi are *inc* genes. At 3h pi, 18 out of the 200 upregulated genes are newly expressed *inc* genes. The last 14 *inc* genes are expressed during the mid-cycle (8-12 h pi) and late (16-24 h pi) time points. The early expression of the majority of *inc* genes suggest that Incs are required for the early developmental cycle, perhaps for the remodeling of the inclusion membrane to evade the endosomal and lysosomal vesicle trafficking pathways. For this reason, Scidmore *et al* characterized some of these early expressed Incs, such as the *inc*D-G cluster (146).

Another example of an early transcribed *inc* is CT147 (18). It is a noteworthy example because it shares significant structural homology with EEA1, a mammalian Early Endosomal Antigen-1 involved in mammalian cell endosome fusion and trafficking and interacts with phosphatidylinositol 3-phosphate and Rab5 GTPase (152). Although CT147 does not have the region necessary for Rab5 interaction,

Belland *et al* hypothesize that CT147 may function in tethering but not fusing with endosomes and, subsequently, with the lysosomes. These results co-incide with the previous findings that while the nascent chlamydial inclusion associate with early endosomes, they do not fuse with each other. This is also an example of host protein mimicry that could interfere with normal host processes and chlamydial interaction with the host endosomal pathway. In addition, CT147 contains the hydrophobic domain motif, characteristic of most Incs with a unique arrangement, such that two *incA*-like genes are connected head-to-tail (7). Its amino acid sequence has a similar hydrophobic profile to another Inc that is expressed early during the infection, CT288 (7). Both Incs localize to the IM in infected cells.

Transcription analysis was also performed in infected samples that were treated with interferon-gamma (IFN-γ) (17). Treatment with this cytokine induces aberrant chlamydial growth via depletion of cytosolic tryptophan pools. Upregulation of several candidate *inc* genes resulted from this treatment including genes that were turned on early, *inc* D-G and CT288, CT345, CT358, and CT434 (17). It is hypothesized that these Incs support chlamydial growth and maintenance of the inclusion during stressful conditions, such as when the host immune system launches an assault on the pathogen.

1.4.2. SECRETION OF INCS TO THE INCLUSION MEMBRANE

Because chlamydiae encode a complete set of genes for the type III secretion (T3S) pathway and Incs do not contain a signal sequence, it was hypothesized that Incs are possibly secreted by and effectors of this pathway (47). One of the ways this

relationship was investigated was by using a heterologous secretion system, whereby Incs are subjected to recognition and secretion by Shigella flexneri T3S (160) or Yersinia T3S (47). Subtil *et al* examined the ability of S. flexneri T3S to recognize the amino-terminal sequences selected from known T3S substrates and Inc proteins for secretion (161). Subtil *et al* determined that six of the eight candidate Inc proteins that they tested were recognized and secreted by the S. flexneri T3S system. These include IncA, IncB, IncC, CP0026, CP0308, and CP0585. Two proteins putative Inc proteins (CP0146 and CP0367) were not secreted by the heterologous system. It may be that other this system does not recognize these two proteins as substrates or other factors may be required, not provided by this system, for secretion.

An interesting paradox in chlamydial development was the detection of early expressed Incs, such as IncC and IncG, while the expression of T3S components occurred during the mid to late developmental stages. Fields et al attempted to resolve this issue by examining if type III components are detected in EBs (47). They used matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and immunoblot analyses of purified EB extracts and detected the core apparatus component CdsJ in both EB and reticulate body (RB) extracts. By immunobloting whole infected-culture extracts and indirect immunofluorescence of infected monolayers, CdsJ was detected prior to expression of *cdsJ* mRNA. They also used a surrogate type III secretion system, *Yersinia pseudotuberculosis*, to determine if early expressed Incs are recognized by the type III apparatus. They determine that early Incs are secreted by type III secretion system and, therefore, suggests that functional chlamydial T3S pathway is present at early stages prior to EB differentiation to RB.

1.5. THE CHLAMYDIAL INFECTIOUS PROCESS AND INTRACELLULAR DEVELOPMENT

There are at least seven general developmental steps involved in chlamydial development: (a) attachment and entry, (b) primary differentiation or the EB to RB transformation, (c) RB cell division, (d) RB multiplication, (e) secretion of chlamydial effectors, (f) secondary differentiation or the RB to EB transformation, and (g) the release of EB to initiate re-infection (1).

1.5.1. ATTACHMENT AND ENTRY OF ELEMENTARY BODIES

Chlamydiae have the ability to successfully infect phagocytic as well as non-phagocytic host cells. This versatility suggests that chlamydiae have conserved adhesins as well as host receptors that these adhesins recognize. Currently, however, no single adhesin or host receptor for attachment or entry has been identified. However, candidate adhesins such as OmpA, OmcB, and Hsp60 have been proposed. Also, C. pneumoniae PmpD is another candidate adhesin (179).

Although chlamydial attachment events are known to occur differently between different chlamydial species and serovars, a general two-step attachment procedure has been proposed (154, 157, 158, 167). First, chlamydiae attach by reversible binding to host heparan- sulfate containing glycosaminoglycans or GAGs via the chlamydial OmpA protein. Second, an irreversible, temperature dependent but heparan-sulfate independent binding stage was demonstrated by use of D4.1-3 cells, a mutant Chinese hamster ovary (CHO) cell line. This permitted the reversible heparan-

sulfate dependent binding of *C. trachomatis* L2 EBs, but inhibits the irreversible heparan-sulfate independent binding step (26). This suggests the possibility that different chlamydial species and serovars use a variety of host cell receptors. However, putative secondary host receptors have not been characterized.

Following the irreversible binding step, a TTSS exported protein, Tarp (Translocated actin-recruiting protein, CT456), is exported into the host cell and rapidly tyrosine- phosphorylated at sites of actin recruitment (32, 33, 79). Tarp is detected in the EB cytoplasm prior to nucleoid decondensation and, subsequently, RB development. This suggests that EB have the ability to facilitate secretion, via type III secretion system, of signaling molecules into the host that may be critical to successful attachment and entry.

1.5.2. THE EB TO RB DIFFERENTIATION

During development, some key events occur to allow the differentiation of an EB to an RB. Morphological observations for such events include a diffusely granular the cytoplasm with a less condensed chromosome, an enlarged EB to approximately three times its original size (~ 1μm), and the presence of actively dividing bacteria (99). Additionally, the surface projections previously observed in EB are also observed in greater density in RB (99).

Molecular mechanisms involved in the EB- to- RB differentiation largely focused on the interaction between the DNA of EBs and the two histone H1 homologs, Hc1 and Hc2 (19, 56, 118, 166). While Hc1 is highly conserved among all chlamydiae, Hc2 is either absent or variable in most chamydial species and strains

(59). By using an *E. coli* expression system, transformation of either histone homolog resulted in a condensed *E. coli* nucleoid structure, suggesting functional roles in the maintenance of nucleoid compaction (13, 19). In addition, expression of these two proteins resulted in a global decrease of transcription and translation, mimicking events that occur when RB differentiate back to EB (12, 116). Recently, Grieshaber *et al* demonstrated that chlamydial histone homolog and EB DNA interactions are disrupted by 2-C-methylerythritol 2,4- cyclodiphosphate, a metabolite that participates in a non-mevalonate pathway (MEP) of isoprenoid biosynthesis and is an antagonist to HctA or Hc1 (53). This unexpected result provides the first molecular clues to the process of EB to RB differentiation.

In addition to chromosome changes and an outer membrane with a less rigid structure, the chamydial chromosome is poised to initiate early chlamydial gene expression. Belland *et al* used a transcription microarray analysis to demonstrate that at least 29 immediate- early genes are transcribed as EB transform into RB (18). Among these genes are those that encode Inc proteins such as *inc*D, E, F, and G, *euo*, and chaperone genes *gro*EL and *gro*ES. Other genes encode proteins that function in metabolite translocation into the bacterial cell and enzymes that participate in metabolic pathways.

Common to all prokaryotic cell division by binary fission is a set of Fts (filamentation temperature sensitive) proteins that facilitate septum formation (132). One of these proteins is FtsZ, a septation- associated protein that localizes to a ring-like structure at the plane of cell division. Surprisingly, the chlamydiae are one of the few microbes that lack an *ftsZ* ortholog in the genome. It has been suggested that RB

may synthesize small amounts of chlamydial peptidoglycan to compensate for the lack of FtsZ during the formation of the nascent division septa (30). Brown et al demonstrated that a unique antigen, termed SEP, (for septum) is localized to a ring-like structure in close proximity to the plane of RB division, an intracellular distribution resembling that of FtsZ in other bacterial species (20).

1.5.3. INTERACTION WITH THE HOST: THE ENDOSOMAL OR LYSOSOMAL PATHWAY

Once EB are internalized, chlamydial modification of the inclusion occurs to render them abilities to avoid fusion with endosomes (early or late) and lysosomes (69, 145, 168, 173). Within the host cell, the compartment in which it initially occupies is derived from the host plasma membrane and can be classified as an early endosome. However, upon closer investigation, there are some differences between this chlamydial compartment and a typical endosome.

First, in this compartment, the endosome pH is not dramatically acidic and retains the endosomal Na+/K+ ATPase (141). In support of this, the chlamydial inclusion does not fuse with lysosomes and is not acidified, as lysosomal markers such as LAMP1/2 and acid phosphatase are not observed inside or around the chlamydial inclusion (49, 68). Additionally, inhibitors of vacuole acidification such as methylamine, ammonium chloride, bafilomycin A1, or chloroquine did not have effect on *C. trachomatis* viability and replication but did restrict growth of *Coxiella burnetii*, a bacterial pathogen occupying a vacuole that fuses with lysosomes (64, 69). However, LAMP1 associates with chlamydial inclusions when dendritic cells or

monocytic cells are infected with *Chlamydia* (113), suggesting perhaps that in these cells, chlamydiae utilize alternative entry pathways that are different from epithelial cells and facilitate a rapid phagosomal/ lysosomal maturation.

Second, the avoidance of chlamydial fusion with the lysosomes may also be attributed to the maintenance of intracellular calcium levels at homeostatic concentration. In this model, annexins III, IV, and V associate with endosomal membrane phospholipids, prevent the chlamydial vacuole from fusing with lysosomes, and enhance fusion between chlamydial vacuoles (96). The inclusion is also not fusogenic with the ferritin-labeled secondary lysosomes (186).

Third, it is not fusogenic with early endosomes as endosomal markers such as the early endosome antigen-1 (EEA1), transferrin receptor and the cation- independent mannose 6-phosphate receptor are not associated with the chlamydial inclusion (69, 143, 145, 168). Transferrin is observed in close proximity to the nascent inclusions but do not fuse with the inclusion membrane (143). The inclusion is also not fusogenic with late endosomes, as late endosomal markers such as cathepsin D, the vacuolar H+-ATPase (69), or Rabs 5, 7, and 9 (135) do not localize to the vacuole surface.

Rab GTPases are examples of a family of proteins that are either recruited to or excluded from the chlamydial inclusion. While most of these proteins are primarily involved with regulation of membrane trafficking, some are involved in receptor recycling, retrograde trafficking, and endosome maturation (38). These proteins regulate membrane traffic in a step-wise fashion as they cycle between a cytoplasmic, GDP-bound and a membrane- associated, GTP- bound states. This regulation includes the facilitation of interaction between transport vesicles at donor membranes and

functions in SNARE recruitment and vesicle tethering by recruiting effector molecules (38).

Rzomp et al transfected cells with GFP (green fluorescent protein)- tagged Rabs, infected cells, and used immunofluorescence to characterize intracellular localization of GFP- tagged Rabs in infected cells (135). From this work, they identified 4 GFP- tagged Rab: Rab1, Rab4, Rab6, and Rab11, to be associated with the *C. trachomatis* inclusion, forming a rim-like staining pattern that appeared similar to the classic chlamydial Inc protein inclusion membrane label. Rab4 and Rab11 are pericentriolar- associated Rab proteins and chlamydiae are known to traffic and develop in the pericentriolar region of the host cell while Rabs 1, 6, and 10 are Golgi-associated Rabs. The co-localization of the Rab11 with the chlamydial inclusion is independent of microtubules. (135).

However, it appears that Rab 4 and Rab 11, markers of the recycling pathway, were localized to the chlamydial inclusion at early time points post- infection (135). Fluid-phase markers such as lucifer yellow are not detected inside the inclusion (69).

In host cells, vesicular fusion is regulated by the identity of docking vesicles and their target sites. The specificity is conferred by the interaction between v-SNARES and t-SNARES, integral membrane proteins of vesicles and their targets, respectively (133). These proteins interact on opposing membranes and form a complex to initiate fusion. This interactive complex recruits soluble protein effectors, *N*-ethylmaleimide sensitive factor (NSF) and soluble NSF attachment protein (SNAP) to facilitate membrane fusion between the vesicles and their targets. The Rab family of GTPases are regulators of vesicle fusion and are involved specifically in regulating,

positively or negatively, the formation of the SNARE complex and membrane fusion (110, 140). In vesicles that mature to terminal lysosomes, the acquisition of Rab proteins are sequentially altered such that some of the markers are no longer detectable (38), possibly a mechanism to evade lysosomal fusion.

The localization of these proteins to the chlamydial inclusion suggests a possible chlamydial- host interaction that may be necessary to for development. Chlamydiae may utilize these signaling proteins to sequester host proteins from normal processes and/ or enable the inclusions to traffic to the intracellular location suitable for optimal development.

1.5.4. INTERACTION WITH THE HOST: THE MULTIVESICULAR BODIES (MVB)

Recently, Beatty identified and characterized the interaction between the chlamydial inclusion and late endocytic multivesicular bodies (MVB) and demonstrates that this interaction is critical to chlamydial development (16). The MVB represent complex organelles, situated along the endosome- lysosome pathway, also intersect the exocytic pathway in a variety of cells. Immunolabeling and confocal microscopy demonstrated that CD63, a protein found in MVB, is found within the inclusion. This represents the first identified host protein found associated with the inner leaflet of the chlamydial inclusion, with direct access to the chlamydiae, and represents a novel pathway of lipid delivery to chlamydiae.

1.5.5. INTERACTION WITH THE HOST: THE AUTOPHAGIC PATHWAY

Al-younes et al hypothesized that the ability of chlamydia to obtain soluble metabolites or nutrient sources that are essential for replication may be attributable to its interaction with the autophagocytic pathway (2). Autophagy is a homeostatic process by which biosynthetic precursors are released from degraded endogenous cellular components (156). While no specific autophagosomal markers (monodansylcadaverine/ MDC) were detected in the chlamydial inclusion, other autophagosome-associated markers such as microtubule associated protein light chain 3 (MAP-LC3) and calreticulin were localized in close proximity to the chlamydial inclusion. Also, addition of exogenous autophagic inhibitors such as 3-methyladenine (3-MA) severely inhibits chlamydial development and disease progression.

1.5.6. INTERACTION WITH THE HOST: THE CYTOSKELETON

Much of the research regarding chlamydial interaction with the host cytoskeleton has been focused largely on chlamydial entry and trafficking to the peri-Golgi region. Microfilament and microtubule function inhibitors, cytochalasin D and vincristine or vinblastine, respectively, also inhibit chlamydial entry (177). Microscopy was utilized by Campbell *et al* to view host cytoskeletal structures that were stained with fluorescent probes in infected cells (25). They noted that the major actin- containing stress fibers do not co-localize with chlamydial inclusions and, instead, are most likely observed with the peri-basal or peri- apical location of the host cell. They also noted that the microtubule network was also distorted in that the nucleus and the inclusions were surrounded by separate rather than a joint network of

microtubules. Disruption of these cytoskeletal networks and subsequent reversal of this disruption did not disturb the separate arrangement between the inclusion and the nucleus.

Schramm *et al* demonstrated that chlamydial response to cytoskeletal inhibitors is serovar- specific (142). While the disruption of cytochalasin D significantly reduced production of *C. trachomatis* serovar E infectious progeny, it did not produce the same effect with *C. trachomati*s serovar L2. In contrast, microtubule inhibitors did not affect serovar E development but produced multiple inclusions with serovar L2 infections that displayed a delayed fusogenic development. These results suggest that chlamydial utilization of the cytoskeletal network is serovar specific and may contribute to their tissue tropism.

Intracellular trafficking of chlamydial inclusions requires an intact microtubule network (31). The co-localization of microtubules with chlamydial inclusions was observed during entry and its migration from the entry site to the perinuclear location at the center of the microtubule network. Inhibitors of dynein, a (+) directed microtubule- associated motor protein results in significant disruption in chlamydial development and infectivity. While dynein co- localizes with *C. trachomatis* inclusions, this association is not observed with *C. pneumoniae* inclusions, suggesting differential cytoskeletal requirements.

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microtubule- associated motor protein results in significant disruption in chlamydial development and infectivity. While dynein co-localizes with *C. trachomatis* inclusions, this association is not observed with *C. pneumoniae* inclusions, suggesting differential cytoskeletal requirements. However, Coombes *et al* determined that an intact actin polymerization is required for *C. pneumoniae* entry (34). In type II pneumocytes, *C. pneumoniae* infection alters microfilament network and modifies uptake, trafficking, and secretion of surfactant (181).

Recently, Grieshaber *et al* determined that chlamydial trafficking from the cell periphery to the microtubule- organizing center (MTOC) is dependent on host cell dynein and independent of dynamitin, a dynein- associated protein (55), suggesting a novel interaction between chlamydia and the host cell cytoskeleton. They also demonstrated that once chlamydia has migrated to the MTOC, it maintains stable association with cellular centrosomes, affects chromosome instability, and effects chromosomal segregation defects (56).

In addition, not only do chlamydiae modify actin and microtubules, they also affect intermediate filament structure. Chlamydial protease/ proteasome-like activity factor or CPAF cleaves keratin 8, an intermediate filament component, as cleavage fragments of keratin 8 are detected in the soluble fraction of chlamydia- infected cells (39).

Chen *et al* recently demonstrated that exogenously expressed CT813, a chlamydial hypothetical protein, in HeLa cells localized to a cytoskeleton-like structure that is similar to intermediate filaments, is an inclusion membrane protein and immunogenic (28). While these structures do not overlap with the intermediate

filaments, they suggest that this protein may be able to polymerize or associate with the cytoskeleton.

Finally, Hackstadt *et al* recently characterized a chlamydial effector that recruits actin filaments at the site of entry, TARP or translocated actin recruiting phosphoprotein, an actin nucleator that does not share homology with other bacterial actin nucleators, such as SipC (67, 79). While TARP is present in all pathogenic Chlamydia species, only the TARP from *C. trachomatis* is phosphorylated. TARP-induced actin filaments associate with activated Arp2/3 during entry and this forms the structure that alters the cytoskeleton to promote chlamydial invasion.

1.5.7 THE RB TO EB DIFFERENTIATION

During the later stages of development, RB differentiate back into EB in preparation to leave the host cell and propagate the infection process. While the molecular signals involved in triggering this differentiation are not fully characterized, previous work and observations provide some clues. First, early micrographs demonstrated that during the asynchronous development, EB are most likely found concentrated in the lumen of the inclusion while RB are usually associated with the inclusion membrane (98). Hackstadt and others suggest this association is essential because RBs need to acquire energy and nutrients from the host cytosolic pools (60). Recently, Wilson et al observed that RBs were tightly associated with projections, thought to be type III secretion (T3S) injectosomes (99) and proposed a mathematical model whereby an increase in binary fission causes a decrease in space inside the inclusion to accommodate accumulating RB (180). This also decreases contact

between the RB, the inclusion membrane, and the injectosome. They hypothesize a contact- mediated activation of RB differentiation such that once the RB detaches from the inclusion membrane, it is signaled to transform into EB. Although these observations are not in agreement with a temporally regulated life- cycle, it does offer a plausible model to characterize the asynchronous nature of chlamydial development.

Additionally, the expression of late-cycle genes, such as *omcA* and *omcB* are involved in initiating the secondary differentiation. Recently, microarray experiments have identified 26 additional late genes that include *hctAB, ltuB,* and *lcrH.1*, most of which are involved in chromosome condensation (18). Proteins encoded by the genes, CT780, CT783, *mtpA*, and *mtpB*, are predicted to be involved in the formation of the highly cross-linked disulfide outer membrane complex. Additionally, components of the TTSS, such as LcrH.1, are also expressed to equip the new EB with genes necessary for the next infectious cycle.

1.5.8 INTERACTION WITH THE HOST: THE EXOCYTIC PATHWAY

The utilization of the fluorescent analog of ceramide, 6-[*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl) aminocaproyl sphingosine] (C6-NBD-ceramide) contributed significantly to the study of chlamydial association with the exocytic pathway (61, 62, 126, 184). C6-NBD-ceramide, a fluorescent ceramide analog with a short-chain fluorescent molecule substituting a long- chain fatty acid, was developed to study lipid trafficking and the Golgi apparatus in live cells (90, 114, 131). Exogenous C6-NBD-ceramide readily traverses through membrane bilayers because it is an uncharged

molecule. Ceramide is processed into sphingomyelin or glucosylceramide in the Golgi apparatus. In contrast to exogenous NBD-ceramide, exogenously added NBD-sphingomyelin is not delivered to the inclusion but is retained in the plasma membrane (61).

Sphingomyelin has an added charged phosphocholine head group and does not readily cross membrane bilayers. Therefore, its incorporation to target membranes must be in the correct orientation. The advantage, however, of the NBD-ceramide (NBD-cer) analogs is that its intercalation into the membranes is not permanent. It can be removed from the plasma membrane by the interaction with another lipid acceptor, such as bovine serum albumin, so that the intracellular probe labeling may be visualized (60). At 4°C, NBD-cer integrates itself into virtually all cell membranes found in the plasma membrane and organelles including the mitochondria, Golgi apparatus, endoplasmic reticulum, and the nuclear envelope (60, 90). At 37°C, however, NBD-cer accumulates, in aldehyde fixed cells, at the Golgi apparatus. In infected cells, continued incubation at 37°C, NBD-cer, the fluorescent probe is observed inside the chlamydial inclusion as incorporated into the chlamydial cell walls. This labeling persists during the chlamydial growth and is not observed to be exchanged or transported (62).

Purified EB extracted from NBD- ceramide labeled infected cells contain labeled sphingomyelin, the Golgi- processed by-product of ceramide (62). In addition, both EB and RB forms contain approximately 1-4% sphingomyelin of the total lipid in their cell walls, a lipid component unusually found in prokaryotic membranes (106, 185). Because the delivery of sphingomyelin from the trans-Golgi to the plasma

membrane is mediated by an exocytic vesicle, a model is proposed whereby the delivery of sphingomyelin to the chlamydiae is mediated by a fusion event between the chlamydial inclusion and the exocytic vesicle carrying sphingomyelin (60).

To distinguish between the transport of sphingomyelin to the plasma membrane or to the chlamydial inclusion, investigators designed experiments that would disrupt normal sphingomyelin transport. First, cellular ATP levels were depleted by addition of sodium- azide and 2-deoxyglucose, or infected cells were incubated at a lower temperature (20°C). Under these conditions, no sphingomyelin accumulated in the inclusion; instead, it was delivered to the Golgi apparatus. Disruption of the Golgi apparatus or mitochondria, by treatment of infected cells with brefeldin A or monensin, also inhibited sphingomyelin delivery to the inclusion (61, 184). This blockage resulted in a decreased inclusion size, but the abundance of infectious progeny was similar to untreated infected cells (61). However, the inhibition of serine palmitoyltransferase, an essential enzyme for sphingomyelin production, also blocked *C. trachomatis* development (175), suggesting that sphingomyelin availability is required for chlamydial growth and development. Collectively, this information suggests that the chlamydial acquisition of sphingomyelin is dependent on time, temperature and energy, similar to requirements of sphingomyelin delivery to the plasma membrane of uninfected cells (82, 131). However, the host pathway involved in trafficking sphingomyelin to the chlamydial inclusion remains unclear.

Other lipids that chlamydiae acquire from the host are cholesterol and glycerophospholipids. Cholesterol and sphingomyelin are observed to co-exist in eukaryotic cell membranes (74) and cholesterol is delivered to the chlamydial

inclusion via the Golgi apparatus (27). Wylie et al utilized radiolabeled isoleucine as a glycerophospholipid tracer to demonstrate that modified glycerophospholipids are trafficked to the chlamydiae (185). Furthermore, they reported that chlamydial-derived lipid precursors are responsible for this modification.

The observation that the chlamydial inclusion is situated distally to the trans-Golgi region in the pathway of exocytic vesicles, suggests a model whereby the chlamydial inclusion is an altered or aberrant exocytic vesicle that is capable of interacting with other exocytic vesicles (61).

While the pathway that traffics the lipids to the inclusion and the regulation of that pathway are largely unknown, various proteins that participate in the exocytic pathways were examined for chlamydial association. It has been demonstrated that chlamydial's interception of vesicles carrying sphingomyelin does not disrupt glycoprotein export. Lectin probes for glycoproteins do not label the incluson membrane (61). Additionally, three proteins, vesicular stomatitis virus glycoprotein G, transferrin receptor, and human major histocompatibility class I antigen demonstrated no disruption for post- translational processing and export in *C. trachomatis*- infected cells (145). While most of these proteins have been studied in non-polarized cells, their regulation in polarized epithelial cells is important and may be different than what has been observed (188).

1.5.9. CHLAMYDIAL RELEASE FROM HOST CELLS

Although the completion of chlamydial intracellular development is different between species, it is typically observed at 36 h but it may be longer. As currently

observed, there are at least two mechanisms utilized by chlamydia for its release: host cell lysis or non-lytic mechanisms when the chlamydial inclusion fuses with the plasma membrane and releases its contents extracellularly. Host cell lysis is general observed with *C. psittaci* and LGV strains of *C. trachomatis* but there are certain strains of *C. trachomatis* that facilitate fusion of their inclusions with the host cell plasma membrane. Still, there may be other ways in which this process occurs as a cytotoxin gene exists in some chlamydial genomes (18). While a seemingly simplistic process, the molecular mechanisms involved with this event are largely uncharacterized.

1.6. CONTEMPORARY APPROACHES TO STUDY INC FUNCTIONS

Chlamydiologists who are examining the function of Incs are faced with the problem that a stable genetic system for introduction of gene and/or function knock-outs is not yet possible in the chlamydial system. Therefore, many investigators have resorted to lesser direct and more creative approaches. One such approach is the utilization of the yeast-two hybrid system, used by Scidmore et al, to determine host proteins that interact with chlamydial proteins (144). From this work, it was demonstrated that chlamydial IncG interacts with 14-3-3β, a host cell protein that functions as an adapter or scaffold protein to carry out complex phosphotransfer processes for proteins that participate in many signaling pathways. The interaction between IncG and 14-3-3β is specific in that mutation of amino acids that are components of the conserved binding motif disrupts the binding specificity. This

study suggests that chlamydial Incs are capable of diverting important host signaling molecules from their designated pathways.

Many investigators have characterized the function of IncA by utilizing different methodologies. One such group utilized microinjection methods (63). From their work, it was determined that the carboxy- terminus of IncA is exposed to the host cell cytosol, formed dimers, and that micro-injection of anti-IncA antibodies resulted in multiple, non-fused inclusions in multiply infected cells.

Other investigators expanded on the idea that the function of IncA can characterized by disrupting its secretion. Fields et al demonstrated that *Chlamydia*-infected cells propagated at 32 °C appear to have reduction of IncA expression within the IM and suggests that the introduction of this lower temperature creates an environment whereby the secretion of IncA is blocked (44). In support of this, Van Ooij et al initially the reduction of IncA expression and localization is responsible for the reduction in the rate of fusion of inclusions (174). Recently, several investigators have expanded on these findings by demonstrating that a drug, INP0400, that inhibits the type III secretion apparatus of *Y. pseudotuberculosis* (104). Treatment with this drug of *Chlamydia*- infected cells inhibits intracellular replication, resulting in small inclusion bodies that contain only one or a few RB. INP0400 given at high concentration prior to partially blocked entry of elementary bodies into host cells. Early treatment also reduced the amount of 14-3-3β recruitment to the IM as IncG secretion to the IM via a T3S mechanism was blocked. IncA, another T3S effector blocked by INP0400, demonstrated reduction in expression and resulted in non-fusion of *Chlamydia*- filled vesicles in multiply infected cells. This drug also caused

premature RB detachment from the inclusion membrane when infected cells were treated during the late developmental stage. While this event is thought to signal RB to EB differentiation (180), drug treatment also reduces infectious particles and infectivity.

Still other investigators have used the approach of expressing Incs in eukaryotic cells to determine their host targets within the infected cells. Recently, Valdivia *et al* devised a homologous recombination-based cloning strategy to express all Chlamydia-specific genes in *Saccharomyces cerevisiae* strains (149). Chlamydial proteins that demonstrated defects in various yeast cellular functions or tropism towards and localization to eukaryotic organelles were identified. In addition, they were also able to identify bacterial factors secreted into the host cell (169). Included in their analyses are 33 putative Inc proteins (only the cytoplasmic domains) and other putative virulence proteins with homology to other bacterial virulence factors. However, because full-length Inc proteins were not expressed in their assays because of their toxicity in yeast, they were not able to demonstrate the effects of Incs in yeast cells. Instead, they expressed these Incs on the surface of yeast endosomes by artificially anchoring the soluble domains of Incs to the transmembrane domain with localization signals of the endosomal SNARE protein Pep12p. In this system, Inc proteins, CT179, CT192, and CT195 caused strong growth defects in yeast suggesting that the close proximity of these chlamydial proteins to membranes influence their function. Although the functions of Inc proteins are not elucidated by this system, they were able to identify other chlamydial proteins that were exported from the

inclusion that influence yeast organelle function or demonstrate tropism to specific eukaryotic organelles such as the mitochondria, the nucleus, and lipid droplets (83).

In addition, Alzhanov *et al* used transfection assays to express *incA*, *incB*, and *incC* in mammalian cells and examine host cell phenotype in the presence or absence of chlamydial infection (4). Expression of *C. caviae* IncA blocked *C. caviae*- filled inclusion formation in cells or produced aberrant inclusion development. However, this ability to block inclusion formation is, thus far, specific to *C. caviae* IncA (4). By utilizing deletion mutagenesis, these studies were able to demonstrate the amino acid residue that is a site of phosphorylation in IncA. Another group of investigators were able to conduct a structural analysis of IncA, which they demonstrated as a tetramer with carboxy termini that is highly coiled-coiled (36).

IncA has also been detected in structures that resemble "fibers" or chains of tiny vesicles emanating from the inclusion or at the margins of the inclusion (21). These fibers are present in cells infected by *C. caviae*, *C. trachomatis*, and *C. pneumoniae*. Under conditions of induced stress, outer membrane proteins such as MOMP or LPS are localized within these fibers. Suchland *et al* expanded this work by attempting to characterize the function of these structures during chlamydial development (163). They used immunofluoresence analysis of mammalian cells infected with a variety of clinical *C. trachomatis* strains. Their work demonstrated that each strain produced different numbers of these fibers. They also discovered that infection by serovar G produced the most fibers and high numbers of vacuoles laden with IncA. These vacuoles are termed "secondary inclusions" because, while IncA can be localized to the IM of these vesicles, they do not contain chlamydial forms. A

model is proposed whereby the primary inclusion utilizes these IncA- laden fibers to deliver RB to these secondary inclusions (163). Eventually, these RB proceed to develop inside these inclusions. As a result, the infected cell now contains two chlamydia- filled inclusions that initially began with one. This suggests that perhaps this mechanism is involved in a novel way of propagating infection. When the host cell divides, this mechanism may distribute other inclusions to daughter cells. Another Inc localized to the fibers is IncG; it is not detectable in secondary inclusions devoid of chlamydial forms (146)

Starnbach et al investigated Inc functions in the context of their ability to be recognized by the immune system (48, 151). In their assays they used a C. trachomatis genomic DNA expression library to identify two chlamydial proteins, CT529 or Cap1 and CT442, characterized as H-2D^{b} –restricted CD8^{+} T cell antigens. Both chlamydial proteins associate with the inclusion membrane and CrpA has a hydrophobic domain typical of most Incs. In contrast, Cap1 does not have the hydrophobicity profile of Incs; instead, it was shown to associate with host actin fibers (21). The induction of CD8^{+} T cells specific to Cap1 or CrpA, as a result of vaccination in mice, confer partial protective immunity. Nevertheless, the recognition of these Incs by the immune system suggest that they are likely targets for CD8^{+} T cells and may be useful in vaccine design.

Another way to study Inc functions is by examining clinical isolates in which a phenotype is associated with a genotype. Chlamydial inclusions exhibit homotypic vesicle fusion, a process that allows them to fuse with other chlamydia-filled inclusions (9). Multiply infected cells develop and replicate within multiple vacuoles

that remain non-fused until approximately 10 hpi, as observed in *C. trachomatis*, at which point the individual vacuoles fuse to generate a large composite chlamydia-filled vacuole (63). Inclusions filled with different chlamydial serovars have also been shown to fuse (100, 125). However, cells multiply infected with different Chlamydia species occupy separate vacuoles and are not known to fuse throughout the course of infection. Some chlamydial strains such as *C. caviae* and some *C. pneumoniae* strains occupy inclusions that do not fuse into a large vacuole (126, 182).

The discovery of natural mutants from clinical isolates of *C. trachomatis* provided opportunity to study this organism for which no stable genetic system for manipulation exists. Suchland and Stamm *et al* collected approximately 12,000 isolates over a 12- year period from the University of Washington *Chlamydia* laboratory. At least 1.5% of these isolates demonstrated chlamydia- filled inclusions that were non- fusogenic throughout the chlamydial developmental cycle (162). By using anti-IncA antibodies in immunofluorescence, the IM of these strains do not contain IncA on the surface and are designated as IncA- negative strains. This is unusual in that virtually all wild-type strains contain IncA on the IM and cells multiply infected with more than one EB contain vesicles that fuse during the developmental cycle. These isolates were identified in both ocular (serovar B) and genital strains (D-K) and designated with (s) symbol next to the serovar designation to differentiate them from the wild- type strain counterparts (162).

The absence of IncA on the IM prompted Rockey et al to sequence *incA* encoded by these non-fusogenic isolates. These studies revealed that the sequence is altered in each of these isolates. The altered *incA* sequence produced a predicted

protein sequence that is significantly truncated (130). Other non- fusogenic isolates encode *incA* that is otherwise intact; an in-frame deletion of 51 nucleotides is observed. The IncA- negative phenotype may have resulted from the inability of the unknown transport or processing mechanisms to recognize the altered *incA* sequence. Because some IncA- positive also contain identical *incA* sequence mutations, these alterations are likely not responsible for the absence of IncA on the IM and perhaps the non- fusogenic phenotype. In contrast to the sequence within *incA* in non-fusing strains, the upstream sequence is identical to the wild- type fusing strains (Rockey et al unpublished results).

Another unusual phenotype produced by these non- fusogenic strains is that some of these strains encode an intact *incA*, produce detectable IncA on the IM, yet produce non-fusogenic inclusions in multiply infected cells (130). However, when cells are co- infected with this non-fusing isolate and another IncA- positive fusing isolate, fusion of inclusions, housing these different strains, is observed. This suggests that, while IncA may primarily be involved in facilitating fusion, there may be other functions that require further investigation.

The significance of studying these natural mutants is at least two- fold. First, with the lack of stable genetic system to manipulate chlamydiae, these strains provide chlamydiologists with an opportunity to study a detectable phenotype with a known genotype. Second, patients infected with these non- fusogenic strains are more likely to have asymptomatic disease than those infected with the wild type fusogenic strains (50). These patients were also less likely to produce more infectious EB. Thus far,

the lack of IncA on the IM is associated with non-fusogenic inclusions, less severe clinical presentation, and shedding fewer numbers of infectious chlamydiae (50).

1.7 CONCLUSIONS

Although chlamydial research lacks a genetic system to manipulate chlamydiae, significant progress has been made in the last couple of decades in characterizing important molecular events in the developmental cycle of this clinically significant microorganism. Researchers in this field continue to utilize contemporary molecular techniques to investigate chlamydial disease from two perspectives, the host and chlamydia. From this work, early microscopical observations of chlamydial development have since translated into characterization of important molecular mechanisms involved in development and its interaction with the host. Two types of large- scale and high- throughput analyses, namely genome sequencing and host or chlamydial microarrays, have contributed significantly in providing research direction to chlamydiologists that characterize chlamydial biology and pathology. The challenge then becomes the integration of the vast information obtained from these techniques, by discerning between important data from irrelevant background noise, and generating pertinent research to address new and old questions.

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CHAPTER 2

Localization of Host Proteins Adducin and Raf-1 is Altered in *Chlamydia*- Infected Mammalian Cells

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In Preparation for Submission

2.1. ABSTRACT

The chlamydial infectious process causes changes in host protein phosphorylation that reflect alterations in host cell biology. To identify unique host cell proteins whose phosphorylation states changed as a result of chlamydial infection, a large- scale analysis of signaling pathways was performed. Phospho- specific antibodies to common eukaryotic kinases and signaling proteins were used as probes of chlamydia- infected or mock-infected cell lysates. Several differences between mock-infected and infected cells were identified in these analyses. One protein that was altered in *Chlamydia trachomatis*- infected cells, relative to mock-infected cells, was adducin, an actin-capping protein affected by the activity of different host cell kinases. The abundance of both the alpha and gamma isoforms of phosphorylated adducin decreased significantly in chlamydia- infected cells when compared to mock-infected cells. Immunoblot analysis demonstrated that adducin abundance is reduced in the Triton-X-100 soluble fraction of *C. trachomatis*- and *C. caviae*- infected cells, but the total cellular adducin remains unaffected by infection. Fluorescence microscopic analysis of chlamydia- infected cells using anti-adducin probes demonstrated that a fraction of cellular adducin localizes in close proximity to the chlamydial inclusion membrane. Additionally, treatment of infected cells with nocodazole or cytochalasin D, disruptors of microtubule and actin, respectively, does not affect adducin localization to the chlamydial inclusion. These results demonstrate that adducin localization is altered as a result of chlamydial infection and may reflect novel cytoskeletal changes associated with the infectious process.

2.2. INTRODUCTION

Chlamydiae are obligate intracellular bacterial pathogens of humans and animals that initiate and complete their development inside the dynamic host cell environment. Among the clinically important chlamydiae are *Chlamydia trachomatis* and *Chlamydophila pneumoniae* that are associated with sexually transmitted diseases and atherosclerosis, respectively (4, 37, 13).

The biphasic life cycle of chlamydiae is initiated by attachment and entry of its infectious form, the elementary body (EB) (27). Within the host, the EBs transform into reticulate bodies (RB) and divide by binary fission inside a non-acidified vacuole, termed an inclusion, that is non-fusogenic with vesicles of the endosomal pathway (32). The accumulating RB population in the inclusion demonstrates a robust metabolic activity, RNA and protein synthesis, and a decrease in chlamydial infectivity.

Successful chlamydial entry and development may require modulation of the host cell processes such as the regulation of signaling pathways that affect downstream effectors. These downstream effectors may be involved in apoptosis (41), vesicular interactions (32), cytokinesis (12), lipid metabolism (15, 33), host cell immunity (23) and perhaps other processes. Among the chlamydial effectors identified thus far include chlamydial protease activity factor or CPAF (36, 44), a protein shown to cleave host transcriptional factors and chlamydial protein associating with death domains or CADD (38), a protein that interacts with the host apoptotic pathways.

Chlamydiae also modulate host processes by recruitment of host proteins to the inclusion to possibly sequester them from host signaling pathways and for re-direction of cargo- carrying vesicles to the inclusion. Included in the list of host proteins that associate with the inclusion are 14-3-3β (34) and selected Rabs (30). Cargo carrying vesicles that interact with the inclusion include exocytic vesicles that carry sphingolipids and cholesterol and multi-vesicular bodies, organelles that traffic protein and lipids along the endosome- lysosome and exocytic pathways (2, 3, 15).

To characterize the host signaling pathways affected during chlamydial development, an array of antibodies specific to phosphorylation sites of the signaling proteins involved in major eukaryotic signaling pathways were used to probe *Chlamydia*- infected or mock- infected cells. Changes in the abundance of these phosphorylated proteins are detected via a slot- immunoblot assay of the Triton X-100 soluble fractions (TS) extracted from infected and mock- infected cells. TS fractions from mock- infected cells or cells infected with *C. trachomatis* serovar J/ UW-36 or a non-fuser *C. trachomatis* $J_{(s)}$ 893 strain (29) were collected and analyzed. Initially, the main objective for this project was to identify differences, if any, that exist in host responses between cells infected by the wild type J/UW- 36 strains and those infected by the nonfuser $J_{(s)}$ 893 strains. However, the kinome analysis revealed that minimal differences were observed between cells infected by the wild- type and non-fuser strains (data not shown). Instead, the significant differences in host response were observed between mock- infected and infected cells. Changes in abundance of phosphorylated proteins in infected cells that have not been previously reported, such

as phospho-α-adducin^{Ser724} and phospho-Raf-1^{Ser259}, were detected from the kinome assay. The results from this analysis were confirmed and expanded in our laboratory.

We demonstrate that the abundance phospho-α-adducin^{Ser724} and phospho-Raf-1^{Ser259} are reduced in the TS fractions of infected cells. However, phosphorylated -α-adducin or phosphorylated Raf-1 is detectable in the total cellular lysates, suggesting that the subcellular localization of either phospho- protein is altered. Immunofluorescence demonstrated that a fraction of α- adducin is also detectable around the chlamydial inclusions. Disruption of microtubules or actin filaments by treatment of infected cells with nocodazole or cytochalasin D, respectively, does not disrupt the localization of α- adducin around the chlamydial inclusions.

2.3. MATERIALS AND METHODS

2.3.1 Materials. Minimum Essential Medium (Gibco) was supplemented with 10% fetal bovine serum (MEM-10) and gentamicin (0.5μg/mL). Protease and phosphatase inhibitors were purchased from Roche (Indianapolis, IN). Anti-phospho-α-adducin^{Ser724}, phospho-Raf-1^{Ser259}, c-Raf1, and phospho- PKB^{Ser473} (protein kinase B) were obtained from Upstate Biotechnology (Lake Placid, NY). To examine the abundance of total α-adducin, antisera to purified carboxy- terminal recombinant human erythrocyte α-adducin was used (21). Chlamydial Hsp60 antibodies were used to label and visualize chlamydial forms (43). Secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) were purchased from Southern Biotechnologies (Birmingham, AL). Secondary

antibodies that are conjugated to IRdye 800 or IRdye 700, such as goat anti- mouse or goat anti- rabbit, respectively, were purchased from Rockland Immunochemicals (Gilbertsville, PA). To label host cell and chlamydial DNA, a DNA- specific fluorescent lable 4',6-diamidino-2-phenylindole dihydrocholoride (DAPI, 2 μg/ml in mounting medium; Vectashield, Vector Laboratories).

2.3.2. *Chlamydia* and cell culture. *C. trachomatis* strains J/ UW-36, D/UW-3, L2, J(s) 893, *C. caviae* GPIC and *C. pneumoniae* were used to separately infect McCoy cells at a multiplicity of infection (MOI) between 3 and 4. Inocula were diluted in 0.25 M sucrose, 10 mM sodium phosphate and 5 mM L- glutamic acid (SPG) buffer. Mock-infected cells were incubated with (SPG) buffer only. Infected and mock-infected cells were centrifuged at 2000 *x* g for 1 h, followed by removal of the inocula and addition of MEM-10 and cycloheximide (1.0 μg/mL). Cells were incubated for 20-24 h at 37°C in 5% CO_2.

2.3.3. Kinetworks™ Phosphorylation- Site Screen (KPSS) kinome analysis. Protein samples prepared for McCoy cells separately infected with two *C. trachomatis* strains (serovar J/ UW-36 and non-fusing serovar J(s) 893) and mock-infected cell lysates were submitted for the KPSS phosphoprotein assays (Kinexus, Vancouver, Canada). Samples were prepared according to the manufacturer's directions. Briefly, infected or mock-infected cells were homogenized in buffer (20 mM Tris-HCl (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate (pH 7.2), 2 mM sodium orthovanadate, 10mM sodium pyrophosphate, 1 mM

phenylmethylsufonyl fluoride, and Triton X-100), kept at 4°C, and ultracentrifuged. The supernatant, Triton X-100 soluble (TS), cell fraction was obtained and used in a Bradford assay (BioRad) for protein content estimation, and the multi- phosphoprotein assay. Approximately 300 μg of the supernatant, TS protein fraction obtained from each sample was equally divided and loaded onto a 20-lane Immunetics multiblotter. Each lane was probed with at least three primary antibodies from a total of 20 phospho-specific antibodies. The immunoblots were then developed by using a chemiluminescent ECL Plus reagent (GE Healthcare, UK) and signals were detected with a Fluor-S-MultiImager and subsequently quantified with Quantity One Software (BioRad).

2.3.4. Immunoblot analysis of host proteins. Infected and mock-infected cells were incubated at 37°C at indicated time points post-infection. Protein samples used were prepared by two methods: whole cell total protein extraction by SDS-PAGE buffer and cell fractionation. For the cell fractionation, infected and mock-infected cells were homogenized in buffer, sonicated, centrifuged at 13,200 rpm and the supernatant and pellet fractions were separated and collected. Protein content in samples was determined by Coomassie blue stain and/or Bradford assay (Bio-Rad). Protein samples were normalized for total protein and electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose. The immunoblots were then blocked with Odyssey™(Licor, Lincoln, NE) blocking buffer and probed with antibodies to phospho-α-adducin (Ser^{724}), phospho-Raf-1 (Ser^{259}), α-adducin, c-Raf1, phospho-PKB (Ser^{473}), or *C. trachomatis* Hsp60. The blots were then probed with goat anti-

rabbit or goat anti-mouse antibodies conjugated to IRdye 700 or IRdye 800, respectively. After the final washes, the Odyssey™ Infrared Imager (160μm resolution, 0mm offset) was used to scan the membranes.

2.3.5. Plasmid constructs and transfection assays. Human α-adducin (1-737aa) was amplified by polymerase chain reaction (PCR) from pRSFDuet-1 template using Platinum Pfx polymerase (Invitrogen, Carlsbad, CA) with the following primers: Addu*Eco*RI-F1 5'CCG**GAATTC**ATGAATGGTGATTCTCGTGCT GCGGTG 3' and Addu*Not*I-R1 5' ATAAGAAT**GCGGCCGC**T CAGGAGTCACTCTTCTTCTTGCTCTT 3' (Sigma Genosys, The Woodlands, TX). The PCR product was cloned into pCDNA™ 4/HisC (Invitrogen, Carlsbad, CA) using restriction enzymes *Eco*RI and *Not*I to generate α-adducin with N-terminal 6x-His tag. After sequence verification, a single clone was used for transfection experiments. HeLa or McCoy cells grown on sterile glass coverslips were transfected with plasmid by using Lipofectamine 2000™ according to manufacturer's directions (Invitrogen). Transfected cells were incubated for 24 h prior to mock- infection or infection with *C. trachomatis* serovar J/UW-36 or *C. caviae* GPIC. Transfected and infected, or transfected and mock-infected cells were fixed in 4% paraformaldahyde at indicated hours post- infection (h pi).

2.3.6. Nocodazole or cytochalasin D treatment of cells. After 24 h pi, infected- and mock- infected cells were treated with 10 μM nocodazole, 10μM cytochalasin D, or 0.1% dimethyl sulfoxide (DMSO) for 2-3 h at 37°C. Cell fixation with 4%

paraformaldehyde was performed as described above. The cells were then probed with monoclonal antibodies to α- tubulin or FITC conjugated- phalloidin and viewed by fluorescence microscopy.

2.3.7. Immunofluorescence. Cell monolayers that were either infected with *Chlamydia* or mock- infected were fixed at designated hours post infection in 4% paraformaldehyde for 1 h. The permeabilized cells were then probed with primary antibodies to chlamydial Hsp60 and phospho-α- adducin (Ser^{724}) or total α-adducin that were diluted in 2% bovine serum albumin (BSA) and incubated for 1h at room temperature. Following washes with 1X PBS, the cells were then incubated for 1h at room temperature with the corresponding FITC or TRITC conjugated antibodies (Southern Biotechnology Associates). To visualize transfected cells by immunofluorescence, an anti-polyhistidine antibody (Invitrogen) to detect N-terminal His-tagged adducin and, subsequently, its corresponding TRITC- conjugated secondary antibodies were used. All images were viewed by using the Leica DMLB microscope (Diagnostics Instruments, Sterling Heights, MI), captured by using Spot Digital Camera software (Diagnostics Instruments), and processed by using the Adobe Photoshop software (San Jose, CA).

2.4. RESULTS

2.4.1. Kinome analysis of *C. trachomatis*- infected cells.

Three screens were performed for protein samples obtained from mock-infected McCoy cells and cells infected with *C. trachomatis* J/UW-36 or $J_{(s)}$ 893.

Each sample was screened for the phosphorylation status of 20 host phospho-proteins. Table 2.1. shows the results of the screens in terms of the relative fold change in phosphoprotein quantity between chlamydia- infected cells and mock-infected control cells. The band intensity for individual phosphoproteins corresponds to the abundance of each phosphoprotein (Fig. 2.1. and Supplementary Data). For a result to be considered significant, a change of phosphoprotein quantity in a sample relative to mock-infected cells must be greater than 25%, as the manufacturer (Kinexus) suggests that any change less than 25% could be attributable to experimental variation. Several phospho- proteins displayed changes in abundance such as, α- adducin, γ- adducin, cAMP response element binding protein (CREB), extracellular regulated kinase 1 and 2 (ERK 1/2), c-Jun oncoprotein, N-methyl-D-aspartate glutamate receptor subunit (NMDA) NR1, p38 α- MAP kinase, MAP kinase 3/6, ribosomal S6 kinase 1, signal transducer and activator of transcription (STAT) 1 and 3 (Table 2.1.). These observed differences were not considered significant between cells infected by the non-fuser *C. trachomatis* $J_{(s)}$ 893 and wild-type *C. trachomatis* J/UW-36 strains. Instead significant changes were observed between the infected cells and the mock-infected cells.

2.4.2 The abundance of phospho- α- adducin in *Chlamydia*- infected cells

The abundance of two phospho- proteins, phospho- α- adducin and phospho-Raf-1, appeared significantly reduced in the TS fractions of infected cells relative to mock- infected cells and were chosen for further analyses (Fig 2.1.). In Fig. 2.2 A, the

Table 2.1. Expression of phospho- protein abundance in infected cells compared to mock-infected cells.

PHOSPHO- PROTEIN			SIGNAL[a]		
Full Name	Abbreviation	Epitope(s)	Control	Fold Change	
				Wild-type	Non-fuser
α-adducin		S724	1	-8.0	-6.76
γ- adducin		S693	1	-0.54	-1.08
Cyclic AMP response element binding protein	CREB	S133	1	-1.78	-0.51
Extracellular regulated kinase 1	ERK1	T202/Y404	1	0.72	1.00
Extracellular regulated kinase 2	ERK2	T185/Y187	1	0.30	0.70
Mitogen activated protein kinase kinase 1/2	MEK1/2	S217/S221	1	-0.72	0.27
N-methyl D-aspartate glutamate receptor	NR1	S896	1	2.89	2.10
Oncogene JUN	Jun	S73	1	-1.88	-0.75
Oncogene Raf-1	Raf-1	S259	1	0	0
p38 α- mitogen activated protein kinase	p38 MAPK	T180/Y182	1	0.88	1.72
Protein kinase Bα (Akt1)	PKBα	S473	1	0.26	0.24
Ribosomal S6 kinase I	RSK1	T359/S365	1	3.19	2.79
Signal transducer and activator of transcription 1	STAT1	Y701	1	6.33	3.83
Signal transducer and activator of transcription 3	STAT3	S727	1	0.62	0.56
Signal transducer and activator of transcription 5	STAT5	Y694	1	-0.51	-0.68

[a]The quantity of each protein is measured by band intensity collected and averaged at individual scan times before saturation occurred. A value of 1 is set for the control (mock- infected cells). A (+) value indicates an increase in phospho-protein abundance detected; a (-) value indicates a decrease in phospho- protein abundance relative to the mock- infected control.

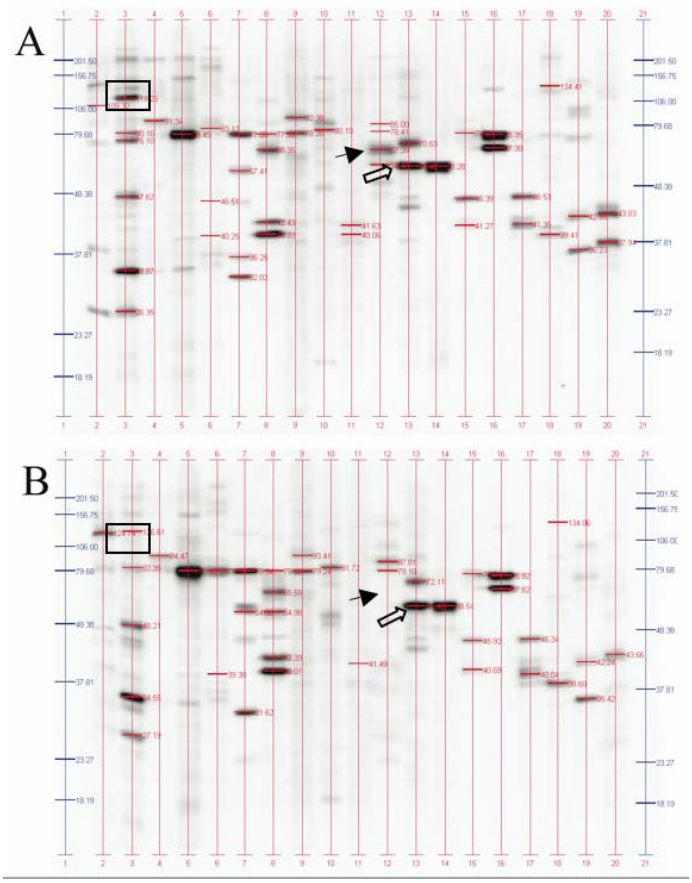

Figure 2.1. Immunoblots represent the multi- phosphoprotein analysis of mock- infected McCoy cells (A) and *C. trachomatis* J/UW-36- infected McCoy cells (B). Each band in the blots represents the accumulated signal for each phospho- protein detected during a given scan time. In each panel, lanes 1 and 21 contain the molecular size standards in kDa. The arrows represent the phospho- proteins discussed in the text such that the black arrow represents Raf1 (S^{259}) in lane 12 and the white arrow represents PKBα (S^{473}) in lane 13. The boxes surround bands corresponding to adducin α (S^{724}) in lane 3. All other phospho- proteins represented in other lanes and not discussed in this manuscript are identified in the Supplementary Material.

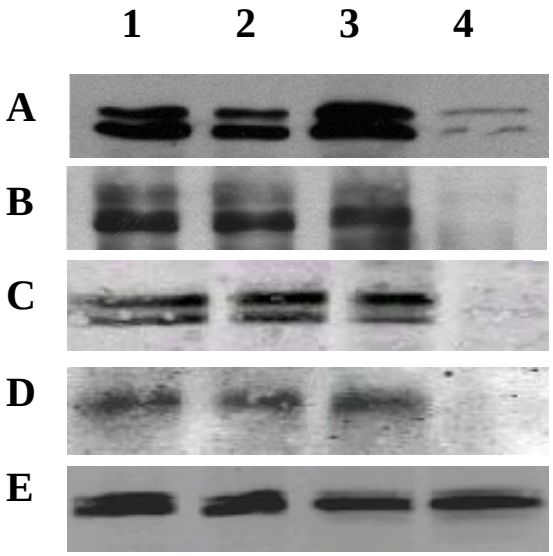

Figure 2.2. Phospho- proteins, α-adducin (Ser^{726}) and Raf-1(Ser^{259}), are depleted in the TS fraction of chlamydia- infected cells. Lanes 1-4 are the same protein lysate samples in panels A-E. Lane representations in all panels are as follows: (1) whole cell protein lysate of mock-infected McCoy cells, (2) whole cell protein lysate of chlamydia- infected McCoy cells (3) TS fraction of mock-infected McCoy cells, and (4) TS fraction of chlamydia- infected McCoy cells. Panel A is probed with anti-phospho- α -adducin (~120 kDa). Panel B is probed with anti- α -adducin (~100 kDa). Panel C is probed with anti- phospho-Raf1 (77 kDa). Panel D is probed with anti- cRaf1 (70 kDa). Panel E is probed with anti- phospho-PKB (66 k Da).

immunoblot demonstrates that phospho- α- adducin is reduced in the TS fractions of cells infected with *C. trachomatis* serovar J/UW-36 relative to the TS fractions of the mock- infected cells. However, the abundance of total cellular phospho- α-adducin of infected cells is unchanged when compared to mock-infected cells. The significant depletion of phospho- α-adducin in the TS fractions of infected cells verifies the results obtained from the KPSS assay (Supplementary Data). Fig. 2.2 B demonstrates that the depletion of phospho- α- adducin in the TS fractions of infected cells is associated with a reduction in total α-adducin and not a function of differential phosphorylation at position Ser724, as the abundance of total α- adducin is also markedly reduced in the TS fractions of infected cells relative to the TS fractions of mock- infected cells. The abundance of another host signaling protein, phospho-Raf1, and total c-Raf1 were also reduced in the TS fraction of *C. trachomatis* serovar J/UW-36 infected cells (Fig. 2.2. C and 2.2. D) relative to mock- infected cells.

Protein lysates obtained from McCoy or HeLa cells infected with other *Chlamydia* strains, such as *C. trachomatis* serovars D/UW-3, L2, or J(s)893 as well as *C. caviae* GPIC were also probed with antibodies to total α- adducin (results not shown) and phospho- α-adducin in the immunoblot assays. In the TS fractions of these infected cells, the abundance of phospho- α-adducin is also depleted (Fig. 2.3. D) relative to the TS fractions of the mock- infected cells. However, the total cellular phospho- α-adducin (Fig. 2.3. B) and α-adducin (results not shown) abundance remain unchanged relative to the mock- infected cells.

To evaluate if chlamydial infection is required for the reduction in phosphoprotein abundance, mock-infected or *Chlamydia*- infected cells were treated with tetracycline. The abundance of α-adducin in the TS fractions of mock- infected or infected cells appeared virtually the same (Fig. 2.4. B). However, in infected cells untreated with tetracycline, α-adducin is depleted as expected (Fig. 2.4. A). In addition, the abundance of phospho- α- adducin the TS fractions of infected cells treated with tetracycline is similar to the mock- infected cells (results not shown).

Temporal analysis of phospho- α-adducin expression in the TS fractions of infected and mock-infected cells was performed. Protein lysates were collected at 6, 12, and 24 h pi. The depletion of phospho- α-adducin in the TS fractions of infected cells was observed at 12 h pi and continued to further decrease at 24 h pi (Fig. 2.4. C). The phospho- α-adducin abundance in the TS fractions of mock- infected cells remained unchanged throughout the time course assay.

2.4.3. The localization of phospho- α-adducin in *Chlamydia*- infected cells.

To determine cellular localization of phospho- α-adducin in infected cells, conventional immunfluorescence and transfection assays were utilized. In Fig. 2.5. , a fraction phospho- α-adducin (Fig. 2.5. A, labeled with TRITC and Fig. 2.5. B, labeled with FITC) was observed in close proximity to the chlamydial inclusions at 24 h pi. In uninfected cells, phospho- α-adducin is observed dispersed throughout the cytoplasm with no specific pattern of accumulation. In cells transfected with N-terminal His-tagged α-adducin (labeled with TRITC), accumulation was observed

around the chlamydial inclusion (Fig. 2.5. D) and throughout the cytoplasm and nucleus of mock- infected cells (Fig. 2.5. C).

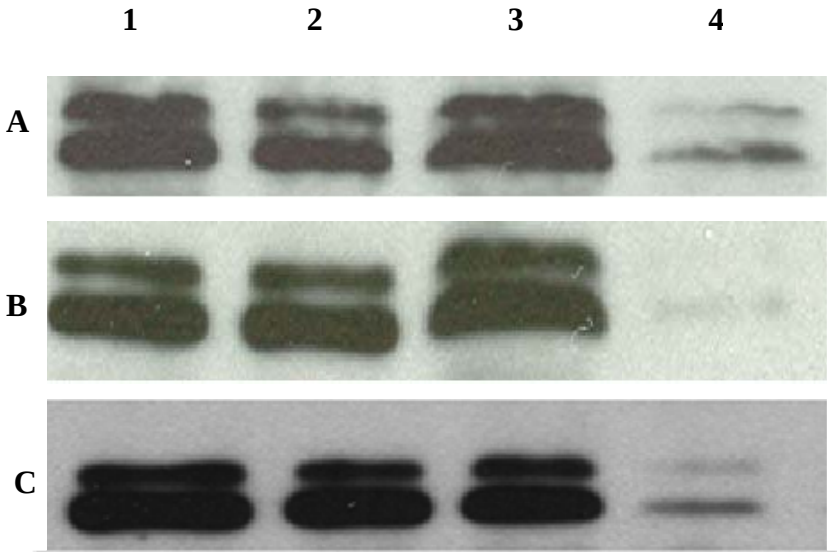

Figure 2.3. The abundance of phospho- α- adducin^{Ser726} is reduced in the TS fractions of cells infected with other chlamydial strains. Lanes 1-4 are (1) total protein lysate of mock- infected McCoy cells, (2) total protein lysates of *Chlamydia*- infected cells, (3) TS fraction of mock- infected McCoy cells, and (4) TS fraction of *Chlamydia*- infected cells. Panel A cells are infected with *C. trachomatis* serovar D. Panel B cells are infected with *C. trachomatis* L2. Panel C cells are infected with *C. caviae* GPIC.

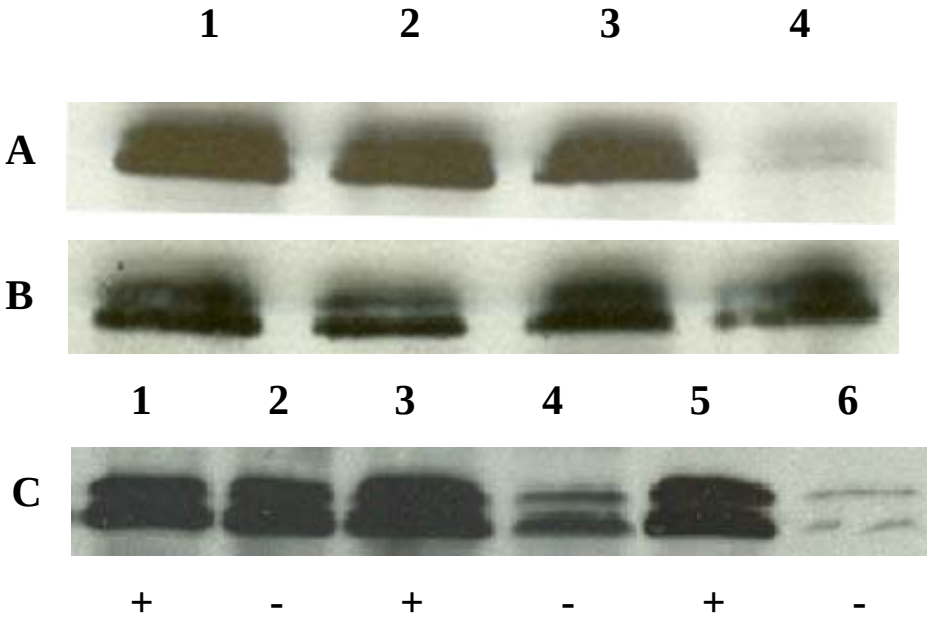

Figure 2.4. Infection, not attachment, results in the depletion of α-adducin in the TS fractions of *Chlamydia*- infected cells. Lane designations are the same in both panels and are as follows: (1) mock- infected whole cell protein lysate, (2) TS fractions of mock-infected cells, (3) *C. trachomatis* J/UW-36 infected whole cell protein lysate, and (4) TS fractions of *C. trachomatis* J/UW-36 infected cells. Panel A represents mock-infected (lanes 1 and 3) and infected (lanes 2 and 4) McCoy cells that are untreated with tetracycline. Panel B represents samples treated with tetracycline. Protein samples are normalized by Bradford assays and/ or Coomassie blue staining. The molecular size of a-adducin is 100 kDa. Panel C represents a time course assay performed to determine accumulation of phospho- a-adducin^{Ser724} at different time points post- infection. (+) lanes are mock- infected TS protein fractions and (-) lanes are *C. trachomatis* J/UW-36- infected McCoy cells TS protein fractions. Numbered lanes represent samples obtained after 6 hpi (lanes 1 and 2), 12 hpi (lanes 3 and 4), and 24 hpi (lanes 5 and 6). The molecular size of phospho- a- adducin^{Ser724} is ~120 kDa.

The microtubules or actin were disrupted by using nocodozale or cytochalasin D, respectively, to characterize the specificity of α-adducin accumulation around the chlamydial inclusions. In infected cells treated with cytochalasin D (Fig. 2.6. B), phospho- α-adducin accumulation around the chlamydial inclusions appear undisturbed by the disruption of the actin filament network, similar to the untreated infected cells (Fig. 2.6. A). Additionally, this association was not altered by treatment of infected cells with nocodazole, a drug used to disrupt microtubules (Fig. 2.6. D).

2.5. DISCUSSION

Chlamydial infection affects a variety of host signaling pathways that facilitate bacterial entry, host- pathogen interaction, and survival. Bacterial interferences in these pathways affect kinase functions, substrate regulation, and downstream events that enable host cells to cater to chlamydial development and replication. A multi- phospho- protein (kinome) analysis using the most common eukaryotic kinases is a useful tool to identify participants of the major signaling pathways affected by chlamydial infection. Our analyses identified participants of at least two signaling pathways affected by infection, namely phospho- α-adducin and phospho- Raf-1. A cytoskeleton- associated protein, α- adducin, is differentially localized in cells infected with various *Chlamydia* strains. Phosphorylation of α-

adducin by various kinases including protein kinase C and Rho-kinase results in regulation that affects its subcellular localization. Phosphorylation by protein kinase C releases α- adducing from actin-spectrin complexes and this phosphorylated adducin can be localized in the host cytoplasm (19, 24).

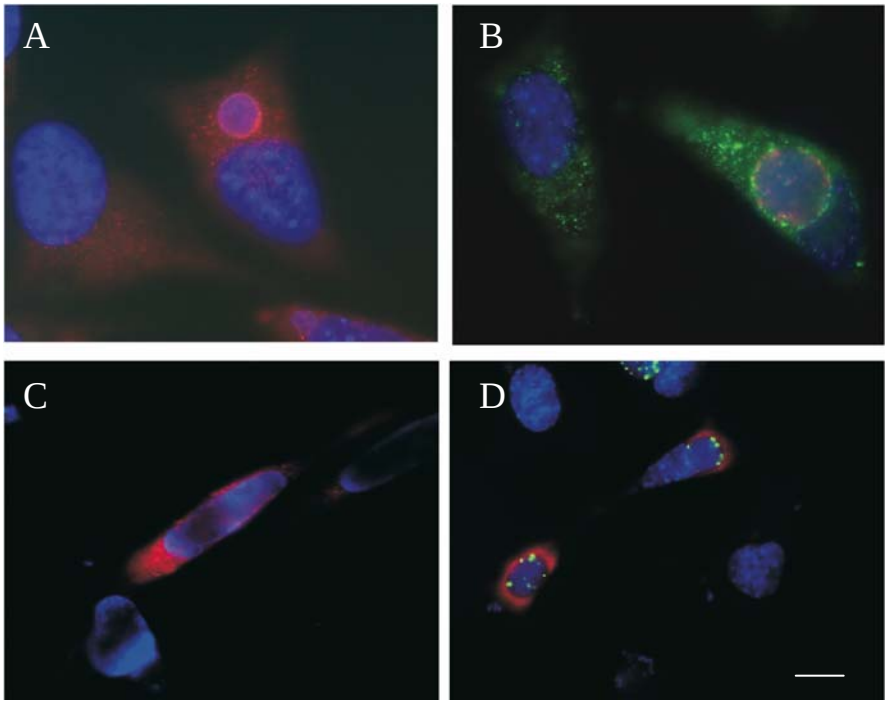

Figure 2.5. α- adducin localization is altered in *C. trachomatis* J/UW-36- infected cells. In panels A, a fraction of phospho- α- adducin (red) appears to localize in close proximity to the inclusion. In panel B, phospho- α- adducin (green) localizes around the chlamydial inclusion. Chlamydial forms, labeled with HSP60 (red) are observed inside the inclusion. In panels C and D, McCoy cells were transfected with mammalian vector, pcDNAHis4.0 with the α-adducin construct, mock- infected (panel C) or infected (panel D), and probed with anti-6XHis (red). Chlamydial forms are labeled with HSP60 (green). Chlamydial and host DNA are labeled with DAPI (blue). Bar = 10 μm.

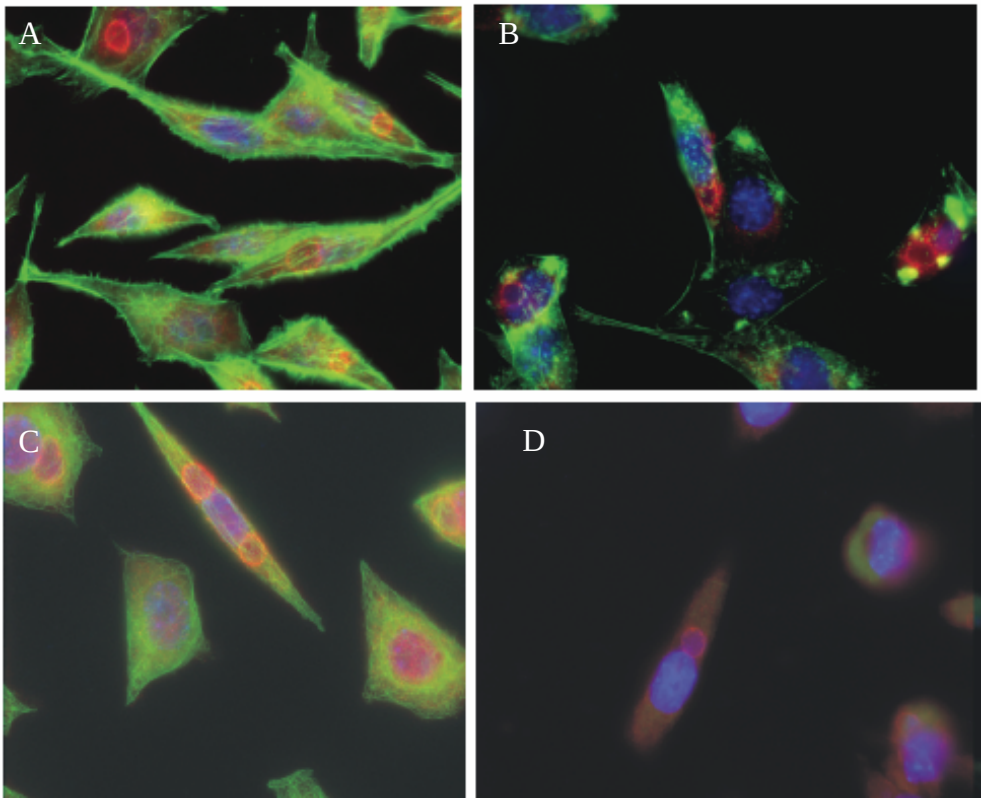

Figure 2.6. The association of phospho- α-adducin with chlamydial inclusions is independent of intact actin or microtubules. Infected McCoy cells at 24 h post infection were treated with DMSO (A and C), 10 μM cytochalasin D (B), or 30 μM nocodazole for 3 h prior to fixation and staining with FITC- phalloidin (A and B), FITC-α-tubulin (C and D) and phospho- α- adducin (A, B, C, and D). Tubulin or nocodazole is labeled green, phospho- α- adducin is labeled red, and DAPI is labeled blue. Bar = 10 μm.

Phosphorylation by Rho-kinase, as observed in platelet activation, causes enhanced association of α- adducin with actin-spectrin complexes (40). We are investigating the Rho kinase- associated phosphorylation patterns of adducin in *Chlamydia*- infected cells.

Phosphorylated Raf-1^{Ser259} is involved in signaling pathways that regulate the MAPK/ ERK pathway, which is critical to regulation of cell proliferation, differentiation, transformation, and apoptosis (1, 9). Dhillon *et al* demonstrated that Ras displaces Raf-1 interaction with 14-3-3 at the N-terminus of Raf-1 at the cell membrane (7, 8). Raf-1 that is phosphorylated at Ser259 is released from the cell membrane to the cytosol to bind with 14-3-3 (7, 8). Raf-1 that is not phosphorylated at Ser259 acts as a positive regulator of MEK phosphorylation (5). Recently, Zhong *et al* determined that for *Chlamydia* to acquire and process host glycerolipids, the activation of the Raf/ MEK/ERK/cPLA2 signaling pathway is required (39). Their experiments demonstrated that phosphorylated Raf-1^{Tyr340} in whole cell protein lysates increased as infection progressed throughout the 44 h time course assay (39). In our experiments, we demonstrate that the abundance of phosphorylated Raf-1 in the TS fractions of chlamydia- infected cells is reduced when compared to mock- infected cells. Because phosphorylated Raf-1 interacts with 14-3-3 proteins in uninfected cells, it is possible that the 14-3-3 interaction with chlamydial IncG (34) in infected cells

results in an altered localization of Raf-1. In addition, Scidmore demonstrated that the interaction between Raf-1 and 14-3-3 is altered as a result of chlamydial infection (31). We are currently investigating the phenotype of phosphorylated Raf-1 in cells infected by *Chlamydia* that lack IncG, such as *C. caviae* GPIC. The depletion of these proteins in the TS fractions of infected cells suggests that their absence in this cellular compartment may influence their ability to interact with downstream substrates, perhaps contributing to chlamydial development and survival inside the host cell.

The host cytoskeletal network is a well-documented cellular component that is affected by chlamydial entry and development. *Chlamydiae* manipulate cytoskeletal structures to facilitate entry (20), re- direct vesicular traffic (32), and maintain chlamydia- filled inclusions in close proximity to the nucleus (13) and other organelles. Tarp is secreted by chlamydia into the host cell cytoplasm to phosphorylate a host protein and induce actin- mediated endocytosis (20). It is not likely, however, that α- adducin is involved in this process as differences in adducin phosphorylation are observed later in development.

While α- adducin and its phosphorylated forms may be involved in a variety of cellular signaling pathways ranging from cytoskeletal arrangements (25) to apoptosis (19), it has also been demonstrated as a component of the dynactin complex (17, 18). Adducin co-immunoprecipitates with components of the dynactin complex, such as $p150^{Glued}$, centractin, as well as dynactin (17, 18). Grieshaber *et al* recently demonstrated that, while chlamydial inclusions traverse intracellularly via dynein-mediated interactions with the microtubules, they can function independently of p50 dynamitin, a component of the dynactin complex (13). The localization of α- adducin

in close proximity to the chlamydial inclusions suggests that a novel interaction may exist between components of the dynactin complex and chlamydial inclusions.

Various host proteins that were reported to associate with the chlamydial inclusion, such as Rabs, accumulate around the inclusion independently of intact microtubules (30). Our data not only demonstrate that phospho- α- adducin localizes to the chlamydial inclusion independently of intact microtubules but also of intact actin filaments. These results are intriguing, as this protein is typically associated with the cytoskeleton, specifically actin and spectrin, and further suggest specific chlamydial interaction with adducin.

In summary, the abundance of two phospho- proteins, phospho- Raf-1^{Ser259} and phospho- α- adducin^{Ser724}, in the TS fractions of chlamydia- infected cells was determined by using the Kinexus Phospho-Site Screen Assay. The localization of both proteins are altered in chlamydia- infected cells, as the abundance of both proteins are depleted in the TS fractions of infected cells and a fraction of α- adducin is detectable around the chlamydial inclusions, most notably later in development. Additional work is required to determine the specific signaling pathways affected by these phospho- proteins in infected cells, the possibility of Raf-1 and/ or α- adducin interaction with the inclusion membrane, and how infection with other chlamydial strains affect the abundance of these phospho- proteins.

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CHAPTER 3

Transcription Analysis of *incA* mRNA in Cells Infected by *Chlamydia trachomatis* Variant Strains

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In Preparation for Submission

3.1. ABSTRACT

Chlamydiae are obligate intracellular pathogens that develop in non-acidified vacuoles termed inclusion in their eukaryotic hosts. Typical development of *Chlamydia trachomatis* strains involves fusion of inclusions in multiply infected cells. IncA is a chlamydial protein that localizes to the inclusion membrane and functions to facilitate fusion events between chlamydial inclusions. A set of clinical isolates of *Chlamydia trachomatis* have inclusions that are non- fusogenic with each other and lack IncA on the inclusion membrane within infected cells. The absence of this protein is associated with stop codons and nonsense mutations introduced within *incA.* In these experiments, we examined transcription of *incA* in wild type and variant isolates. Initial experiments using reverse-transcriptase polymerase chain reaction (RT- PCR) indicated that *incA* is expressed in both wild type and variant strains. Quantification of the *incA* transcripts by Northern Blot analyses and real-time RT-PCR show a reduction of *incA* transcripts in non-fusogenic strains versus the wild type strains. Additionally, 5' RACE experiments demonstrate that the transcriptional start site of *incA* mRNA is similar in both non-fusogenic and wild- type strains. These data suggest that the while these non-fusogenic strains are capable of transcribing *incA* transcripts, the abundance of these transcripts is below the threshold of IncA production.

3.2. INTRODUCTION

Chlamydia trachomatis is the bacterial pathogen that causes the most common form of sexually transmitted disease in the United States (4, 8, 19). Additionally, the pathogen causes blinding trachoma, in many underdeveloped countries(19). The chlamydiae have a biphasic life cycle that consists of two forms: elementary bodies (EB) are infectious but metabolically inactive, and reticulate bodies (RB) are non-infectious but metabolically active (13). Host cells are initially infected when EB are endocytosed inside a vesicle, termed an inclusion (13). The inclusion grows in size to accommodate dividing reticulate bodies (RB) (13). Chlamydial inclusions are not recognized by and do not fuse with endosomes or lysosomes (5, 10, 21).

Chlamydiae produce a collection of proteins, termed Incs, that localize to the inclusion membrane (2, 15, 16, 17). Genes encoding Incs can be identified within the chlamydial genome because they typically have a large hydrophobic domain consisting of approximately 40-70 amino acids (1). This domain is predicted to target the protein to the inclusion membrane (1). From the *C. trachomatis* and *C. pneumoniae* genome, at least 40 candidate Inc proteins have been identified (1). Recent work using a heterologous secretion system by Fields *et al* and Subtil *et al* have shown that Incs are substrates of the type III secretion system in *Shigella flexneri* (6, 24, 25). Some Incs are expressed and secreted as early as 2 hours post infection and

could have a putative function for remodeling the IM to ensure chlamydial survival (3). To date, however, no specific functions have been assigned for a majority of the Incs.

One specific Inc, IncA, is an important protein for homotypic vesicle fusion within infected cells (9). Recently, a collection of nonfusing variants represented in *C. trachomatis* serovars B, D-, E, F, G, H, Ia, J, and K were observed in ~1.5% of isolates obtained from genital infections diagnosed at clinics in the Seattle-King County Department of Public Health during 1988 to 1999 (26). Cells multiply infected with the non-fusing variants have *Chlamydia*- filled inclusions that fail to fuse throughout the developmental cycle (18, 26). Sequence analysis reveals polymorphisms within *incA* of the non-fusing variants (18). Further characterization of IncA by immunoblotting and immunofluorescent assays reveal that 24 out of 27 variants that lack IncA on the IM are non-fusers while 3 out of 27 are non-fusers but have IncA on the IM (18). Inclusions produced from co-infections with an IncA-negative and IncA-positive variant do not fuse (26). Epidemiological studies in patients infected by these non-fusing serotypes reveal that these strains are associated with a less aggressive disease (7).

Because genetic manipulation systems in chlamydia are currently not available, we used contemporary molecular techniques to determine if the absence of IncA is attributable to transcription regulation. We used reverse-transcriptase polymerase chain reaction (RT-PCR) to detect *incA* transcripts, Northern Blot analysis to determine *incA* abundance and transcript size, 5' RACE (rapid amplification of cDNA ends) to determine transcription start site, and real-time RT-PCR to quantify

incA transcripts. This work presents evidence that the accumulation of *incA* mRNA is significantly less in the IncA-negative, non-fusing strains relative to the wild-type and IncA-positive non-fusing strains.

3.3. MATERIALS AND METHODS

3.3.1 Cell culture and *Chlamydia*.

Mock- or *Chlamydia*- infected murine epithelial McCoy cell cultures were grown at 37° C in the presence of 5% CO_2 in Minimal Essential Media (Life Technologies, Baltimore, MD) supplemented with 10% fetal bovine serum, L-glutamine, and gentamicin (MEM-10). Wild-type *C. trachomatis* serovars D/ (UW-3), J/ (UW-36), G/ (UW-57), and non-fuser IncA- negative variants $D_{(s)}$ 2923, $J_{(s)}$893, $J_{(s)}$1980, $E_{(s)}$ 1986, $H_{(s)}$5642, $Ia_{(s)}$ 2197, $F_{(s)}$4022, and $F_{(s)}$ 8063 (26) stored in 0.25M sucrose, 10mM sodium phosphate, 5mM L-glutamic acid (SPG) buffer, pH 7.2, were used to infect McCoy cells for RNA extraction and isolation.

3.3.2. Immunofluorescence**.**

Monolayers of McCoy cells on glass coverslips were infected separately with wild- type or variant strains and incubated at room temperature for 1h on a rocking platform. After removal of the inocula, the cells were washed with Hank's Balanced Salts Solution (HBSS; Life Technologies). Infected cells were incubated at 37° C for 24 h, fixed in 100% methanol for 10 min, rinsed with Hank's Balanced Salt Solution (HBSS), and incubated in fluorescent antibody (FA) block (2% BSA in PBS) for 20 min. Monolayers were then incubated for 1h with primary antibodies (IncA, LPS,

Hsp60) diluted in FA block and rinsed three times with PBS. FITC or TRITC conjugated secondary antibodies were then added, and following a second 1h incubation, the cells were rinsed with PBS. Coverslips were rinsed briefly with deionized water and inverted onto a drop of non-photobleaching agent (Vector Laboratories, Burlingame, CA) with DAPI (4',6-Diamidino-2-phenylindole) on a microscope slide. A Zeiss fluorescent microscope was used to visualize the stained slides. Images were observed under a 100X oil immersion objective and digitally collected with a SPOT camera (Diagnostic Instruments). Photoshop (Adobe Software) and Canvas (Deneba Software) were used to process the images.

3.3.3. RNA Extraction.

Total RNA was harvested by treating mock-infected or infected cells with TriZol reagent (Life Technologies) according to manufacturer's directions. Amplification grade RQ1 DNase (Promega, Madison WI) was added to remove residual genomic DNA. The RNA mini kit (QIAGEN, Valencia, CA) protocol was used to remove residual DNAse and contaminants. RNA integrity was verified electrophoretically by ethidium bromide staining and by $A_{260/280}$ absorption reading in a UV spectrophotometer (Biorad, Hercules, CA).

3.3.4. Reverse-transcriptase (RT-PCR).

Reverse transcriptase (RT)- PCR analysis of total RNA extractions was used to detect *incA* transcripts via an Access RT-PCR kit (Promega, Madison, WI) with gene-specific oligonucleotide sets (Table 1) according to manufacturer's directions. Avian

myeloblastosis virus (AMV)- reverse transcriptase was used to transcribe total RNAs into cDNAs. This reaction was incubated for 45 min at 48 °C. PCR amplification of cDNAs was accomplished by initial denaturation of templates for 2 min at 94C and 35 cycles consisting of 30 s at 94 °C, 45 s at 52 °C, and 45 s at 72 °C. To control for genomic DNA contamination, RNA samples amplified without the RT enzyme were used as templates in PCR. Amplification products were then electrophoresed through 1.5% agarose gels stained with ethidium bromide and visualized.

3.3.5. Northern Blot analysis.

The NorthernMax-Gly kit was used for Northern analysis (Ambion) according to manufacturer's directions. Briefly, 30 μg of total RNA obtained from infected or mock- infected cell lysates were mixed with glyoxal dye (50% glyderol, 10mM NaPO4, 0.25% bromphenol blue, 0.25% xylene cyanol FF), incubated at 50 °C for 30 min, and electrophoresed through 1.5% agarose gel. RNA was then blotted on a positively charged nylon membrane followed by UV- crosslinking. DNA probes (a 316-bp *incA* DNA and a 320- bp *16S rRNA* PCR products) were labeled by PCR amplification with digoxigenin (DIG) -labeled dNTPs (Roche) according to manufacturer's directions. Hybridization of DIG- labeled probes was performed at 52 °C for 12-16 h. The membrane was washed twice in 2 X SSC (1 X SSC= 0.15 M NaCl/ 15 mM sodium citrate) at room temperature and once in 0.5 X SSC at 52 °C for 30 min and incubated with anti- DIG antibody prior to autoradiography.

3.3.6. 5’ RACE.

To map the transcriptional start site of *incA* , the protocol for 5’ rapid amplification of cDNA ends was performed according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The cDNA was generated from reverse transcription of 5 μg of total RNA using Superscript II and a gene-specific primer, RCE1 (Table 1). Briefly, the cDNA samples were “tailed” by addition of cytosine residues at the 3’end using terminal deoxynucleotidyl transferase (Tdt). The “tailed” samples were amplified by PCR using a nested gene- specific primer RCE2 (Table 1) and an oligo (dG)- linked amplification primer. PCR conditions used were 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 52 °C, and 1 min extension at 72 °C. PCR products were purified (Qiagen, Valencia CA) and diluted for amplification using another nested gene specific primer, SEQ1 (Table 1) and an oligo-dG primer, AAP (Invitrogen, Carlsbad, CA). Secondary PCR products were purified and used to transform *E. coli* according to manufacturer’s directions (Invitrogen, Carlsbad, CA). Sequencing of PCR products was performed at the Center of Gene Research and Biotechnology Core Laboratories, Oregon State University, Corvallis, OR.

3.3.7. Real-time RT- PCR.

cDNA from total RNA (5 ug) was produced by using the protocol described in Superscript II™ First Strand Synthesis System (Invitrogen, Carlsbad, CA). Briefly, DNAse-I treated total RNA was incubated with a Superscript II™ RT with the gene-specific primers listed in Table 1. PCR conditions used were 65 °C for 5 min, 50 °C for 50 min, 80 °C for 5 min, and 37 °C for 20 min. The cDNAs were then used as

templates for real-time PCR. Real- time PCR was performed in the iCycler thermocycler (Biorad, Hercules, CA) as cDNA templates were subjected to denaturation at 95 °C for 3 min, and 40 cycles consisting of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Separate PCR amplification reactions were used for target cDNAs from experimental and control samples. Control samples to normalize DNA content are cDNA amplified with *16S rRNA* and/ or *hsp60* primers. To determine transcript copy numbers of experimental samples, the C_T values and corresponding copy numbers for the controls were used. Data obtained from a standard curve generated by PCR amplification of several dilutions of amplicons with known quantity were compared to copy numbers obtained from PCR reactions of the target experimental samples. All assays were performed in triplicate to verify results.

3.4. RESULTS

3.4.1. Immunofluorescence and immunoblot of IncA in non-fusogenic strains.

Non- fusing, variant *C. trachomatis* strains either lack or possess detectable IncA on the IM (18). Fig. 3.1. illustrates immunofluorescence labeling that allowed analysis of the two IncA phenotypes, a non- fuser with undetectable IncA on the IM (panels D and E) and a non- fuser with detectable IncA (panel F). IncA is detected on the IM of wild- type strains (panels A, B, and C). To verify detection of IncA by immunofluorescence, protein lysates from infected (Fig. 3.2., lanes 1-4) and mock-infected (Fig. 3.2., lane 5) were extracted and used as samples for immunoblotting experiments. Antibody to Hsp60 (Fig. 3.2. panel B, lanes 1-5) was used to control for

chlamydial protein content in each sample lysate. IncA is detectable in wild- type, fusing strains (lanes 1 and 2) but not in variant, non-fusing strains (lanes 3 and 4). No detectable protein bands specific for chlamydial IncA and Hsp60 are detected in protein lysates of mock- infected McCoy cells.

3.4.2. Transcription analyses of *incA*.

Transcription analysis was performed to determine if *incA* mRNA is transcribed and accumulates in cells infected with the non-fusing strains. Initially, RT-PCR was performed to determine if *incA* transcript is detectable in these strains. Fig. 3.3. demonstrates that *incA* (316 bp) transcript is detectable in cells infected with either wild- type (lane 2) or non-fusing strains (lanes 3, 4, and 5). No DNA contamination is detected in the RNA samples transcribed without the RT enzyme (lanes 9-14) that were used as templates for RT- PCR. Total RNA obtained from the mock- infected cells did not produce detectable *incA* transcripts (lane 6).

Northern blot analysis was performed to determine the size and abundance of these transcripts. To control for chlamydial RNA loading, *hsp60* transcript levels were normalized in all RNA samples used. Fig. 3.4. demonstrates that at 20-24 hpi two *incA* transcripts are detected, the top band is approximately 0.8 kb and the bottom band is approximately 0.6 kb. The abundance of *incA* transcripts is different in cells infected with the non- fusing strains (lanes 3 and 5) relative to those infected with the wild- type strains (lanes 2 and 4) or the mock- infected (lane 1) cells.

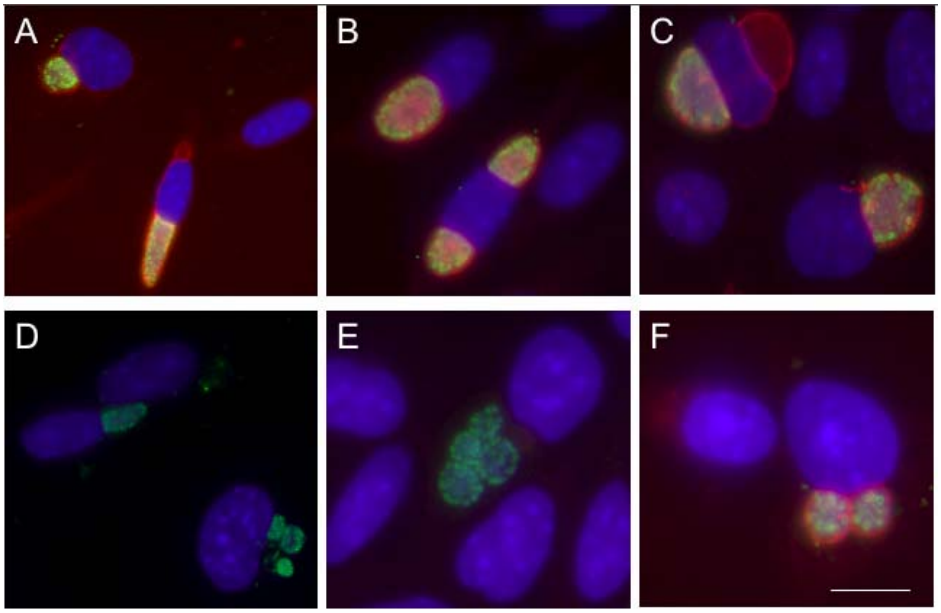

Figure 3.1. McCoy cells infected with wild type strains D/ UW-3 in panel **A**, J/ UW-36 in panel **B**, and G/UW-57 in panel **C** have detectable IncA on the inclusion membrane. Non-fusing variants lack detectable IncA on the inclusion membrane (**D**: $D_{(s)}2923$ and **E**: $J_{(s)}$ 893) or have detectable IncA (**F**: $G_{(s)}459$) but retain the non- fusing phenotype. Green: LPS. Red: IncA. Blue: DAPI (DNA). Bar = 10 μm.

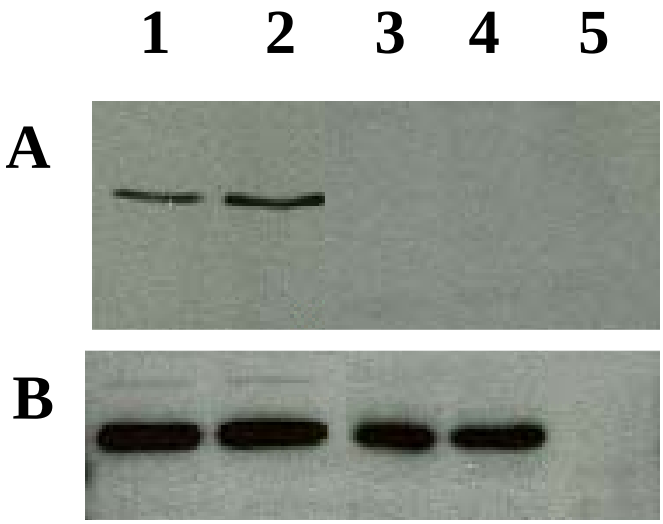

Figure 3.2. IncA (29 kDa) is not detected in protein lysates of cells infected with the non-fusing strains. Protein lysates of cells infected with wild- type strains, D/UW-3 and J/UW-36 (lanes 1 and 2, respectively), and non-fusing strains, $D_{(s)}$ 2923 and $J_{(s)}$ 893 (lanes 3 and 4, respectively) are probed with monoclonal antibody to (A) IncA or (B) Hsp60 (57 kDa).

Although the results from the Northern blot analyses reflected similarity in transcript sizes, the transcription start sites (TSS) may be different in *incA* transcripts produced by the different strains. 5' RACE was used to determine the transcription start site of the *incA* transcript. Fig. 3.5. shows that the TSS for *incA* is 27 or 28 nucleotides upstream of the predicted translation start site. The wild- type and non-fusing strains both utilize an identical TSS. Upstream of the TSS are the putative –10 promoter element, TAGGAT, and a –35 promoter element, TGCTTT. Also notable is a characteristic A/T spacer (20) sequence immediately downstream of the –35 promoter, AGAAAA.

3.4.3. Quantification of *incA* transcripts.

Three independent real- time RT-PCR experiments were performed in triplicate to quantitatively verify northern blot results. Total RNA samples obtained from mock- infected cells or cells infected with either the wild- type or non- fusing strains were reverse- transcribed into cDNA with gene- specific primers for *incA* and chlamydial *hsp60*. All samples were normalized to contain similar amounts of *hsp60* transcripts. Cells infected with the non- fusing strains that lack IncA contain

approximately 50 to 70% less *incA* transcripts relative to cells infected with the wild- type strains or IncA- positive non- fusing strains (Fig. 3.6.). The abundance of *incA* transcripts in cells infected by wild type strains is similar to cells infected by *C. trachomatis* G(s) 459, an IncA- positive non-fuser strain.

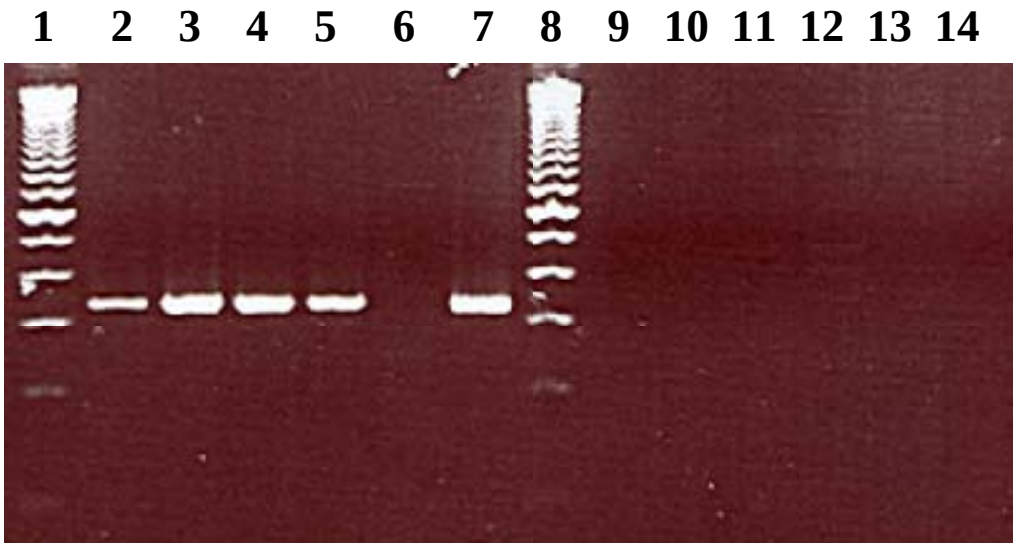

Figure 3.3. Transcript for *incA* is detected in wild type and non-fusing variant strains. Lane 2 contains the positive RT-PCR control with D/UW-3 genomic DNA as template. Lanes 3, 4, and 5 represent RT-PCR products from *incA* transcripts obtained from McCoy cells infected with $D_{(s)}$ 2923, $J_{(s)}$ 893, and $J_{(s)}$1980, respectively. Lane 6 is the mock-infected McCoy cell control. Lane 7 contains the RT-PCR product obtained from *incA* transcript of a wild type strain D/UW-3. In the same order as lanes 2 -7, lanes 9 –14 represent RT-PCR products without the reverse-transcriptase enzyme in the reaction.

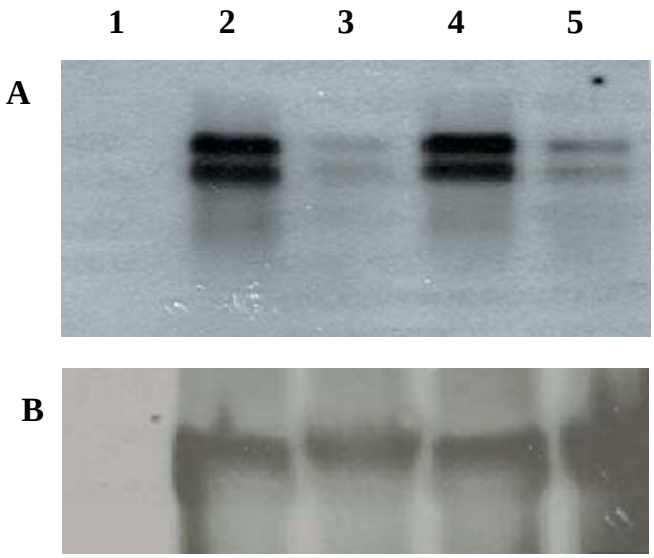

Figure 3.4. Accumulation of *incA* transcripts in total RNA lysates from cells infected with non-fusing variants appear significantly reduced relative to the cells infected with the wild-type strains. Northern blots of total RNA lysates are probed with DIG- labeled *incA* (panel A) or DIG- labeled *hsp60* (panel B). Lane 1 contains total RNA lysate from mock- infected McCoy cells. Lanes 2, 3, 4, and 5 contain total RNA lysates from McCoy cells infected with D/UW-3, $D_{(s)}$ 2923, J/UW-36, and $J_{(s)}$ 893. The size of *incA* transcript is 0.8 kb (top band) and 0.6 kb (bottom band). The size of *hsp60* transcript is 2.2 kb.

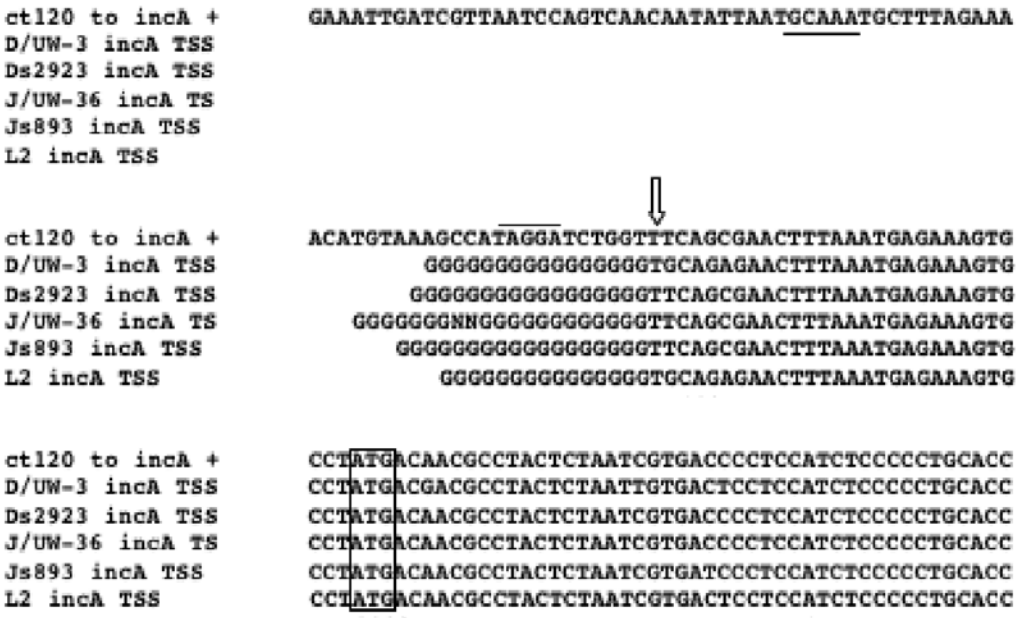

Figure 3.5. 5' RACE map of *incA* transcription start site (TSS). The *C. trachomatis* strains are listed on the left. Transcription begins at a T that is 29 nucleotides upstream of the predicted translational start site (box). The translation start site, ATG, is boxed in. The putative -10 and -35 regions are indicated with a bar on top of the nucleotides and underlined, respectively.

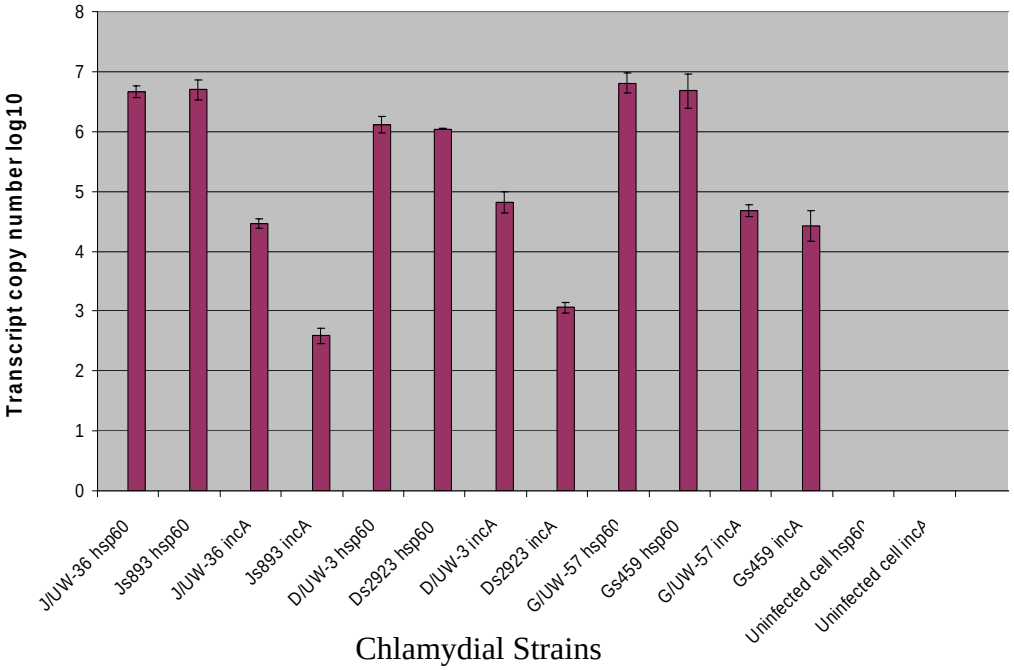

Figure 3.6. Abundance of *incA* mRNA detected by quantitative RT-PCR. Purified total RNA samples were obtained from cells infected by wild- type C. trachomatis (J/UW-36, D/UW-3, and G/UW-5), mutant strains (Js893, Ds2923, and Gs459), and uninfected McCoy cells. Each sample was used in a quantitative RT-PCR assay with TaqMan primer/ probe sets that are specific for *incA* or *hsp60*. The transcripts for each sample was normalized with chlamydial *hsp60*. The abundance of *incA* demonstrates at least a ten-fold reduction in cells infected by non-fuser, IncA negative, mutant strains (Js893 and Ds2923) relative to those infected by the wild- type (J/UW-36, D/UW-3 and G/UW-57) and the IncA- positive mutant strain (Gs459).

3.5. DISCUSSION

In this report, we show that transcripts for *incA* are detectable in total RNA lysates of cells infected with the non- fusing strains that lack IncA on the IM. Previously, it was reported that the *incA* sequence in non- fusing strains contain polymorphisms that lead to premature stop codons, large deletions, and/ or nucleotide changes that change the IncA amino acid sequence (18). We were interested to characterize transcription of *incA* from this strain because its sequence is very similar to the wild- type *incA* sequence. However, we recently re-sequenced *incA* from $D_{(s)}$ 2923 and discovered a deletion of nucleotide 389 and this introduced a frameshift mutation in addition to the 3 nucleotide changes that were previously described (data not shown). While it is possible that the sequence polymorphisms in the non- fusing variants contribute to the lack of IncA expression, the transcription assays demonstrate that the *incA* transcript is detected in cells infected by these strains. Although two transcript bands are detected on the northern blots in both non- fusing and wild-type strains, only one TSS was determined by the 5' RACE assays. The TSS is approximately 27 nucleotides upstream of the translation start site. The TSS for the smaller transcript is not detected by 5' RACE. It may be that there is another functional transcription site or that post- transcription processing of *incA* occurs at 24 hpi. An alternative method, primer extension analysis, may facilitate detection of a possible second transcription initiation site. To demonstrate that this start site and putative –10 and –35 promoters are functional and recognized by the chlamydial transcription machinery, *in- vitro* transcription assays may be required, where

chlamydial transcription components are utilized in a heterologous transcription system (12).

Quantification of transcripts by real-time RT-PCR demonstrated the reduction of *incA* transcripts in cells infected by non- fusing variants and verified the results obtained from the northern blot assays. However, variability observed in reduction may be attributable to the differences in detection sensitivity of each technique. The nature of real-time RT-PCR assays offer a significantly more sensitivity in detection than the Northern blots.

There are a number of possibilities to offer explanations for the reduction of the *incA* mRNA in cells infected by the non- fuser strains. First, it may be that the production of the *incA* mRNA is impaired, such that a transcription factor encoded by another gene is absent in the genome of or non- functional in non- fuser strains. The upstream regions of the *incA* mRNA sequences that correspond to the ribosome binding sequences and transcription initiation sequences (putative -10 and -35 RNA polymerase recognition regions) are identical in the non-fusers and the wild- type strains (data not shown). The transcription initiation start site, as demonstrated by the 5' RACE experiments, is identical in D/UW-3 and $D_{(s)}$ 2923. Therefore, it is plausible that there is an additional factor, involved in transcription, that is missing in $D_{(s)}$ 2923. Several transcription factors have been identified in chlamydia including ChxR, a stage- specific transcription regulator (11). Second, the *incA* mRNA produced by the non- fuser strains may have mutations that perturb the sequence required for signaling through the T3S system. Schneewind *et al* recently demonstrated that some mutations that alter the mRNA sequence for a T3S substrate but does not alter codon specificity

affected secretion of some T3S substrates (22). It will be important to determine if secretion of IncA to the inclusion membrane requires an intact consensus sequence found within the *incA* mRNA.

Recently, it was shown that Incs are recognized as substrates of the type III secretion (T3S) system (6, 23, 24, 25). Additionally, inhibitors of the chlamydial T3S system by treatment of cells with INP0400, a drug used to inhibit T3S system in *Yersinia*, resulted in multiple, non- fusogenic inclusions when cells are multiply infected with *C. trachomatis* serovar L2 (14).

It will be important for future work with non-fusers to determine if the genes encoding the T3S systems are intact and functional and if additional Incs that are identified from the ongoing sequencing and genome analyses of new chlamydia strains are involved in the fusogenic phenotype.

Because the non- fusing phenotype has an associated clinical presentation of a less aggressive disease, it will be important to identify additional factors, host and/or bacterial associated, involved in the development and survival of the non-fusers. Currently our laboratory is in the process of sequencing and annotating one non-fuser strain, *C. trachomatis* $D_{(s)}$ 2923, to determine sequence polymorphisms in the genome to perhaps identify these additional factors.

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CHAPTER 4

Phosphorylated PKB is Depleted in *Chlamydophila caviae* GPIC- Infected Mammalian Cells

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In Preparation for Submission

4.1. ABSTRACT

Chlamydiae are obligate intracellular pathogens that develop and multiply in non-acidified vacuoles within host cells. Their intracellular development and survival require manipulation of host signaling pathways that are involved in many processes including apoptosis, vesicle trafficking, proliferation, and perhaps many others. Phosphorylation events between participants of host signaling pathways are affected by chlamydial infection such that activity of kinases and/ or their substrates are altered. This can lead to significant differences in cellular phenotype in response to environemental change. In this study, a large kinome analysis that utilized phospho-specific antibodies to common eukaryotic kinases and their substrates was used to determine their abundance in *Chlamydia trachomatis*- infected or mock- infected cells. From these analyses, it was demonstrated that the abundance of phosphorylated protein kinase B (PKB) at position Ser473 was similar between the infected and mock-infected cells. This phospho- protein was then subsequently used to normalize protein content in infected or mock- infected protein samples used in other assays in the laboratory. However, an unexpected finding in *Chlamydophila caviae* GPIC- infected cells revealed that this phosphoprotein is depleted at later time points post infection while the total PKB abundance remains intact. Fluorescence microscopic analysis demonstrated alterations in both phospho-PKB abundance and intracellular distribution.

4.2. INTRODUCTION

Chlamydiae are a group of obligate intracellular bacteria that inhabit a diverse group of organisms that range from simple living cells such as amoeba to complex organisms such as animals and humans (5, 6). In humans, *Chlamydia trachomatis* is involved in sexually transmitted diseases or trachoma (17, 38, 42). *Chlamydophila pneumoniae* is associated with respiratory conditions and, most recently, atherosclerosis (25, 26), and *Chlamydia psittaci* is a zoonotic pathogen that commonly infects avian species (28, 31).

Chlamydiae alternate between two morphological forms, the elementary body (EB) and the reticulate body (RB) during their developmental cycle. Infection is initiated by attachment and entry of the infectious, yet metabolically inactive, EB (29). Within the host cell, the EB is housed inside a non-acidified vacuole, termed an inclusion, that exhibits minimal to no apparent interaction with the endosomal and the lysosomal pathways (19, 40). Intracellular EB then develop into RB, the non-infectious and metabolically active form of chlamydiae (29) that will continue to divide by binary fission and eventually occupy the inclusion and differentiate back into EB and be released outside of the host cell (46).

The study of chlamydial disease has several animal model systems (20, 33). One such model involves infection of cells with *Chlamydophila caviae* GPIC, an infection that mimics naturally occurring *C. trachomatis* infection and disease in humans (33). Typically, inclusions housing *C. trachomatis* in multiply infected cells fuse into one large inclusion during the developmental cycle while *C. caviae* inclusions are not fusogenic with each other and not only undergo RB division but

also partitioning of the inclusion (35). Although *C. caviae* is used in animal models of *C. trachomatis* infections, its genome shares a closer phylogenetic relationship to *C. pneumoniae* (34).

Host signaling pathways affected during chlamydial infection may be species-specific. For example, *C. caviae* infection requires intact host signaling pathways in order to enter host cells unlike infection by other *Chlamydia* spp. (44). One of these pathways is the phosphatidylinositol 3' kinase (PI3K) pathway. Inhibitors of this pathway, such as wortmannin and LY294002, also inhibit *C. caviae* infectivity and entry into host cells but do not inhibit entry of other *Chlamydia* spp. (44).

The study of PKB/Akt regulation is important because it is involved in pathways that promotes sequestration of pro-apoptotic proteins (9), prevents transcriptional activators that induce transcription of pro-apoptotic genes (36), and is associated with pathways involved in cell transformation and carcinogenesis (24).

The PI3K pathway is characterized by a complex interaction between signaling proteins that are intracellularly activated as a response to hormones and growth factors that function as ligands of cell surface receptors (14). PI3K interacts with proteins on the plasma or endosomal membrane to generate 3' phosphorylated phosphoinositides (10, 11). These phosphoinositides, in turn, interact with proteins that contain specific phosphatidylinositol 3' phosphate (PIP3) binding motifs such as those that contain the pleckstrin homology (PH) or FYVE domains (12). These proteins are translocated to the plasma (PH) or endosomal (FYVE) membranes where they interact with and are activated by PIP3 binding (23). Two specific proteins that have PH domains are phosphoinositide- dependent kinase 1 (PDK1) and (protein kinase B or Akt) PKB, a

known substrate of PDK1 (3). Among the substrates of PKB are other pro-apoptotic proteins, such as BAD, and glycogen synthase kinase-3 (GSK-3), a protein that targets transcription factors, structural proteins and metabolic enzymes (1,32).

There are 3 isoforms of PKB (α,β, and γ) (41) and each one contains important protein domains such as the N-terminal PH domain, a C-terminal hydrophobic domain, a kinase domain and an HM domain(41). The HM domain functions as a docking site for other kinases such as PKC, p70 S6K, SGK and RSK to interact with PDK1 (2). Currently, there is much debate on the role of PDK1 in phosphorylating PKB at Ser 473 and the identity of an additional kinase responsible for phosphorylation at this position. However, candidate kinases that are being explored include integrin-linked kinase (ILK), PKCβII, and DNA-dependent protein kinase (DNA-PK) (15, 30).

Stratford *et al* recently analyzed the interaction of ceramide with the PI3K pathway (43). In a variety of cell types, PI3K commonly supports cell survival while ceramide signaling is involved in apoptosis (18). However there is some evidence that suggest that ceramide does not directly modulate PI3K enzymatic activity (37, 45, 46, 50) and does not inhibit the accumulation of PIP2 and PIP3 (43). Stratford *et al* verified that C2-ceramide inhibits PKB phosphorylation by blocking its recruitment to the plasma membrane, a translocation needed for phosphorylation (43). Besides translocation, ceramide may also be involved in activation of a specific phosphatase that could inhibit Ser-473 phosphorylation or dephosphorylate at Ser 473 (39).

Recently, protein samples obtained from chlamydia- infected and mock-infected cells were submitted for kinome analysis, through the use of an assay that quantitatively measures the abundance of phospho-proteins that participate in major eukaryotic signaling pathways. From this assay, it was determined that significant differences in host response were observed between mock- infected cells and chlamydia- infected (*C. trachomatis* serovar J/UW-36 or J(s) 893) cells. However, phospho- PKB/Akt^{Ser473} abundance in both infected and mock- infected samples was unremarkable and this phospho- protein was subsequently used to normalize protein content in both samples in immunoblotting experiments to verify the kinome analysis results. However, a differential phosphorylation pattern of PKB/Akt^{Ser473} was observed unexpectedly between *C. caviae* - and *C. trachomatis*- infected cells at later time points post infection. Furthermore, immunofluorescence analysis of *C. caviae*-infected cells showed minimal labeling with phosphorylated PKB/Akt relative to uninfected or *C. trachomatis*- infected cells.

4.3. MATERIALS AND METHODS

4.3.1. Materials.

Minimal Essential Medium (Gibco) was supplemented with 10% fetal bovine serum, L-glutamine, and gentamicin at (MEM-10). Protease and phosphatase inhibitors were purchased from Roche (Indianapolis, IN). Polyclonal Anti-phospho-PKB^{Ser473} and Anti-PKB/ Akt were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal Hsp60 antibodies specific for chlamydial forms were obtained as described (49). Fluorescein isothiocyanate (FITC) or

tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibodies were purchased from Southern Biotechnologies (Birmingham, AL).

4.3.2. *Chlamydia* and cell culture.

Purified *Chlamydia trachomatis* serovars J/UW-36, D/UW-3, L2, and *Chlamydophila caviae* GPIC EB were used to separately infect McCoy cells at a multiplicity of infection (MOI) between 1 and 2. Inocula were diluted in 0.25 M sucrose, 10 mM sodium phosphate and 5 mM L-glutamic acid (SPG) buffer. Mock-infected cells were incubated with SPG buffer only. Infected and mock- infected cells were centrifuged at 2000 x *g* for 1h, followed by removal of inocula and addition of MEM-10 and cycloheximide at 1 μg/mL. Cells were incubated for 20-24 h at 37 °C with 5% CO_2.

4.3.3. Immunoblot analysis of host proteins.

Protein samples used were prepared by two methods: whole cell total protein extraction with SDS-PAGE buffer and cell fractionation. For the cell fractionation, infected or mock-infected cells were homogenized in buffer (20 mM Tris-HCl (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate (pH 7.2), 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100), sonicated, centrifuged at 13,200 rpm at 4 °C, and the supernatant (TS fractions) and pellet fractions were separated and collected. Protein content in samples was normalized via Coomassie blue stain and/or via the Bradford assay (Bio-Rad). Protein

samples were then electrophoresed through 12% SDS-PAGE, blotted on nitrocellulose, blocked with Odyssey™ blocking buffer (Licor, Lincoln, NE), and probed with antibodies to PKB/ Akt, phospho-PKB (Ser^{473}), or chlamydial Hsp60. The blots were then probed with goat anti-rabbit or goat anti-mouse antibodies conjugated to IRdye 800 or IRdye 700 (Rockland Immunochemicals, MD). After the final washes, the Odyssey™ Infrared Imager was set to 160μm resolution, 0mm offset parameters and used to scan the membranes (Licor).

4.3.4. Immunofluorescence.

Cell monolayers that were either infected with *Chlamydia* or mock- infected were fixed at designated hours post infection (hpi) in 4% paraformaldehyde for 1 h. The permeabilized cells were treated with 0.1% Triton X-100 and subsequently probed with antibodies to chlamydial Hsp60, phospho-PKB (Ser^{473}) or total PKB that were diluted in 2% bovine serum albumin (BSA) and incubated for 1h at room temperature. Following washes with 1X PBS, the cells were then incubated for 1h at room temperature in the dark with the corresponding secondary FITC or TRITC conjugated antibodies. All images were viewed by using a Leica DMLB microscope (Diagnostics Instruments, Sterling Heights, MI). Images were captured by using Spot Digital Camera software (Diagnostics Instruments), and processed for publication using the Adobe Photoshop software (San Jose, CA).

4.4. RESULTS

4.4.1. Immunoblot analyses of PKB in *Chlamydia*- infected cells.

Initial immunoblot assays performed to verify the kinome analysis data for protein lysates from mock- infected or *C. trachomatis*- infected McCoy cells demonstrated similar abundance of phosphorylated PKB/ Akt^{Ser473}. Therefore, the abundance of phosphorylated PKB/ Akt^{Ser473} was used as a loading control to normalize protein content for all samples analyzed. However, the abundance of phosphorylated PKB/ Akt^{Ser473} in *C. caviae* GPIC infected cells at 24 h post infection was significantly reduced in the total protein lysate sample (Fig. 1D) and in the TS fractions relative to similar protein fractions in mock- infected cells. Additionally, protein samples from *C. trachomatis* serovars J/UW-36 (Fig. 1I), L2 (Fig. 1H), or D/ UW-3 (results not shown) obtained at 24 h post infection demonstrated phosphorylated PKB/ Akt^{Ser473} abundance that did not significantly differ from those obtained from the mock- infected control cells.

4.4.2. Temporal immunoblot analyses of PKB in *C. caviae*- infected cells

To determine if the difference in abundance was influenced by the total PKB protein content, the total protein and TS fractions of *C. caviae* infected or mock-infected cells were probed with polyclonal antibodies to total PKB. At 24 h and 48 h post infection (Fig. 1E and G, respectively), the total PKB abundance in all chlamydia- infected cells or mock- infected cells is remarkably similar.

The entry of *C. caviae* requires an active PI-3K signaling pathway (43). Therefore, it was important to determine the temporal association between chlamydial infection and the depletion of phosphorylated PKB/ Akt^{Ser473} in *C. caviae*- infected cells. The total protein and TS fractions were separately obtained from *C. caviae*-

infected cells at different time points post infection including 3, 6, 12, 24, and 48 h. The protein content in each sample was normalized by the total PKB concentration. In Fig. 1 A-C, the abundance of phosphorylated PKB/ Akt^{Ser473} in the total protein lysate and TS fractions of the infected cells at early time points (3, 6, and 12 h pi, respectively) appeared virtually similar to the samples obtained from mock- infected cells. However, at 24 h to 48 h post- infection (Fig. 1 D and F), a significant decrease of phosphorylated PKB/ Akt^{Ser473} was observed while no significant changes were observed in the protein fractions of the mock- infected cells. Additionally, there were no significant changes in the abundance of total PKB observed in both protein fractions obtained from the infected or mock- infected cells (Fig. 1 E and G).

4.4.3. Localization of PKB in *C. caviae*- infected cells.

Immunofluorescence was then used to microscopically visualize the cellular localization of phosphorylated PKB/ Akt^{Ser473} in chlamydia- infected or mock-infected cells. McCoy cells were infected with *C. trachomatis* serovar J/UW-36, *C. caviae* GPIC or both strains simultaneously. The inclusions occupied by the *C. trachomatis* serovar J/UW-36 strain appear morphologically distinct from the ones

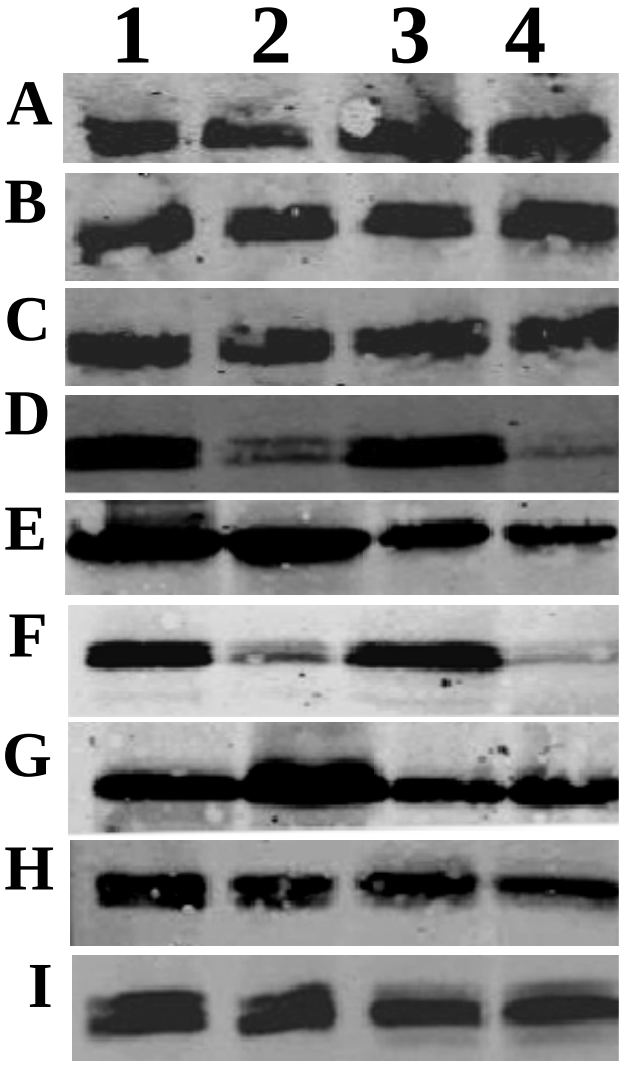

Figure 4.1. Immunoblot analyses of *C. caviae* GPIC - infected cells demonstrate depletion of phosphorylated PKB/Akt^{Ser473} during the late time points post infection. Lane designations for all panels are as follows, (1 and 3) total protein lysates and Triton X-100 soluble fractions of mock- infected McCoy cells, respectively, (2 and 4) total protein lysates and Triton X-100 soluble fractions obtained from *Chlamydia*- infected McCoy cells. Protein samples in all panels are probed with polyclonal antibodies specific to phosphorylated PKB/Akt^{Ser473}, except those in panels E and G which are probed with polyclonal antibodies specific to total PKB/ Akt. In the *Chlamydia*- infected samples in panels A-G, *C. caviae* EB were used to infect McCoy cells and protein samples were obtained at 3 h (A), 6 h (B), 12 h, (C) 24 h (D and E), and 48 h (F and G) post infection. In panels H and I, the chlamydia- infected protein lysates were obtained from cells infected with *C. trachomatis* L2 and *C. trachomatis* J/UW-36, respectively, 24 h post- infection. The molecular weight for total PKB/ Akt and phosphorylated PKB/Akt^{Ser473} are ~ 60 kDa and ~ 66 kDa, respectively.

occupied by the *C. caviae* GPIC strains. Typically, the *C. caviae* inclusions are non-fusogenic, resembling grape-like clusters, that persist throughout the developmental cycle while the *C. trachomatis* serovar J/UW-36 chlamydial forms are housed inside one large inclusion.

In Fig. 4.2.A and C, the phosphorylated PKB/ Akt^{Ser473} (green) labeling of the uninfected cell appear diffused throughout the host cell cytosol while in the *C. caviae*-infected cell (A), fixed at 24 h post infection, this protein is minimally detectable. However, in cells infected with *C. trachomatis* J/UW-36 (C) phosphorylated PKB is distributed similarly with the uninfected cells. However, when infected or mock-infected cells were probed with polyclonal antibodies to total PKB, no significant changes in labeling patterns were observed between cells infected by *C. caviae* or *C. trachomatis* and uninfected cells (Fig. 4.2. B and D).

4.5. DISCUSSION

C. caviae is the etiological agent of guinea pig inclusion conjunctivitis and, while phylogenetically distant from *C. trachomatis*, is used in animal models to study *C. trachomatis* infection in animals and humans (34). In order to understand the observed differences in host response associated with chlamydial infection, it is essential to discuss previous findings where *C. caviae* infected cells responded differently from those infected by other chlamydial strains. Most relevant to this work is the recent finding that PI-3K pathway inhibitors, such as Wortmannin and LY294002, impaired infectivity and entry of *C. caviae* (44) or *C. pneumoniae* (7), but

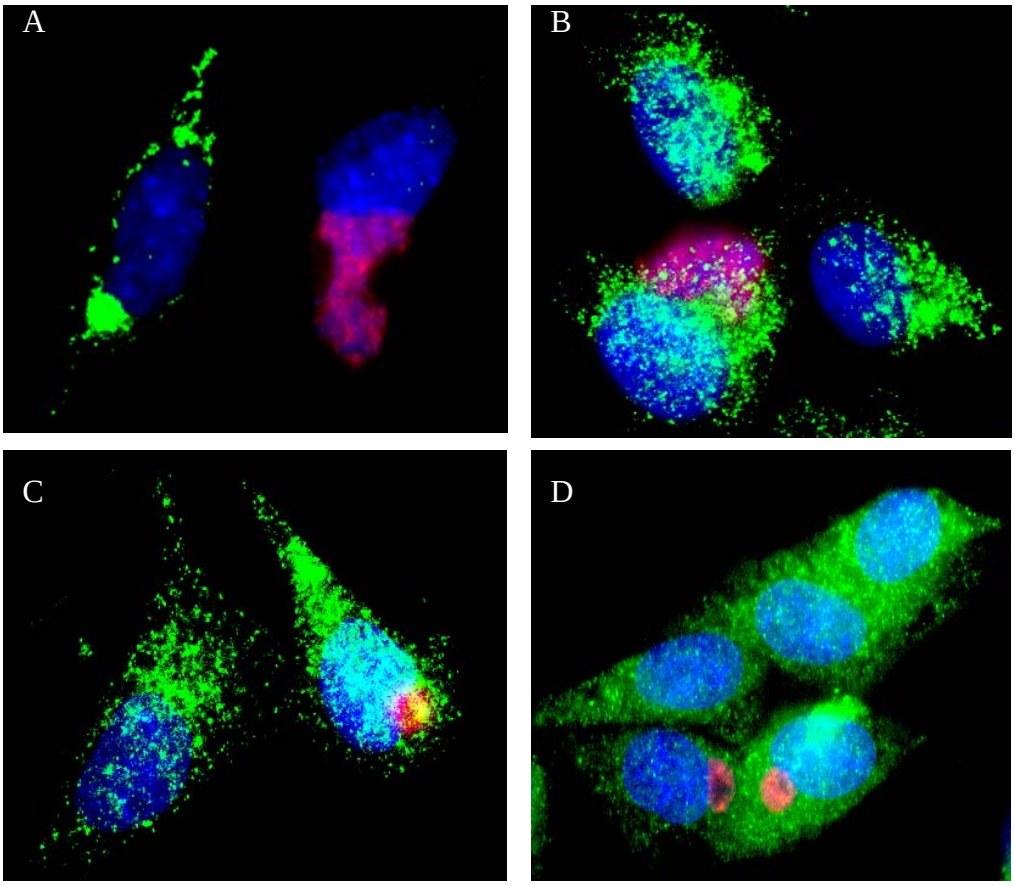

Figure 4.2. Phosphorylated PKB^{Ser473} does not accumulate in cells infected with *C. caviae* GPIC. In panels A and B, McCoy cells are infected with *C. caviae* GPIC and fixed at 24 h post infection while in panels C and D, McCoy cells are infected with *C. trachomatis* J/UW-36. Panels A and C cells and probed with antibodies to phosphorylated PKB/Akt^{Ser473} (green) and chlamydial Hsp60 (red). Panels B and D cells are probed with antibodies to total PKB (green) and chlamydial Hsp60 (red). Chlamydial and host DNA are stained with DAPI and appears blue.

not *C. trachomatis* L2 (44), into host cells. Our results also demonstrate that in *C. caviae*- infected cells, the PI3K pathway is active in that a downstream target PKB/ Akt, is phosphorylated during early time points post infection. In macrophages, PI3K catalyze the D3-phosphorylation of phosphoinositides, effectors that participate in this pathway are involved in phagosome formation and maturation (47), and important facilitators of recruiting membrane from intracellular pools and enabling pseudopod extension to accommodate ingested particles (8). Subtil *et al* suggest, however, that since chlamydial EBs are much smaller particles, it is unlikely that chlamydial entry requires the PI3K-dependent membrane recruitment for EB endocytosis (44).

While previous work suggests a function of PI3K *C. caviae* for entry, it does not offer explanation for the depletion of phosphorylated PKB/ Akt^{Ser473} during the late time points (24- 48 h) post infection. Perhaps the regulation of this pathway in *C. caviae* - infected cells during the later stages of development may function in one of several possibilities including (i) maintenance of the non-fusogenic nature of the its inclusions, (ii) acquisition of lipids from host cells such as sphingomyelin, or (iii) facilitation of fusion between chlamydial inclusion and the plasma membrane during exocytic release of *C. caviae* EB.

Recently, Alzhanov *et al* demonstrated that treatment of chlamydia- infected cells with C6-NBD-ceramide produced a fluorescence activated cell sorter (FACS) profile that distinguished between cells infected by *C. caviae* and *C. trachomatis* (2). They reported that *C. caviae*- infected cells displayed a brighter fluorescence profile when compared to cells infected by other *Chlamydia* spp, suggesting that *C. caviae* retained more fluorescent label. In light of these previous results, our data suggest that

C. caviae may induce ceramide production in infected cells that may contribute to PKB dephosphorylation events or inhibtion of phosphorylation at position Ser473.

Ceramide functions as a signaling molecule that is involved in a variety of cellular events relevant to cell growth and differentiation (18, 21, 27). Stratford *et al* reported that C2- ceramide inhibits PKB phosphorylation at position Ser473 in NIH3T3 cells (fibroblast cell line), primarily, by blocking its recruitment to the plasma membrane and preventing occurrence of phosphorylation events (43). In some cell types, such as TF-1 cells (factor- dependent human erythroleukemic cell line), ceramide promotes the de-phosphorylation of the Ser473 position of PKB by activating a phosphatase that is sensitive to calyculin and okadaic acid (39).

Collectively, these results demonstrate that phosphorylated PKB^{Ser473} is depleted in *C. caviae*- infected cells at later time points post infection. Although previous findings suggest that ceramide production is involved in PKB phosphorylation status, it will be important to determine if there is a correlation between the microscopic observations and PKB phosphorylation in *C. caviae*- infected cells and if additional downstream host signaling pathways are affected as a result of chlamydial infection.

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5. CONCLUSIONS

Despite the lack of a genetic system to manipulate chlamydiae, chlamydial research has expanded significantly in the last decade. Advanced technology has enabled chlamydiologists to utilize high-throughput instruments to sequence more chlamydial genomes in a shorter amount of time and at more reasonable costs. Newer streamlined contemporary molecular tools and techniques are being utilized to explore host- chlamydial interactions and the mechanisms involved in these processes. Specifically, these new methods contribute to the identification and characterization of host proteins and chlamydial proteins involved in the infectious process. While some emerging data offer answers to some basic chlamydial biology questions, other findings challenge old notions and introduce new questions.

Within the last few years, I examined aspects of chlamydial biology from two perspectives: the host and the chlamydiae. To characterize differences in host processes affected as a result of infection by wild- type or variant chlamydial strains, a kinome analysis was performed in Chapter 2 in which host phosphoprotein abundance was quantified in the TS fractions of protein samples obtained from infected cells. Although no significant differences were observed from this initial assay, differences in phosphoprotein abundance between mock- infected and chlamydia- infected cells were observed. The abundance of two host proteins, adducin and Raf-1, were depleted in the TS fractions of infected cells relative to mock- infected cells. However, the abundance of these phosphoproteins in the total protein lysates of infected cells is similar to the total protein lysates of mock- infected cells, suggesting that that the subcellular localization of these proteins is altered in as a result of

infection. Furthermore, a fraction of adducin is detected at the margin of chlamydial inclusions and that this localization is independent of intact actin or microtubules. Host proteins that localize to the chlamydial inclusion may serve a purpose in facilitating chlamydial intracellular survival and such interactions need further investigation.

The non- fusogenic, natural variant strains of *C. trachomatis* clinical isolates offered a unique opportunity to study chlamydial biology in the absence of a genetic system to manipulate this pathogen. In Chapter 3, previous work that characterized IncA as a participant in chlamydial vesicle fusion was expanded to investigate transcription of *incA* mRNA in IncA- negative or IncA- positive non- fuser strains. Included in these analyses was D(s) 2923, an IncA- negative nonfuser strain that contained an otherwise intact *incA* sequence and did not produce detectable IncA. All IncA- negative nonfusers produced *incA* transcripts with similar transcription initiate sites relative to IncA- positive non- fusers and wild- type chlamydial strains. However, the abundance of these transcripts in the non-fuser strains was significantly reduced, as demonstrated by Northern Blot and real- time RT-PCR assays. It is currently unknown if the reduction of transcripts is attributed to decreased production or degradation of *incA* mRNA. Because infections by these variant chlamydial strains are largely asymptomatic, it will be important to identify additional factors that contribute to the lack of IncA in non- fuser strains and determine advantages this phenotype offers chlamydiae. Recently, our laboratory has completed sequencing and annotating the genome of *C. trachomatis* variant strain D(s) 2923. It will be important

to focus on the genotypic differences observed between the wild- type and non-fuser strains.

In Chapter 4, I initiated the characterization of an unexpected result obtained from probing protein lysates obtained from other chlamydial strains, such as *C. caviae* GPIC, with the phospho- specific antibodies used in the kinome assay. In *C. caviae*-infected cells, phosphorylated PKB is depleted in the protein samples obtained from both the TS fractions and the total protein lysates. However, the abundance of total PKB in both protein samples of infected cells is similar to the protein samples obtained from mock- infected cells. These results suggest that *C. caviae*- infection affects PKB phosphorylation events, specifically during the later stages of intracellular development. It will be important to investigate the mechanisms involved in these alterations because they may be important in facilitating late stage events such as the exit of EB from cells or RB to EB maturation.

Collectively, this work has contributed more questions than answers pertaining to chlamydial biology, specifically host- pathogen interactions. Further investigation of chlamydial interaction with these proteins may fill some gaps in previously proposed models of chlamydial- host interactions. Or they may direct other investigators to look at other possibilities, not previously examined, regarding chlamydial virulence and, and perhaps, infection control and/ or vaccine development.

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