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Nectin-1 is Degraded in *Chlamydia trachomatis*-Infected Genital Epithelial Cells and is Required for Herpes Simplex Virus Co-Infection-Induced *C. trachomatis* Persistence.

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by
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ABSTRACT

Nectin-1 is Degraded in *Chlamydia trachomatis*-infected Genital Epithelial Cells and is Required for Herpes Simplex Virus Co-infection-induced *C.trachomatis* Persistence

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The obligate intracellular bacterium *Chlamydia trachomatis* is the most common bacterial STD agent in the US. This bacterium has a unique biphasic developmental cycle in which the infectious elementary body (EB) infects a host mucosal epithelial cell and differentiates into the replicative form (the reticulate body or RB) within a modified vacuole called an inclusion. The RB later divides and develops back into an EB and is released, perpetuating the infectious cycle. When developing chlamydiae are exposed to unfavorable environmental conditions, they deviate from the normal developmental cycle into a non-infectious but viable state termed persistence. Previous data from our laboratory indicate that i) during *C. trachomatis*/HSV co-infection, the chlamydiae become persistent and ii) HSV gD interaction with host cell surface is sufficient to induce this response. During viral entry, HSV gD interacts with one of four host co-receptors, one of which is the host adhesion molecule nectin-1. Interestingly, Western blotting demonstrated that nectin-1 is significantly decreased in *C. trachomatis*-infected HeLa cells. Additional studies indicated that active *C. trachomatis* replication is required for nectin-1 down-regulation and nectin-1 is likely down-regulated post-translationally. CPAF, a chlamydia-secreted protease, is responsible for degrading several host proteins.

Both *in vivo* experiments using CPAF-specific chemical inhibitors and cell-free cleavage assays using recombinant CPAF indicate that nectin-1 is degraded by CPAF in *C. trachomatis*-infected cells. Further studies suggest that nectin-1 is the most likely candidate involved in triggering HSV-induced chlamydial persistence. Co-infection experiments using nectin-1-specific HSV-1 mutants suggest that nectin-1 is, indeed, required for persistence induction. Additional studies in single co-receptor-expressing CHO cells demonstrate that, despite the fact that HSV-1 enters both HVEM- and nectin-1-expressing cells, viral co-infection reduces chlamydial infectivity only in the CHO-nectin-1 cell line. These data confirm that HSV/nectin-1 interaction is sufficient for chlamydial persistence induction. Although nectin-1 ligation is known to activate Cdc42, pull-down assays indicate that Cdc42 is not activated in co-infected HeLa cells. Taken together, these data suggest that: i) HSV gD-nectin-1 binding activates a novel host epithelial cell pathway that restricts chlamydial development and ii) the chlamydiae may degrade nectin-1 to evade this inhibitory host response.

DEDICATION

This manuscript is dedicated to my husband Dr. Yiyong Liu. Thank you for always being there to support me. Thank you for always encouraging me to never give up on my dreams. Thank you for always inspiring me to aim high and accomplish.

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INTRODUCTION

Chlamydia trachomatis

Members of the genus *Chlamydia* are the etiologic agents of several important human diseases. The species *Chlamydia pneumoniae* is a widespread respiratory tract pathogen that primarily causes community-acquired pneumonia. According to reports from the Centers for Disease Control and Prevention (CDC), in the Unites States, approximately 50% of young adults and 75% of elderly persons have serological evidence of previous *C. pneumoniae* infection (CDC, 2005). It is estimated that 300,000 cases of *C. pneumoniae* pneumonia occur each year. Recently, seroepidemiological studies have indicated a possible association between chronic *C. pneumoniae* infection and cardiovascular disease (Kuo *et al.*, 1993; Saikku *et al.*, 1988) as well as late-onset Alzheimer's disease (Balin *et al.*, 1998). In addition, there is evidence linking unresolved respiratory *C. pneumoniae* infection with asthma (Hahn *et al.*, 1991) and chronic obstructive pulmonary disease (Blasi *et al.*, 1993). The species *Chlamydia psittaci*, a zoonotic pathogen common in birds, causes psittacosis in humans. Although the illness caused by psittacosis is usually mild to moderate in severity, it can be life threatening if not properly diagnosed and treated (Kirchner, 1997).

The third major human species of genus *Chlamydia* is *Chlamydia trachomatis*, which includes two biovars: the Lymphogranuloma Venereum (LGV) biovar and the trachoma biovar. The LGV biovar is subdivided into 4 serovars: L1, L2, L2a, and L3. They primarily affect lymphatic tissue after invasion through an epithelial surface, causing an invasive sexually transmitted disease. LGV infections usually present with genital ulcers, papules, or inguinal lymphadenopathy (buboes) (Mabey & Peeling, 2002). The trachoma biovar is subdivided into 14 serovars (A-K, Ba, Da, and Ia), based on antiserum specificity to the variable region of the major outer membrane protein (MOMP). Serovars A, B, Ba, and C are the causative agents of trachoma, the world's

leading cause of infectious blindness (Thylefors *et al.*, 1995). It is estimated that active trachoma affects approximately 84 million people, causing an estimated loss of $2.9 billion in productivity annually (Kumaresan & Mecaskey, 2003), and that about 1.3 million people are blinded by these bacteria (Wright *et al.*, 2008). Serovars D-K are considered the most common sexually transmitted bacterial pathogens worldwide (Gerbase *et al.*, 1998), with an estimated 4 million new cases each year in the United States. Initial *C. trachomatis* genital tract infections are often asymptomatic, which allows the bacteria to ascend the genital tract undetected, and thus untreated. Eventually, this will lead to severe complications, including epididymitis and prostatitis in men as well as salpingitis, endometritis, pelvic inflammatory disease, ectopic pregnancy and infertility in women (Darville, 2000a). Infections by *C. trachomatis* serovars D to K also cause neonatal conjunctivitis (Schachter *et al.*, 1986) and pneumonia (Beem & Saxon, 1977) by vertical transmission through an infected birth canal.

The Chlamydial Developmental Cycle

All chlamydiae are Gram-negative obligate intracellular bacteria and share a unique biphasic developmental cycle (Fig. 1.1). *C. trachomatis* alternates between two morphologically distinct forms. First, the extracellular, infectious but metabolically inert form (the elementary body or EB, 0.3µm diameter) attaches to and enters mucosal epithelial cells via receptor mediated endocytosis (Wyrick, 2000). EBs are characterized by a highly compacted nucleoid due to the condensation by the chlamydial histone-like proteins HctA and HctB (Barry *et al.*, 1992; Brickman *et al.*, 1993). EBs have little or no peptidoglycan in their cell walls (Ghuysen & Goffin, 1999). Instead, structural rigidity is provided by the highly cross-linked outer-membrane complex. Several chlamydial ligands (such as MOMP, heat shock protein 70, the outer membrane complex protein OmcB, and heparan sulfate-like glycosaminoclycans) and host cell receptors (such as the heperan sulfate receptor, the mannose and mannose-6-phosphate receptors, and the estrogen receptor) have been proposed to be involved in attachment and entry of chlamydiae (Chen & Stephens, 1997; Davis & Wyrick, 1997; Davis *et al.*, 2002; Raulston *et al.*, 2002; Stephens *et al.*, 2000; Stephens *et al.*, 2001; Su *et al.*, 1996; Wyrick,

2000; Zhang & Stephens, 1992). After entry, EB-containing endosomes avoid fusion with host cell lysosomes, intercept sphingomyelin and cholesterol from the exocytic pathway and are rapidly trafficked to the perinuclear region of the host cell (Hackstadt *et al.*, 1996; Wolf & Hackstadt, 2001). Following these actions, EBs differentiate into larger (1µm diameter), metabolically active but non-infectious reticulate bodies (RBs) within an enlarged endosomal sac, termed an inclusion. The mature chlamydial inclusion is established by insertion of multiple chlamydial proteins and host cell-acquired lipids, suggesting chlamydiae actively modify the inclusion and manipulate the host cell to provide a protected intracellular niche (Hackstadt *et al.*, 1996; Hackstadt *et al.*, 1997; Wylie *et al.*, 1997). Using ATP and metabolites from the host cell, RBs grow and divide by binary fission. After 8-12 rounds of replication, the RBs mature into infectious EBs, which are released from the host cell (Wyrick, 2000).

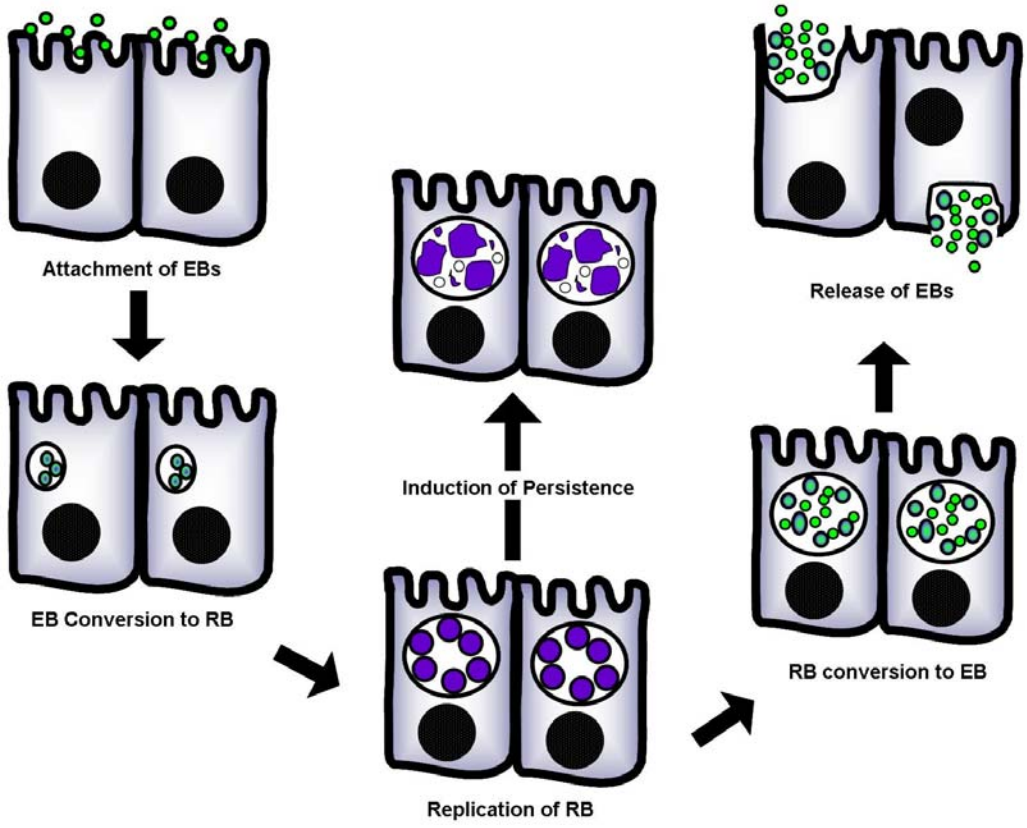

Figure 1.1 The *Chlamydia trachomatis* developmental cycle. (Courtesy Jennifer Vanover)

Chlamydial Persistence

The normal chlamydial developmental cycle can be interrupted when developing chlamydiae are exposed to unfavorable environmental factors (Beatty *et al.*, 1994a; Darville, 2000a; Gerard *et al.*, 2001; Raulston, 1997). In this situation, the chlamydiae deviate from the normal developmental cycle into a state termed persistence (Hogan *et al.*, 2004) and can remain in this state for weeks or months (Galasso & Manire, 1961). Although chlamydial persistence *in vivo* has been difficult to demonstrate, recent studies strongly suggest that chlamydiae do become persistent in humans (Dean *et al.*, 2000; Fortenberry *et al.*, 1999; Gerard *et al.*, 2001; Gerard *et al.*, 2002; Patton *et al.*, 1994). Development of persistence is thought to be a mechanism used by the chlamydiae to ensure survival under stressful conditions of growth and development (Harper *et al.*, 2000; Mpiga & Ravaoarinoro, 2006).

Persistent chlamydiae have been traditionally described as a viable but culture-negative form of chlamydiae (Beatty *et al.*, 1994b) (Fig. 1.2). During the persistent state, chlamydiae continue to synthesize unprocessed 16S rRNA and replicate chromosomes (Gerard *et al.*, 1998; Gerard *et al.*, 2001). The RBs continue to enlarge but neither undergo binary fission nor differentiate back into EBs, thus resulting in enlarged RBs and decreased EB infectivity (Beatty *et al.*, 1993; Beatty *et al.*, 1994a). At the ultrastructural level, persistent chlamydiae appear as swollen, misshapen, diffuse RBs. Inclusions containing persistent chlamydiae often contain large numbers of membrane blebs. In addition, it is reported that, during persistent state, accumulation of MOMP is decreased while the accumulation of the immunodestructive antigen HSP60 is stable or increased (Beatty *et al.*, 1993; Beatty *et al.*, 1994b). Finally, because they are still viable, persistent chlamydiae have ability to re-enter and complete the normal developmental cycle once the persistence “inducer” is removed.

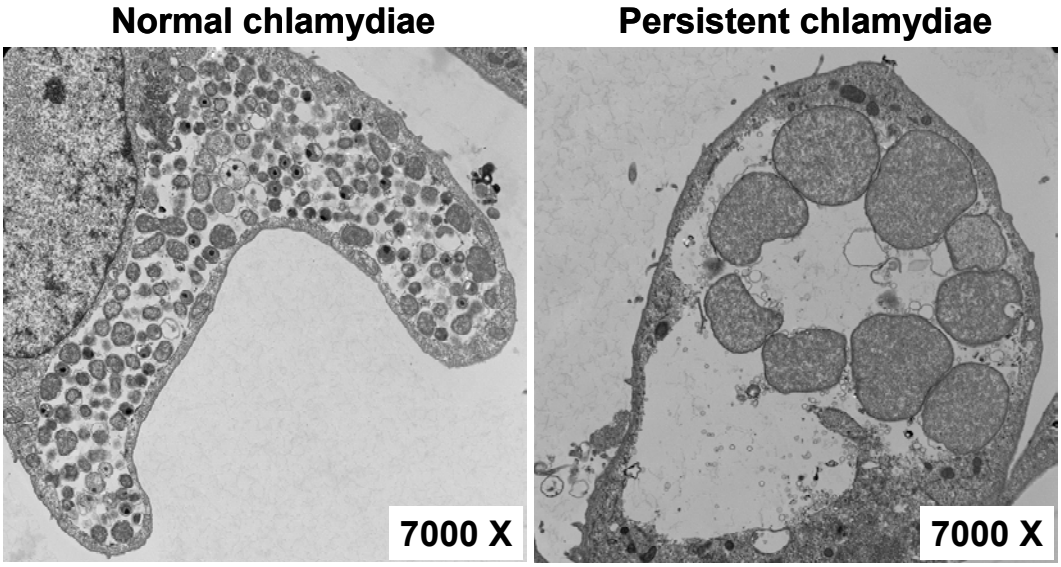

Figure 1.2 Transmission electron microscopy (TEM) pictures of normal and persistent chlamydiae.

Since chlamydial persistence represents a long-term relationship between the chlamydiae and their host cells, there has been a great amount of interest within the field to further define this phenomenon. So far, several different factors have been discovered to induce chlamydial persistence *in vitro*, including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), or penicillin G exposure as well as amino acid, iron, or glucose deprivation. Persistence has also been reported to develop following phage infection of chlamydiae (Hsia *et al.*, 2000) or chlamydial infection of monocytes (Koehler *et al.*, 1997). Spontaneous development of chlamydial persistence has also been found to occur in continuous cultures without presence of any stresses (Shatkin *et al.*, 1985).

Propagation of chlamydiae in host cells deprived of amino acids, glucose, or iron has been shown to induce persistence. Under iron restriction, the inclusions of *C. trachomatis* serovar E are small and surrounded by electron-dense material (Raulston, 1997). The RBs within these inclusions become abnormal with loose and wavy outer

membranes (Raulston, 1997). Minor reductions in amino acids or glucose concentration in culture media also induce persistence (Coles *et al.*, 1993; Harper *et al.*, 2000). In all cases where nutrient restriction has been used to induce persistence, the chlamydiae re-enter the normal developmental cycle following supplementation with the appropriate nutrients (Coles *et al.*, 1993; Harper *et al.*, 2000; Raulston, 1997).

Of the cytokine-induced persistence models, IFN-γ-induced persistence has been best characterized. For example, infection of HeLa cells with *C. trachomatis* serovar A followed by exposure to 0.2 μg/ml IFN-γ produces small chlamydial inclusions containing enlarged and abnormal RBs (Beatty *et al.*, 1993). The mechanism behind this phenomenon is that IFN-γ exposure increases host cell indoleamine 2,3-dioxygenase (IDO) expression, leading to the conversion of tryptophan to N-formylkynurenine. The resultant tryptophan deficiency drives the developing chlamydiae into the persistent state. Removal of IFN-γ from the culture media or addition of tryptophan to the culture media allows the persistent chlamydiae to recover and resume normal growth (Beatty *et al.*, 1993; Byrne *et al.*, 1986). In murine cells, IFN-γ exposure induces chlamydial persistence via a different mechanism. IFN-γ activates the p47 GPTase, Iigp1, which inhibits chlamydial development by restricting vesicular trafficking to the developing inclusions (Nelson *et al.*, 2005).

Finally, many researchers have also observed that exposure of chlamydia-infected cells to antibiotics, such as penicillin, ofloxacin, or ciprofloxacin, induces development of greatly enlarged and aberrant RBs (Dreses-Werringloer *et al.*, 2000; Johnson & Hobson, 1977; Segreti *et al.*, 1992). The RBs resume normal growth after removal of antibiotics. Generally, agents that target chlamydial RNA or protein synthesis inhibit chlamydial differentiation from EB to RB or from RB to EB, while those that target DNA or peptidoglycan synthesis inhibit only RB to EB differentiation (Moulder, 1991). These observations indicate that inappropriate antimicrobial therapy may be a significant cause of persistent chlamydial infection *in vivo*.

Chlamydia Protease-like Activity Factor (CPAF)

The unique, biphasic chlamydial developmental cycle involves a complex interaction between the pathogen and its obligate host. To ensure their successful intracellular replication and long-term survival within infected host cells, chlamydiae have evolved various strategies to evade host defense systems and to modulate host cell functions. For example, during chlamydial infection, chlamydiae prevent productively and persistently infected host cells from undergoing apoptosis, which may allow them to maintain long-term survival within infected cells (Dean & Powers, 2001; Dong *et al.*, 2005a; Fan *et al.*, 1998; Fischer *et al.*, 2004; Geng *et al.*, 2000; Pirbhai *et al.*, 2006; Rajalingam *et al.*, 2001). Chlamydiae alter host cell-cell contacts to facilitate chlamydial growth (Prozialeck *et al.*, 2002). Chlamydiae can also modify host cell cytoskeletal arrangement and signal transduction to benefit chlamydial intravacuolar replication (Clausen *et al.*, 1997; Majeed & Kihlstrom, 1991; Majeed *et al.*, 1993; Schramm & Wyrick, 1995; Xia *et al.*, 2003).

Another strategy that chlamydiae use to survive in their intracellular habitat is the secretion of chlamydia protease-like activity factor (CPAF). CPAF is synthesized within the chlamydial inclusion and subsequently secreted into the host cell cytoplasm (Fan *et al.*, 2002; Zhong *et al.*, 2001). CPAF has been shown to be produced by the two human pathogens *C. trachomatis* and *C. pneumoniae* as well as by the three non-human pathogens, *C. muridarum*, *C. psittaci* and *C. caviae* (Dong *et al.*, 2005b; Fan *et al.*, 2002; Zhong *et al.*, 2001). Further studies demonstrated that there is little or no difference in CPAF expression between persistent and productive chlamydial infections, suggesting that CPAF production may be important in both types of infection (Heuer *et al.*, 2003; Shaw *et al.*, 2002). CPAF is encoded by a single open reading frame (ORF) in the chlamydial genome, but the main purified product consists of two fragments. CPAF is initially produced as an inactive proenzyme of 70 kDa and rapidly processed into two fragments, a 35 kDa C-terminal portion (designated CPAFc) and a 29 kDa N-terminal portion (designated CPAFn), which must form intramolecular dimers to acquire proteolytic activity (Dong *et al.*, 2004a; Dong *et al.*, 2004b). Studies by Dong *et al*

further confirmed that cleavage of CPAF is both necessary and sufficient for CPAF activity (Dong *et al.*, 2004a). The proteolytic activity of CPAF is inhibited by the cytosolic proteasome inhibitor lactacystin but not by other proteasome inhibitors such as MG132 and MG115, or by broad spectrum protease inhibitors, such as PMSF and pepstatin A (Zhong *et al.*, 2000; Zhong *et al.*, 2001).

A number of studies suggest that CPAF plays important roles in promoting chlamydial replication and protecting infected cells from host immune detection. For example, CPAF has been shown to cleave RFX5 and USF-1, two eukaryotic transcription factors required for both major histocompatibility complex (MHC) class I and II antigen expression (Zhong *et al.*, 1999; Zhong *et al.*, 2000). This process may help infected cells escape immune detection so that the chlamydiae have sufficient time to complete their replication in infected hosts. Similarly, CPAF has been found to be responsible for degrading keratin-8 (Dong *et al.*, 2004c), pro-apoptotic BH-3 only proteins (Pirbhai *et al.*, 2006), and CD1d glycoprotein (Kawana *et al.*, 2007). Keratin-8 is a key subunit of intermediate filaments in epithelial cells. Cleavage of keratin-8 would increase the solubility of the host cell cytoskeleton, thus facilitating the expansion of chlamydia-laden inclusion, which is essential for chlamydial intracellular replication. Pro-apoptotic BH-3 only proteins are required for apoptosis induction in response to cytotoxic stimuli or exogenous stress, and they function by neutralizing their pro-survival counterparts or activating pro-apoptotic Bcl-2 family members Bax and Bak (Bouillet & Strasser, 2002). Down-regulation of BH-3 only proteins contributes to chlamydial anti-apoptotic activity which may help chlamydiae evade CTL-mediated host cell apoptosis induction and, thus, benefit chlamyidal long-term survival within infected host cells (Pirbhai *et al.*, 2006). CD1d glycoprotein is an MHC-like protein that presents lipid antigens to natural killer T cells. Degradation of CD1d protein may prevent recognition or elimination of chlamydiae from the host innate immune system (Kawana *et al.*, 2007). Taken together, CPAF, the first example of a cytoplasmically localized chlamyidal protease, seems to execute its functions by degrading host cell proteins to either facilitate chlamydial intravacuolar replication or help chlamydiae evade host immune surveillance.

Nectins

In epithelial cells, intercellular adhesions are intimately involved in maintaining cell structure, transmitting information, regulating cellular migration, and imparting strength and rigidity to tissues (Farquhar & Palade, 1963; Gumbiner, 1996; Takeichi, 1995). Intercellular adhesions are mainly maintained by cell-cell adherens junctions (AJs) and tight junctions (TJs) (Farquhar & Palade, 1963). Importantly, the organization and functions of TJs are dependent on the formation and maintenance of AJs (Tsukita & Furuse, 1999). TJs are formed at the apical side of AJs and regulate selective diffusion of solutes and water along the paracellular pathway. Thus, TJs act as a fence between the apical and basolateral plasma membrane domains in epithelial cells (Tsukita *et al.*, 1999; Tsukita *et al.*, 2001). At TJs, three cell adhesion molecules (CAMs) {claudin, occludin, and junctional adhesion molecule (JAM)} have been identified, and they are linked to the actin cytoskeleton through peripheral membrane proteins such as ZO-1, -2 and -3 (Tsukita *et al.*, 2001). AJs are located immediately at the basal side of TJs and connect the lateral surfaces of adjacent epithelia cells. AJs form complexes with actin and myosin filaments to internally brace cells and thereby control their shape. AJs play important roles not only in the formation and maintenance of TJs, but also in concentrating biologically active molecules such as cell surface receptors, signaling molecules, and oncoproteins (Gumbiner, 2000; Nagafuchi, 2001; Perez-Moreno *et al.*, 2003; Tsukita *et al.*, 1992; Van Aelst & Symons, 2002). E-cadherin, an important component of AJs, is a Ca^{2+}-dependent cell-cell adhesion molecule found in epithelial cells (Takeichi, 1991). E-cadherin is a member of the cadherin superfamily, which consists of more than 80 members. Most cadherins are single-membrane-spanning molecules and are associated with the actin cytoskeleton through peripheral membrane proteins, including α- and β-catenins, vinculin, and α-actinin (Gumbiner, 2000; Nagafuchi, 2001; Takeichi, 1995). It was previously believed that AJs were formed by cadherins, but recent evidence indicates that AJs are formed by both cadherins and nectins (Irie *et al.*, 2004; Nakanishi & Takai, 2004; Ogita & Takai, 2006a; Sakisaka & Takai, 2004; Shimizu & Takai, 2003; Takai *et al.*, 2003; Takai & Nakanishi, 2003; Takeichi, 1991).

Nectins are Ca^{2+}-independent, immunoglobulin (Ig)-like cell-cell adhesion molecules with three extracellular Ig-like loops, a single transmembrane region (except for nectin-1γ) and a cytoplasmic tail. The nectin family currently contains at least 4 members (nectins-1~4) (Sakisaka & Takai, 2004; Takai & Nakanishi, 2003). Nectin-1, nectin-2 and nectin-3 are widely distributed in a variety of tissues and cell types, such as epithelial cells, neurons, and fibroblasts (Takai & Nakanishi, 2003). Nectin-2 and nectin-3 are also found in blood cells and spermatids, where cadherins are not expressed (Bouchard *et al.*, 2000; Lopez *et al.*, 1998; Ozaki-Kuroda *et al.*, 2002). Human nectin-4 appears to be primarily distributed in placenta (Reymond *et al.*, 2001). Each nectin first forms *cis*-dimers homophilically and then the *cis*-dimers homophilically or heterophilically interact in *trans* with each other to form *trans*-dimers. The heterophilically formed *trans*-interactions are stronger than the homophilically formed *trans*-interactions (Sakisaka & Takai, 2004). In contrast, cadherins only can form homophilic *trans*-dimers (Takeichi, 1995). In addition to interacting with each other, nectins also interact in *trans* with other Ig-like molecules such as nectin-like molecules (Necls), CD226/DNAM-1, and CD96/Tactile (Bottino *et al.*, 2003; Ikeda *et al.*, 2003; Kakunaga *et al.*, 2005; Seth *et al.*, 2007; Shingai *et al.*, 2003). The cytoplasmic tail of each nectin member binds to afadin, an actin filament (F-actin)-binding protein, which connects nectin to the actin cytoskeleton (Takai & Nakanishi, 2003). Afadin has multiple domains and acts as an adaptor protein by binding many scaffolding proteins and F-actin-binding proteins, associating nectins with other cell-cell adhesion and intracellular signaling pathways (Takai *et al.*, 2008). This novel nectin-afadin adhesion system is required not only for the formation of AJs but also for the subsequent formation of TJs, as well as for the formation of functional cell-cell junctions, acting cooperatively with or independently of cadherins (Takai & Nakanishi, 2003). Additionally, this nectin-afadin adhesion system has been shown to be novel mediator of many other cellular events including movement, proliferation, differentiation, polarization, and the entry of viruses by interacting with other CAMs and cell surface receptors. For example, *trans*-interactions of nectins induce the activation of c-Src. The activated c-Src stimulates the small G proteins Cdc42 and Rac, which are eventually involved in cell-cell adhesion through reorganization of the actin cytoskeleton, gene expression by activation of the

JNK pathway, and cell polarization by cell polarity proteins (Honda *et al.*, 2003; Kawakatsu *et al.*, 2002). Furthermore, this adhesion system regulates the formation of synapses in neurons and the organization of heterotypic junctions between Sertoli cells and spermatids in the testis (Bouchard *et al.*, 2000; Mizoguchi *et al.*, 2002; Ozaki-Kuroda *et al.*, 2002).

The functions of nectin-1 and nectin-2 in mediating the entry of viruses into host cells have been extensively studied. The cDNAs of nectin-1 and nectin-2 were originally described as encoding poliovirus-receptor-related proteins, and, therefore were named PRR-1 and PRR-2 (Eberle *et al.*, 1995; Lopez *et al.*, 1995). However, neither PRR-1 nor PRR-2 has been shown to serve as a poliovirus receptor. Because they were later demonstrated to serve as receptors for α-herpes viruses, they were renamed HveC and HveB, respectively (Geraghty *et al.*, 1998; Warner *et al.*, 1998). Finally, they were renamed nectin-1 and nectin-2, respectively, due to their roles in cell-cell adhesion. Nectin-1 and nectin-2 have different affinity specificities for wild-type alphaherpesviruses. Nectin-1 mediates entry of all α-herpes viruses tested so far, including herpes simplex virus type I (HSV-1) and type 2 (HSV-2), as well as pseudorabies virus (Campadelli-Fiume *et al.*, 2000; Spear *et al.*, 2000). In contrast, nectin-2 primarily mediates the entry of HSV-2 into host mucosal epithelial cells (Campadelli-Fiume *et al.*, 2000; Spear *et al.*, 2000). It remains unknown whether nectin-3 and nectin-4 serve as receptors for viruses.

Herpes Simplex Virus (HSV)

HSV-1 and HSV-2 are members of the viral family *Herpesviridae* and cause a range of infections from mild uncomplicated mucocutaneous infection to life threatening disease such as meningitis. Although both HSV-1 and HSV-2 can infect either oral or genital sites, HSV-1 is more often associated with oral infections such as cold sores or fever blisters, while HSV-2 is considered the primary etiological agent of genital herpes. HSV can infect many cell types, but the two most important cellular targets are mucosal/skin epithelial cells and neurons. After primary infection of epithelial cells, the

virus establishes latent infection within sensory and autonomic neurons whose axons extend to the locale of the lesions. Latent infection can be periodically reactivated in response to many stimuli such as colds and influenza, emotional and physical stress, immune suppression, exposure to bright sunlight, gastric upset, fatigue, or injury. Reactivated virus transports, via the nerve's axon, back to the site of original infection to cause recurrent lesions. Most people suffer an average of 5 reactivations per year, during which lesions and virions are present (Corey *et al.*, 1983). Evidence also suggests that HSV-2 is commonly shed in the absence of detectable symptoms (Mertz *et al.*, 1992).

The HSV virion has a large double-stranded DNA genome about 150 kbp. The genome is packed within a capsid, an icosahedral protein cage, containing 162 capsomers or morphological elements (Spear, 2004). The capsid is in turn coated with a protein layer (the tegument) and, subsequently, an envelope composed of lipids and more than a dozen viral proteins and glycoproteins. Several envelope glycoproteins are essential for viral entry (Spear, 2004). HSV-1 and HSV-2 each contain at least 74 genes within their genomes, encoding proteins involved in forming the viral capsid, tegument and envelope, as well as controlling the replication and infectivity of the virus (Dolan *et al.*, 1998).

Entry of HSV (Fig. 1.3A) into host cells is initiated when the virion binds to the cell surface receptor heparan sulfate using the viral envelope proteins gC and/or gB (to a lesser extent). Although this binding of gC or gB with heparan sulfate significantly increases the efficiency of HSV infection, it is not absolutely essential (Spear, 2004). After initial binding, the viral gD envelope glycoprotein interacts with one of 4 cellular co-receptors including HVEM (herpes virus entry mediator), nectin-1, nectin-2, or 3-O sulfated heparan sulfate (3-O-S-HS) (Spear, 2004). HVEM is a member of the tumor necrosis factor (TNF) receptor family which is present on B and T lymphoctyes, other leucocytes, fibroblasts, and epithelial cells, but not on neurons (Hsu *et al.*, 1997; Kwon *et al.*, 1997; Marsters *et al.*, 1997; Montgomery *et al.*, 1996). HVEM can be used as a co-receptor by both HSV-1 and HSV-2 with equally high efficiency (Spear, 2004). Nectin-1 and nectin-2, as described in the nectins section, are two cell adhesion molecules belonging to the immunoglobulin superfamily which are expressed on fibroblasts,

neurons, and epithelial cells. Nectin-1 mediates entry of both HSV-1 and HSV-2, while nectin-2 primarily mediates entry of HSV-2 (Spear, 2004). 3-O-S-HS is wildly distributed on human cells and primarily mediates HSV-1 entry (Spear, 2004). Binding of gD to any one of the these co-receptors facilitates the fusion of viral envelope with the host cell plasma membrane. Viral envelope-membrane fusion also requires assistance from viral gB and gH/gL proteins (Spear *et al.*, 2000; Spear, 2004). HSV was previously believed to enter cells only via envelope-membrane fusion, but recently it has been reported that HSV entry can also occur through direct penetration of the cell plasma membrane or by endocytic pathways, depending on the host cell line used.

After fusion occurs, the virus releases the capsid-tegument structure into the cytoplasm (Fig. 1.3B). Subsequently, the capsid is transported to the host nucleus where the viral DNA genome circularizes and is transcribed. Immediate early genes (or α-genes) are first transcribed which are involved in transcriptional regulation and are not found in the mature virion. They also promote transcription of early genes (or β-genes). Early proteins include DNA polymerase and enzymes, which are mainly involved in DNA replication. Late genes (or γ-genes), transcribed after DNA synthesis, are translated in the cytoplasm and then transported back to the nucleus. Viral envelope glycoproteins are synthesized in the rough endoplasmic reticulum where they obtain high mannose sugar chains. They are then processed by the host secretory pathway and localized to the nuclear and cytoplasmic membranes, where they are surface exposed. New virions are assembled in the nucleus, bud through the nuclear membrane via areas in which the viral envelope glycoproteins are enriched, and egress the host cell by vesicular transport (Roizman, 2001b).

Numerous studies have demonstrated that HSV infection can modulate host cell signal transduction pathways. Yokota *et al* has reported that during the early stage (2 to 3 hour post infection) of HSV-1 infection in human amnion FL cells, the IFN signal pathway is suppressed by inhibiting IFN-induced phosphorylation of STATs and Janus kinases (Yokota *et al.*, 2001). In HSV-infected T cells, T cell receptor (TCR) signal transduction is suppressed by inhibition of the activation of LAT (linker for activation of

T cells) and the steps distal to LAT in the TCR signal cascade, including inhibition of calcium flux and multiple MAPK (Sloan *et al.*, 2006). In human monocyte cell lines, interaction of gD with HVEM is involved in NF-κB-dependent protection against apoptosis (Sciortino *et al.*, 2008; Teresa Sciortino *et al.*, 2007). HSV infection also induces the expression of host cellular suppressor of cytokine signaling-3 (SOCS3), a negative regulator of the JAK/STAT pathway (Yokota *et al.*, 2005), conferring efficient viral replication. Additionally, Hoppe *et al* have demonstrated that the small Rho-like GTPases Rac1 and Cdc42 are activated at 15 to 30 minutes after HSV-1 infection of epithelial MDCK canine kidney cells (Hoppe *et al.*, 2006). Inhibition of Cdc42 activity using a dominant-negative inhibitor decreased both Cdc42 activation and subsequent viral replication. Since Rac1 and Cdc42 are members of the nectin-1 *trans*-interaction induced signaling pathway, it is speculated that HSV-1/nectin-1 interaction is responsible for activation of Rac1 and Cdc42 (Hoppe *et al.*, 2006).

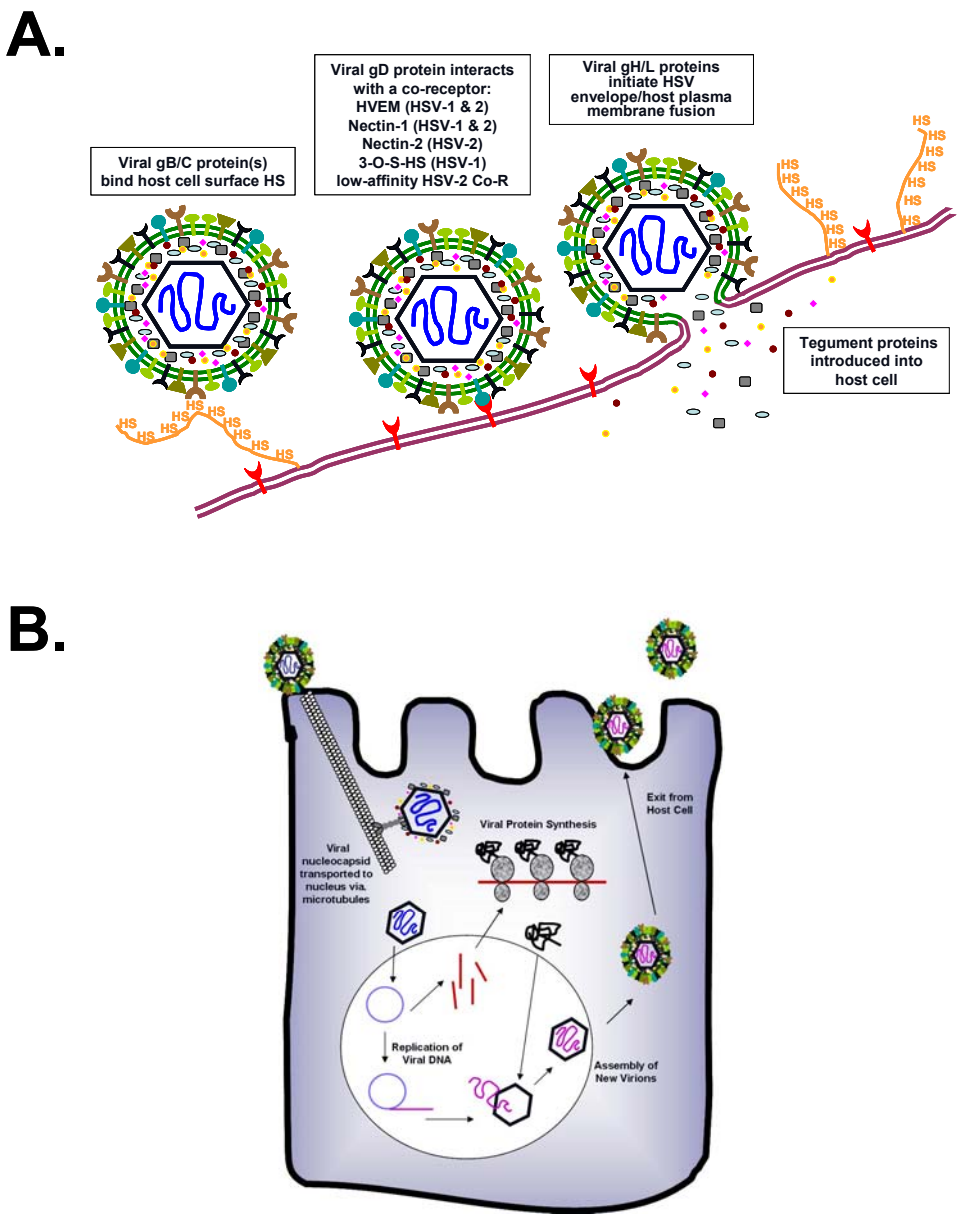

Figure 1.3 Replication cycle of HSV. A. Attachment and entry of HSV virions into the host cell. B. Replication and release of HSV from the host cell.

HSV and *C. trachomatis* Co-infection

Despite substantial improvement in the prevention of sexually transmitted diseases (STDs), they still represent a substantial fraction of the disease burden. According to reports from the CDC, STDs are among the most common infections in the United States. Approximately 19 million new cases of STDs occur each year in the United States, almost half of them among people ages 15 to 24 (CDC, 2006; Weinstock *et al.*, 2004). In addition to the health consequences of STDs, these diseases also cause a severe economic burden. The direct medical costs of treating these diseases were estimated at up to 14.7 billion dollars in the United States in 2006 (CDC, 2006). Moreover, epidemiological studies have demonstrated that infection with one STD increases the chance for individuals to acquire others (Cameron *et al.*, 1989; Laga *et al.*, 1993; Plummer *et al.*, 1991). For instance, HSV-2 infection increases the risk for acquiring Human Immunodeficiency Virus (HIV) infection by 3-fold (Freeman *et al.*, 2007).

In the United States, two of the most common STD agents are *C. trachomatis* (serovars D-K), causing 4 million new infectious diseases annually and HSV (primarily HSV-2), causing 200,000-500,000 new infectious cases annually (Johnson *et al.*, 1989). A number of studies have shown that *C. trachomatis* and HSV-2 co-infections occur *in vivo*. Both pathogens have been simultaneously isolated from the genital tract of women afflicted with endometritis and salpingitis (Paavonen *et al.*, 1985) or cystitis (Tait *et al.*, 1985). Serological studies indicate that HSV-2 positive individuals are likely to be *C. trachomatis*-positive as well (Paroli *et al.*, 1990; Silins *et al.*, 2002; Vetter *et al.*, 1990). In addition, seropositivity rates of > 50% for both HSV-2 and *C. trachomatis* have been reported, implying that some participants had been exposed to both pathogens (Duncan *et al.*, 1992; Wagner *et al.*, 1994). Although IgG seropositivity can not be used to establish concurrent infection, it is likely that both organisms were present simultaneously in the genital tracts of some individuals, since *C. trachomatis* often causes long-term infections and HSV-2 reactivates on average 5 times per year.

Several groups have also established HSV/*C. trachomatis* co-infection in cell culture. Chiarini *et al* demonstrated that the number of cells positive for chlamydiae by immunofluorescence was reduced when HeLa cells were co-infected with HSV-2/*C. trachomatis* serovar D (Chiarini *et al.*, 1996). Pontefract *et al* observed that Vero cells co-infected with *C. trachomatis* serovar L2 and HSV-2 contained swollen inclusions with few RBs or EBs (Pontefract *et al.*, 1989). HSV-2 pre-infection of HT-1376 human bladder cells reduced production of infectious *C. trachomatis* EBs by about 15-fold (Superti *et al.*, 2001). These observations indicated that HSV-2 co-infection might alter the chlamydial developmental cycle in a way similar to that observed during persistent infections.

Summary of Previous Co-infection Studies from Our Laboratory

Although previous studies on HSV-2/*C. trachomatis* co-infection suggested that HSV-2 co-infection alters chlamydial development, these studies did not examine the co-infection process in detail. Recently, we developed a tissue culture model of HSV-2/*C. trachomatis* serovar E co-infection. Data from this model indicate that HSV-2 super-infection of *C. trachomatis*-infected genital epithelial cells induces chlamydial persistence, as evidenced by significantly decreased infectious chlamydial EB production, abnormal RB morphology, increased membrane blebs and vesicles within inclusions, as well as continued accumulation of chlamydial genomic DNA and unprocessed RNA, but no infectivity (Deka *et al.*, 2006).

Furthermore, co-infection of HeLa or HEC1B cells with *C. trachomatis* serovar E and either HSV-2 or HSV-1 reduces chlamydial EB titer, alters inclusion ultrastructure but does not reduce chlamydial DNA copy number, indicating that induction of chlamydial persistence by HSV is neither host cell type nor virus strain specific (Deka *et al.*, 2007). UV-inactivated HSV (HSV-2_{uv}) attaches to and enters host cells but does not replicate (Moxley *et al.*, 2002). Cyclohexamide, a well known eukaryotic protein synthesis inhibitor, inhibits *de novo* synthesis of host cell proteins (Vasquez, 1979) and all kinetic classes of HSV proteins as well as preventing HSV productive replication

(Fenwick & Walker, 1978; Swanstrom *et al.*, 1975) but does not affect *C. trachomatis* development (Ripa & Mardh, 1977). Co-infection of HeLa cells with HSV-2_{uv} or with replication-competent HSV-2 in the presence of cyclohexamide continues to drive *C. trachomatis* into a persistent state, suggesting productive HSV replication is not required for induction of chlamydial persistence, and HSV attachment or entry is sufficient for this effect (Deka *et al.*, 2007).

Global deprivation of amino acids, iron, or glucose can cause developing chlamydiae to enter persistence (Harper *et al.*, 2000; Hogan *et al.*, 2004; Raulston, 1997). However, supplementation of HSV-2/*C. trachomatis* co-infected cells with excess amino acids, iron-saturated transferrin, glucose, or a combination of amino acids and glucose does not rescue EB infectivity, suggesting that viral co-infection-induced chlamydial persistence is not due to amino acid, iron, or glucose deprivation (Vanover *et al.*, 2008). Luminex assays indicate that IFN-γ, IFN-α and TNF-α, three well-studied inducers of chlamydial persistence, are not released from co-infected cells. Semiquantitative RT-PCR experiments demonstrate that IFN-β, IFN-γ, IDO, lymphotoxin-α, and inducible nitric oxide synthase (iNOS) are not expressed during co-infection. These data indicate that viral-induced persistence is not stimulated by any cytokine currently known to induce host anti-chlamydial responses (Vanover *et al.*, 2008). Also, inclusions within co-infected cells continue to enlarge and incorporate C6-NBD-ceramide, indicating that HSV-2 co-infection does not inhibit vesicular transport to the developing inclusion (Vanover *et al.*, 2008). Therefore, taken together, these data demonstrate that HSV co-infection-induced chlamydial persistence is not mediated by any currently identified persistence inducer or anti-chlamydial pathway (Vanover *et al.*, 2008).

Studies show that persistent chlamydiae re-enter the normal developmental cycle and re-gain infectivity following removal of persistence inducers or replacement of deficient/depleted nutrients (Harper *et al.*, 2000; Matsumoto & Manire, 1970; Raulston, 1997). Our unpublished data indicate that chlamydiae can recover infectivity within 44hr during long-term co-infection with UV-inactivated HSV-2, demonstrating that, like other persistence inducers, HSV-induced chlamydial persistence is reversible (Vanover, 2009b).

Moreover, contact between paraformaldehyde-fixed, HSV-2-infected cells and viable *C. trachomatis*-infected cells induces chlamydial persistence, indicating that HSV entry is not required for chlamydial persistence induction and suggesting that interaction between HSV surface glycoproteins and a host cell receptor is sufficient for triggering chlamydial persistence (Vanover, 2009b). More importantly, subsequent studies in our laboratory determined that HSV glycoprotein D interaction with host cell surface renders sufficient stimulus to induce chlamydial persistence, probably via a novel host anti-chlamydial pathway (Vanover, 2009b).

Questions to be Answered in the Studies

Upon infection, chlamydiae modulate host cell functions in a variety of ways in order to ensure their replication, dissemination, and long-term survival within infected hosts. It has been reported that *C. trachomatis* infection disturbs cell-cell contacts between polarized epithelial cells in culture (Wyrick *et al.*, 1989). Notably, release of *C. trachomatis*-infected epithelial cells has been observed *in vivo* as well (Doughri *et al.*, 1972; Soloff *et al.*, 1985). In both studies by Doughri *et al* and Soloff *et al*, the authors hypothesized that polymorphonuclear neutrophils (PMNs) responding to chlamydial infection promote detachment of chlamydia-infected cells from the mucosal surface, resulting in the release of intact, infected cells into the cervical lumen (Doughri *et al.*, 1972; Soloff *et al.*, 1985). However, in the absence of PMNs, Prozialeck *et al* have observed that chlamydial infection also loosens cell-cell contacts by promoting breakdown of the N-cadherin/β-catenin complex (Prozialeck *et al.*, 2002), suggesting that the developing chlamydiae may contribute directly to the release of observed *in vivo* of infected cells from the infected mucosa. Recently, it has been elucidated that the nectin-based adhesion system is required not only for the formation of AJs but also for the subsequent formation of TJs as well as for the formation and maintenance of functional cell-cell junctions, acting cooperatively with or independently of cadherins (Takai & Nakanishi, 2003). To find out whether *C. trachomatis* infection alters nectin-1 in genital epithelial cells, we have systematically examined the accumulation and expression level of nectin-1 using Western blotting, immunofluorescence assay and RT-PCR analyses.

Our data indicated that nectin-1 accumulation is significantly decreased at the post-transcriptional level in *C. trachomatis*-infected genital epithelial cells and suggested that *C. trachomatis* may disrupt AJs, at least in part, by diminishing nectin-1 expression. This work has been published in *Microbiology* (2008, 154:1290-1299) (Sun *et al.*, 2008) and is presented here as Chapter 2.

CPAF, a chlamydia-secreted protease, has been reported to degrade a number of host cell proteins during chlamydial infection, including transcription factors RFX5 and USF-1, keratin-8, pro-apoptotic BH-3 only proteins, and CD1d glycoprotein (Dong *et al.*, 2004c; Kawana *et al.*, 2007; Pirbhai *et al.*, 2006; Zhong *et al.*, 1999; Zhong *et al.*, 2000). Given the broad range of cellular proteins that CPAF targets, we hypothesized that CPAF may degrade nectin-1 in *C. trachomatis*-infected genital epithelial cells. To elucidate the molecular mechanism by which chlamydiae down-regulate nectin-1, we determined whether or not CPAF degrades nectin-1. This work has been published in *Microbes and Infection* (2009, 11:12-19) (Sun, 2009) and is presented here as Chapter 3.

HSV gD is the main determinant of cell recognition for viral entry. Fusion of the HSV envelope and host cell membrane is mediated by the binding of HSV gD to one of its co-receptors (HVEM, nectin-1, nectin-2 and 3-O-S-HS). Unpublished data from our laboratory indicate that HSV glycoprotein D (gD) interaction with the host cell surface is sufficient to induce chlamydial persistence (Vanover, 2009b). Subsequently, we determined that virus interaction with one of the known co-receptors is sufficient to alter the chlamydial developmental cycle. This work has been summarized in a manuscript "Nectin-1 is required for Herpes simplex virus (HSV) co-infection-induced *Chlamydia trachomatis* persistence" and is presented here as Chapter 4.

CHAPTER 2

THE HOST ADHERENS JUNCTION MOLECULE NECTIN-1 IS DOWN-REGULATED IN *CHLAMYDIA TRACHOMATIS*-INFECTED GENITAL EPITHELIAL CELLS

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Running title: *C. trachomatis* down-regulates nectin-1 accumulation.

Keywords: *Chlamydia trachomatis*, Nectin-1, Adherens Junction.

Abbreviations: AJ, adherens junction; TJ, tight junction; DS, desmosome; h.p.i, hours post infection; RIN, RNA Integrity Number; Camp, chloramphenicol; penG, penicillin G; STD, sexually transmitted disease; EB, elementary body; RB, reticulate body; m.o.i, multiplicity of infection; FAK, focal adhesion kinase; MOMP, major outer membrane protein; IFU, inclusion-forming units; CPAF, chlamydial protease/proteasome-like activity factor; CAMP, cationic anti-microbial peptide; JNK, c-Jun N-terminal kinase.

Microbiology (2008) 154, 1290-1299.

Summary

Nectin-1, a member of the immunoglobulin superfamily, is a Ca^{2+}-independent cell adhesion protein implicated in the organization of E-cadherin-based adherens junctions (AJs) and claudin-based tight junctions (TJs) in epithelial cells. Nectin-1 also regulates cell–cell adhesion and cell polarization in a Cdc42- and Rac-dependent manner. Western blot analyses demonstrated that accumulation of host nectin-1 is decreased by 85 % at 48 hours post-infection (h.p.i.) in *Chlamydia trachomatis* serovar E-infected HeLa cells. Time-course experiments demonstrated that this decrease was sustained to 60 h.p.i. Nectin-1 downregulation in *C. trachomatis*-infected cells was prevented by both chloramphenicol exposure and prior inactivation of the chlamydiae with UV light, demonstrating that active *C. trachomatis* replication was required. Penicillin G-exposure studies demonstrated that nectin-1 accumulation was also altered during persistent infection. Finally, RT-PCR analyses indicated that chlamydial infection did not alter accumulation of any nectin-1 transcripts, demonstrating that nectin-1 accumulation is reduced at a post-transcriptional level. Intesrestingly, N-cadherin-dependent cell–cell junctions can be disrupted by *C. trachomatis* infection, as reported by Prozialeck *et al.* (2002)✦. Because interaction of nectin molecules on adjacent cells is essential for AJ formation, these data suggest that *C. trachomatis* may disrupt AJs, at least in part, by diminishing nectin-1 accumulation. Notably, release of chlamydiae-infected epithelial cells has been observed both *in vitro* from polarized monolayers and *in vivo* from tissues, suggesting that chlamydia-modulated downregulation of adhesion molecules and the subsequent disruption of host cell adherence may be involved in chlamydial dissemination or pathogenesis.

Introduction

The obligate intracellular bacterium *Chlamydia trachomatis* is a significant Gram-negative human pathogen, with 4 million new cases per year in the USA. Long-term *C. trachomatis* genital tract infections are often asymptomatic and associated with severe ascending complications, including epididymitis, prostatitis, ectopic pregnancy, salpingitis, and infertility (Darville, 2000a). The chlamydial developmental cycle begins when the elementary body (EB) binds to and enters mucosal epithelial cells. Once internalized, the EB remains within an inclusion and differentiates into a reticulate body (RB). Using host cell nutrients, the RB grows, divides by binary fission and eventually produces more EBs, which are released by host cell lysis (Wyrick, 2000). When developing chlamydiae are confronted with nutrient deficiency or penicillin exposure, they deviate from the normal developmental cycle and enter into a state called persistence, which is characterized by formation of aberrantly enlarged, viable but non-infectious chlamydial RB (Hogan *et al.*, 2004). Notably, the chlamydiae can re-enter and complete the normal developmental cycle once the persistence "inducer" is removed.

Upon infection, chlamydiae alter host cellular functions in a variety of ways. Chlamydial infection prevents host cell apoptosis, induces reorganization of the actin cytoskeleton, and alters host cellular signaling mechanisms (Hackstadt, 1999; Hatch, 1999). *C. trachomatis* infection also disturbs cell-cell contacts between polarized epithelial cells in culture (Wyrick *et al.*, 1989). This cell-cell disassociation was later shown to be due, at least in part, to disruption of N-cadherin-dependent cell-cell junctions and breakdown of the N-cadherin/β-catenin complex (Prozialeck *et al.*, 2002). Notably, infected epithelial cell release has also been observed i*n vivo* (Doughri *et al.*, 1972; Soloff *et al.*, 1985). Doughri *et al* further suggest that release of chlamydiae within intact host cells might protect the organisms from anti-chlamydial antibodies (Doughri *et al.*, 1972). In both studies, the authors hypothesize that PMNs responding to chlamydial infection promote detachment of chlamydia-infected cells from the mucosal surface, resulting in the release of intact, infected cells into the cervical lumen (Doughri *et al.*, 1972; Soloff *et al.*, 1985). The seminal observation that chlamydial infection in the absence of PMNs

loosens cell-cell junctions by promoting breakdown of the N-cadherin/β-catenin complex (Prozialeck *et al.*, 2002) suggests that the developing chlamydiae may also directly contribute to the release of infected cells from the infected mucosa observed *in vivo*.

In epithelial cells, junctional complexes, which are composed of tight junctions (TJs), adherens junctions (AJs), and desmosomes (DSs), play critical roles in forming intercellular adhesions. Intercellular adhesions, in turn, are intimately involved in maintaining cell structure, transmitting information, regulating cellular migration, and imparting strength and rigidity to tissues (Farquhar & Palade, 1963; Gumbiner, 1996; Lauffenburger & Horwitz, 1996; Takeichi, 1995). Importantly, the organization and function of TJs and DSs are dependent on the formation and maintenance of AJs (Tsukita & Furuse, 1999). AJs are located at the basal side of TJs and connect the lateral surfaces of adjacent epithelia cells. AJs form complexes with actin and myosin filaments to internally brace cells and thereby control their shape. E-cadherin and claudin are two well-studied cell-cell adhesion molecules found in AJs and TJs, respectively.

A novel cell-cell adhesion system consisting of nectin and afadin has recently been identified at AJs in epithelial cells, neurons, and fibroblasts (Takai & Nakanishi, 2003). Nectins are Ca^{2+}-independent, Ig-like cell-cell adhesion molecules with three extracellular Ig-like loops, a single transmembrane region (except for nectin-1γ) and a cytoplasmic tail. The nectin family currently contains 4 members (nectins-1~4) (Sakisaka & Takai, 2004; Takai & Nakanishi, 2003). Afadin is a nectin- and F-actin-binding protein that connects the *trans*-membrane domain of nectin to the actin cytoskeleton (Takai & Nakanishi, 2003). This nectin-based adhesion system is required not only for the formation of AJs but also for the subsequent formation of TJs (Takai & Nakanishi, 2003). Furthermore, activation of Cdc42 and Rac small G proteins through c-Src by *trans*-interaction of nectins has been implicated in controlling cell-cell adhesion and cell polarization (Takai *et al.*, 2003).

In this study, Western blot analyses demonstrate that accumulation of host nectin-1 is significantly decreased in *C. trachomatis* serovar E-infected cervical epithelial cells.

This down-regulation requires active *C. trachomatis* protein synthesis and/or replication. RT-PCR analyses further show that reduced expression of nectin-1 likely occurs post-transcriptionally. Because the nectin-afadin adhesion system is critical for formation of functional E-cadherin based AJs and subsequent claudin-based TJs, and because chlamydial infection has been previously shown to disrupt cell:cell junctions (Prozialeck *et al.*, 2002), our data suggest that *C. trachomatis* may disrupt AJs, at least in part, by diminishing nectin-1 expression.

Methods

Cell culture, chlamydial infection and titrations.

HeLa cells, a human cervical adenocarcinoma epithelial cell line (ATCC No. CCL2), were cultured as described (Deka *et al*, 2006). A human urogenital isolate of *C. trachomatis* E/UW-5/CX was originally obtained from S.P. Wang and C.C. Kuo (University of Washington, Seattle, WA). The same standardized inoculum of *C. trachomatis* serovar E EBs, propagated in McCoy cells, was used for all experiments (Wyrick *et al.*, 1996). For infection experiments, HeLa cells were either grown in standard tissue culture plates or were polarized on 3.0 μm, Collagen IV-coated chamber inserts (Biocoat 4544, Becton Dickinson) as described (Wyrick *et al.*, 1989). Host cells were infected at a multiplicity of infection (MOI) of 1 using crude EB stock, which infects ~ 90% of the HeLa cells, for 1 hour at 35°C. In some experiments, HeLa cells were infected with an equivalent amount of UV-inactivated *C. trachomatis* serovar E (*C. trachomatis*-UV). Mock-infected host cells were treated similarly except they were exposed to 200 μl of 2SPG (0.2 M sucrose, 6 mM NaH_2PO_4, 15 mM Na_2HPO_4, 5 mM L-glutamine, pH 7.2). After infection, the inoculum was aspirated and the infected cells were refed with growth medium and incubated at 35°C for the indicated times. Chlamydial titrations were performed as previously described (Deka *et al.*, 2007). Each experiment was repeated three times and each repeat contained three biological replicates.

Generation of *C. trachomatis*-UV.

A UV cross-linker (Spectrolinker XL1500, Spectronics Corporation) was used to generate stocks of UV-inactivated, replication-incompetent *C. trachomatis* (*C. trachomatis*-UV). Stock *C. trachomatis* serovar E was thawed on ice and 200 µl was aliquoted into each well of a 24-well plate. The plate was placed on a 4ºC heat sink during UV exposure to prevent heat inactivation of the samples. EB inactivation was assayed by performing chlamydial titrations (Deka *et al.*, 2007). A UV dose of 1.0 J cm^{-2} was sufficient to completely inactivate undiluted *C. trachomatis* stocks (data not shown).

RNA and DNA isolation.

Total RNA and DNA were isolated from mock- or *C. trachomatis*-infected HeLa cells at 48 hpi and 60 hpi using the method previously described by Deka *et al* (Deka *et al.*, 2006). Total RNA and DNA preparations were quantified by measuring optical density (OD) at 260 and 280 nm. All samples had $OD_{260/280}$ ratios > 1.9. The quality and concentration of each RNA sample were further confirmed by analysis on a 2100 Bioanalyzer instrument (Agilent) using the RNA 6000 Nano LabChip kit. All samples had RNA Integrity Numbers (RINs) between 9.0-10.0 (data not shown).

PCR, Reverse transcription and RT-PCR.

Total cellular DNA was used as a template to amplify human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for determination of host genome copy number. Total cellular RNA samples were subjected to reverse transcription as previously described (Deka *et al.*, 2006), except that two micrograms of total RNA was used. RT-PCR reactions were than carried out using RT(+) and RT(–) cDNA reactions as templates, as appropriate. Experimental template DNAs/cDNAs were used in PCR reactions at dilutions ranging from 1/10 to 1/1000 (in ddH_2O), such that each reaction was in the linear amplification range. The amplification conditions were based on those previously described (Deka *et al.*, 2006). Published primer sets included human β-actin (Nelson *et al.*, 2005) and human GAPDH (Deka *et al.*, 2006). Specific primers and positive oligonucleotide amplification controls for human nectin-1α, nectin-1β and nectin-1γ were designed using Oligo 4.0 software. They are listed in supplementary Table S1. Most

reactions were cycled as follows: 94°C, 1 min; 60°C, 1 min; 72°C, 1 min for 35 cycles. Human β-actin reactions were performed under identical conditions but were cycled 30 times; human GAPDH PCR reactions were performed for 35 cycles and annealed at 67°C. The resulting PCR products were separated by electrophoresis on 2.5% agarose/TBE gels and stained with ethidium bromide. A Bio-Rad Chemi Doc XRS Image Capture System with Quantity One V4.5.0 software was used to visualize and quantify amplimers.

SDS-PAGE and Western blotting.

Monolayers were lysed and denatured as described (Deka *et al.*, 2006). Samples were then normalized for total protein content using SYPRO Ruby stain (BioRad) according to the manufacturer's instructions. Samples were diluted to identical concentrations, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and Western blotted (Deka *et al.*, 2006). Primary antibodies used included mouse monoclonal anti-nectin-1 CK6 (1:200 dilution, sc-21722, Santa Cruz Biotechnology), mouse monoclonal anti-nectin-1 CK8 (1:200 dilution, 37-5900, Invitrogen-Zymed), rabbit polyclonal anti-human focal adhesion kinase (FAK) C-20 (1: 2000 dilution, sc-558, Santa Cruz Biotechnology), and goat polyclonal anti-MOMP (1:5000 dilution, B65266G, BioDesign International). Bound primary antibodies were detected using corresponding secondary antibodies conjugated to horseradish peroxidase and visualized using SuperSignalWest Pico reagent (Pierce). Specific bands were quantified using an FX phosphorimager and Quantity One V2.5.0 software (Bio-Rad). To control for small variations in cell number and gel loading between sample lanes, nectin-1 quantity in each sample was normalized to the amount of FAK protein detected in that same lane. Previous studies from our laboratory have demonstrated that FAK accumulation is not altered by chlamydial infection and , therefore, makes an ideal internal control (Deka *et al.*, 2006).

Statistical analyses.

Statistical analyses were performed using Microsoft Excel. A two-sample *t*-test for independent samples was used for comparison of means. Significance was defined as $P \leq 0.05$.

Results

Nectin-1 accumulation is reduced in *C. trachomatis*-infected HeLa cells.

Nectin-1 is an important cell-cell adhesion molecule involved in the formation and maintenance of AJs and TJs. Previous studies demonstrated that cell:cell contacts are disrupted in *C. trachomatis*-infected cervical epithelial cells (Prozialeck *et al.*, 2002). To determine whether chlamydial infection modulates nectin-1 expression, monolayers of non-polarized HeLa cells were either mock- or *C. trachomatis* serovar E-infected at a MOI of 1. Cell lysates were collected 48 hpi and processed for SDS-PAGE analyses. Duplicate gels were either stained with SYPRO Ruby (data not shown) or Western blotted (Fig. 2.1a) using anti-nectin-1 CK6 mAb, anti-*C. trachomatis* MOMP or anti-FAK as described (Deka *et al.*, 2006). Nectin-1 bands were quantified, normalized to FAK and plotted in Fig. 2.1(b). Focal adhesion kinase (FAK) was chosen as an internal control protein because FAK accumulation is not altered by chlamydial infection (Deka *et al.*, 2006). As expected, MOMP expression was detected only in *C. trachomatis*-infected cells. Nectin-1 accumulation was decreased by up to 85% in *C. trachomatis*-infected HeLa cells compared to that in mock-infected controls. Sequential immunoprecipitation and blotting experiments with a different nectin-1-specific monoclonal antibody (Supplementary Fig. S2.1b) confirmed that the protein detected on the blots in Fig. 2.1 was indeed nectin-1. Additionally, little or no nectin-1 was immunoprecipitated from chlamydia-infected cells harvested at 48 hpi by either antibody, confirming the observations described above.

In vivo, polarized epithelial cells differentially distribute proteins and lipids to the plasma membrane, creating two distinct surfaces -- the apical and basolateral domains. Previous studies have shown that polarized and non-polarized cells differ in *C. trachomatis* infection efficiency, duration of the *C. trachomatis* developmental cycle and their response to IFN-γ (Arno *et al.*, 1990; Kane & Byrne, 1998; Tam *et al.*, 1992). Thus, we wanted to investigate whether or not the polarity of epithelial cells would affect the observed nectin-1 reduction. Western blotting analyses (Supplementary Fig.S2.2a) showed that nectin-1 was also down-regulated in *C. trachomatis*-infected polarized cells.

At 48 hpi, nectin-1 accumulation was decreased as much as 88% in *C. trachomatis*-infected polarized HeLa cells compared to that in mock-infected controls (Supplementary Fig. S2.2b). Because similar results were obtained from both non-polarized and polarized HeLa cells, non-polarized cells were used in subsequent experiments.

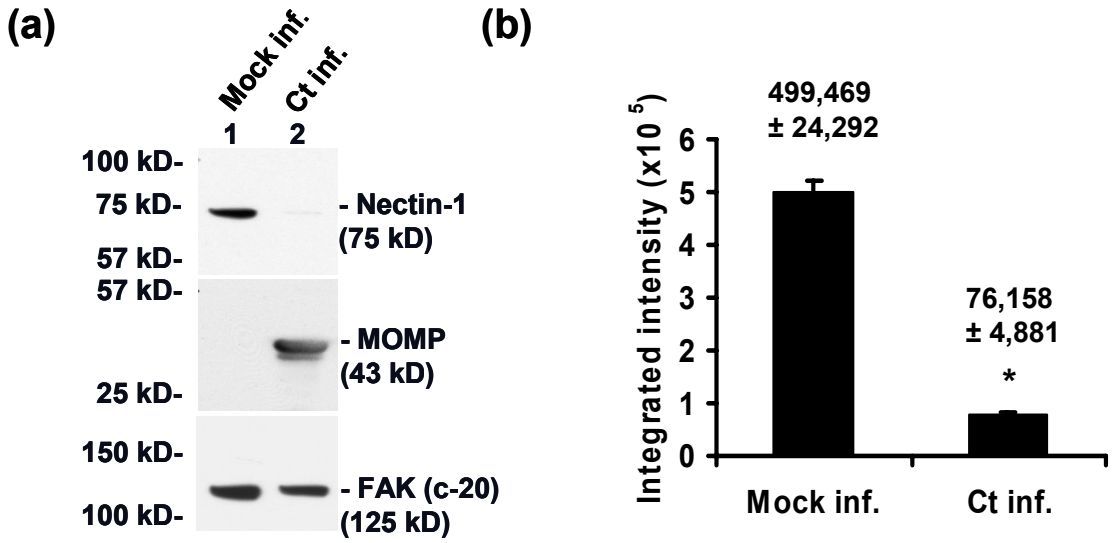

Figure 2.1 Nectin-1 accumulation is reduced in *C. trachomatis*-infected HeLa cells. HeLa cells were either mock- or *C. trachomatis* serovar E-infected at a MOI of 1. Cell lysates were collected 48 hpi and Western blotted using anti-nectin-1 CK6, anti-*C. trachomatis* MOMP or anti-FAK (C-20). Nectin-1 bands were quantified, normalized to the internal control protein FAK and plotted in (b). Results are expressed as the average of three biological replicates ± SEM. $P \leq 0.05$ was considered significant. Groups significantly different from the mock-infected controls are indicated by asterisks (*). These data are representative of three independent experiments.

Nectin-1 accumulation is reduced in *C. trachomatis*-infected HeLa cells from 36 to 60 hpi.

To more precisely define when down-regulation of nectin-1 occurs during the *C. trachomatis* developmental cycle, an infection time course ranging from 0 to 60 hpi was conducted. HeLa cells were either mock- or *C. trachomatis*-infected. Cell lysates were collected at 0, 24, 36, 48 and 60 hpi and Western blotted with (i) anti-nectin-1 (CK6), (ii) anti-FAK (C-20) and (iii) anti-MOMP. A representative blot is shown in Fig. 2.2(a); the

quantification of this blot is shown in Fig. 2.2(b). The nectin-1 quantity observed in *C. trachomatis*-infected cells from three independent experiments was expressed as a percentage of that observed in mock-infected control cells at each time interval and plotted (Fig. 2.2c). These data demonstrate that *C. trachomatis* infection significantly reduced accumulation of nectin-1 starting at 36 hpi and the reduction was maximal at 60 hpi.

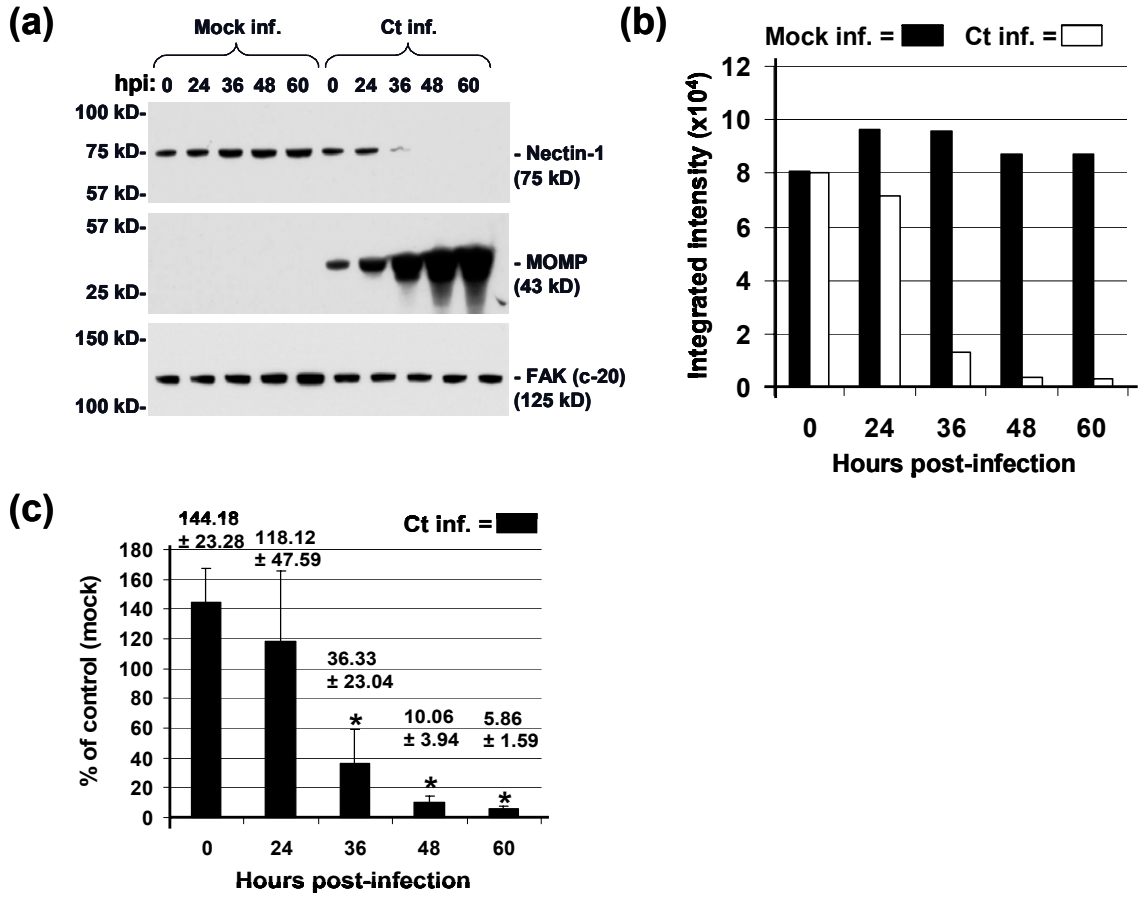

Figure 2.2 Nectin-1 accumulation is reduced in *C. trachomatis*-infected HeLa cells from 36 to 60 hpi. (a) Cell lysates from mock- or *C. trachomatis*-infected HeLa cells were collected at 0, 24, 36, 48, 60 hpi and Western blotted using anti-nectin-1 CK6, anti-*C. trachomatis* MOMP or anti-FAK (C-20). (b) The quantity of nectin-1 detected in panel (a) was normalized to the internal control protein FAK and plotted. (c) Nectin-1 quantity observed in *C. trachomatis*-infected cells was expressed as a percentage of that observed in mock-infected control cells at each time interval. Results are expressed as the average of three biological replicates ± SEM. $P \leq 0.05$ was considered significant. Groups

significantly different compared to *C. trachomatis*-infected cells at 0 hpi are indicated by asterisks (*). These data are representative of three independent experiments.

Nectin-1 down-regulation requires *C. trachomatis* protein synthesis and/or replication.

To extend these findings, we next investigated whether *C. trachomatis* protein synthesis and/or replication was required for nectin-1 down-regulation. Nectin-1 expression in persistently-infected cells was also examined. HeLa cells were either mock- or *C. trachomatis*-infected and refed immediately after infection with growth medium plus ddH_2O, medium plus 60 μg chloramphenicol ml^{-1} (Camp, which specifically inhibits function of bacterial 50S ribosomal subunits) or medium plus 20 units penicillin G ml^{-1} (PenG, a known inducer of chlamydial persistence). Cell lysates were harvested at 48 hpi for Western blot analyses (Fig. 2.3a, b). Nectin-1 quantity observed in *C. trachomatis*-infected cells from three independent experiments was expressed as a percentage of that observed in mock-infected control cells under that set of experimental conditions (Fig. 2.3c). As a control to confirm that each antibiotic was working as expected, duplicate cultures from each drug-exposure group were subjected to chlamydial sub-passage titration analyses. The number of inclusion-forming units (IFU) in the undiluted inocula was then calculated and expressed as IFU ml^{-1} (Fig. 2.3d). Nectin-1 down-regulation in *C. trachomatis*-infected cells was prevented by chloramphenicol exposure (Fig. 2.3a, lanes 5 and 6), demonstrating that *C. trachomatis* protein synthesis and/or replication is required for this effect. Nectin-1 levels also decreased in the presence of penicillin G (Fig. 2.3a, lanes 7 and 8), although to a lesser degree than that observed in diluent-exposed, chlamydiae-infected monolayers (Fig. 2.3b, c). These data indicate that nectin-1 accumulation can also be reduced by penicillin-induced, persistent *C. trachomatis* infection. Chlamydial sub-passage experiments demonstrate that both chloramphenicol and penicillin G exposure significantly reduced recovery of infectious EB, as expected (Fig. 2.3d).

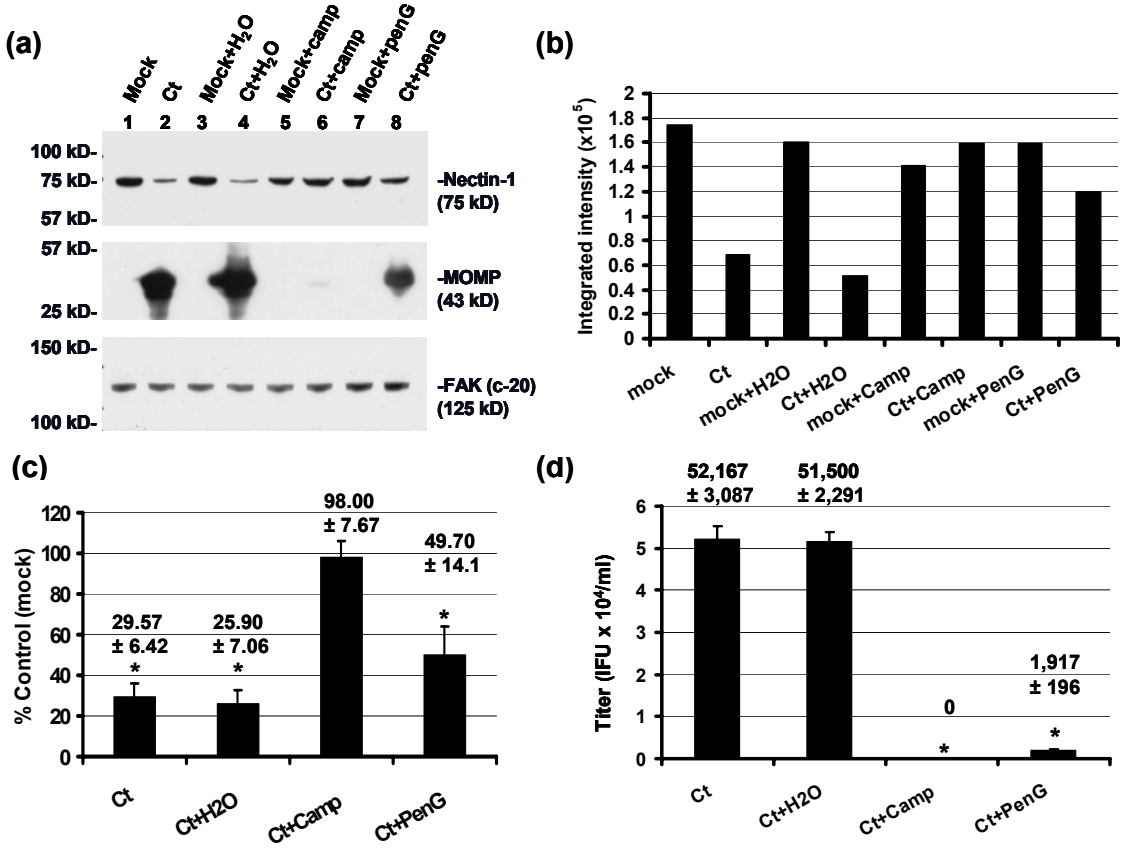

Figure 2.3 *C. trachomatis* protein synthesis and/or replication is/are required for nectin-1 down-regulation. (a) Cells were refed immediately after *C. trachomatis*-infection with growth medium + ddH_2O, 60 µg chloramphenicol ml^{-1} (Camp) or 20 units penicillin G ml^{-1} (PenG). Cell lysates were harvested at 48 hpi and Western blotted using anti-nectin-1 CK6, anti-*C. trachomatis* MOMP or anti-FAK (C-20). (b) The quantity of nectin-1 detected on the blot in panel (a) was normalized to FAK and plotted. (c) Nectin-1 quantity observed in *C. trachomatis*-infected cells was expressed as a percentage of that observed in mock-infected control cells under that set of experimental conditions. The average from three biologic replicates ± SEM is shown. $P \leq 0.05$ was considered significant. Groups significantly different compared to Camp-exposed, *C. trachomatis*-infected cells are indicated by asterisks (*). (d) Duplicate cell cultures from each drug-exposure group were subjected to chlamydial sub-passage titration. Results are expressed as the average of three biological replicates ± SEM. Significance was defined as $P \leq 0.05$. Groups significantly different compared to non-drug exposed, *C. trachomatis*-infected cells are indicated by asterisks (*). All data shown here are representative of three independent experiments.

Viable *C. trachomatis* EBs are required for nectin-1 down-regulation.

To determine if viable *C. trachomatis* EBs are required for down-regulation of nectin-1, UV-inactivated *C. trachomatis* was used to infect HeLa cells. Previous studies have demonstrated that UV-inactivation of *C. trachomatis* renders the bacteria completely replication-incompetent (Eissenberg *et al.*, 1983). HeLa cultures were mock-, *C. trachomatis*-, or *C. trachomatis*-UV-infected, collected at 48 hpi and processed for Western blot analyses (Fig. 2.4a). Average nectin-1 accumulation was quantified, normalized and plotted (Fig. 2.4b). Nectin-1 accumulation was reduced in *C. trachomatis*-infected cells but not in cultures infected with an equivalent quantity of UV-inactivated *C. trachomatis* EB, demonstrating that nectin-1 is down-regulated only if cells are infected with viable EBs.

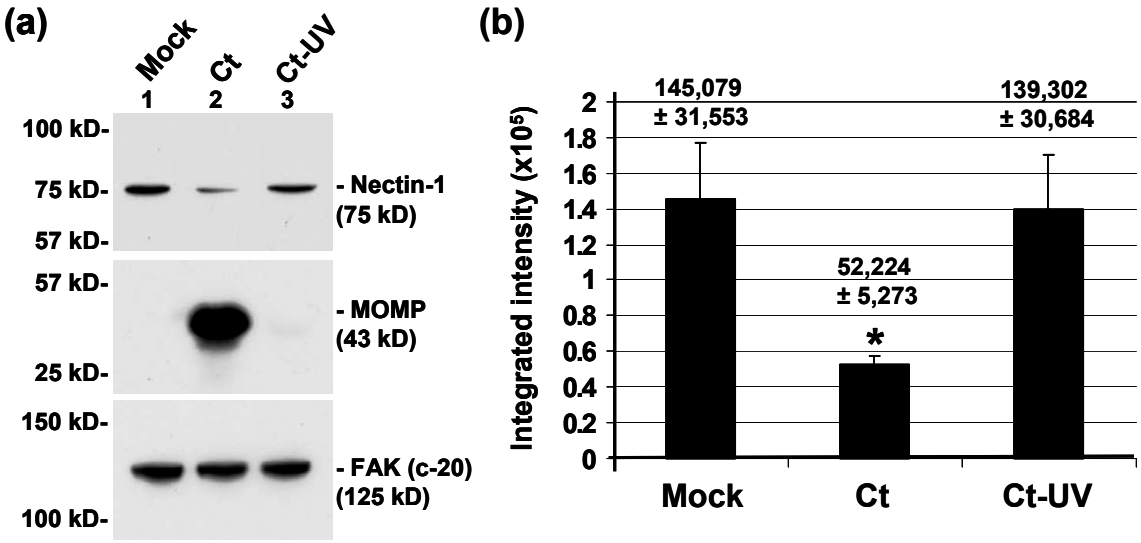

Figure 2.4 Viable *C. trachomatis* EBs are required for nectin-1 down-regulation. (a) HeLa cells were mock-, *C. trachomatis*-, or *C. trachomatis*-UV-infected. Cells were collected at 48 hpi and Western blotted using anti-nectin-1 CK6, anti-*C. trachomatis* MOMP or anti-FAK (C-20). (b) The quantity of nectin-1 was normalized to internal control protein FAK and plotted. Results are expressed as the average of three biological replicates ± SEM. Significance was defined as $P \leq 0.05$. Groups significantly different from the mock-infected controls are indicated by asterisks (*).The data shown here are representative of three independent experiments.

C. trachomatis infection of HeLa cells does not alter accumulation of nectin-1α, β and γ transcripts.

Nectin-1 has three splice variants: nectin-1α, nectin-1β, and nectin-1γ. All three nectin-1 isoforms encoded by these transcripts associate with other adhesion molecules and play important roles in the formation of cell-cell junctions. Although the nectin-1α and nectin-1β proteins both contain the CK6 and CK8-reactive epitopes (Supplementary Fig. S2.1a), the observed molecular weight (75kD) is most consistent with the reported size for nectin-1α (Struyf *et al.*, 2005) . To aid in elucidating the mechanism by which host cell nectin-1 expression is altered in response to *C. trachomatis* infection, we investigated whether down-regulation of nectin-1 occurs at the transcriptional level. Total RNA from mock- or *C. trachomatis*-infected cells was isolated and subjected to RT-PCR using primers specific for human β-actin, nectin-1α, nectin-1β, and nectin-1γ transcripts. Parallel total DNA samples were amplified using GAPDH-specific primers to determine host genome copy number. All amplimers were the expected size (Fig. 2.5a) and the identity of each was confirmed by DNA sequencing (data not shown). In each experiment, a six log dilution series of known DNA template controls was used to generate amplification standard curves. Experimental samples were only quantified if they fell within the linear range of the PCR. Amplification products were not observed in template-negative samples (Fig. 2.5a, lane 1) or in RT(-) controls (data not shown). The quantity of nectin-1α, β and γ products was similar in mock- compared to *C. trachomatis*-infected samples at both 48 and 60 hpi (Fig. 2.5a, lanes 2-5). All amplimers were quantified, normalized to host genome copy number as ascertained by PCR with human GAPDH-specific primers, and plotted in Fig. 2.5(b). Statistical analyses showed that there was no significant difference in accumulation of any of the nectin-1 transcripts in mock- versus *C. trachomatis*-infected cells (Fig. 2.5b). These data indicate that expression of nectin-1 was not regulated at the transcriptional level and, thus, was likely regulated post-transcriptionally.

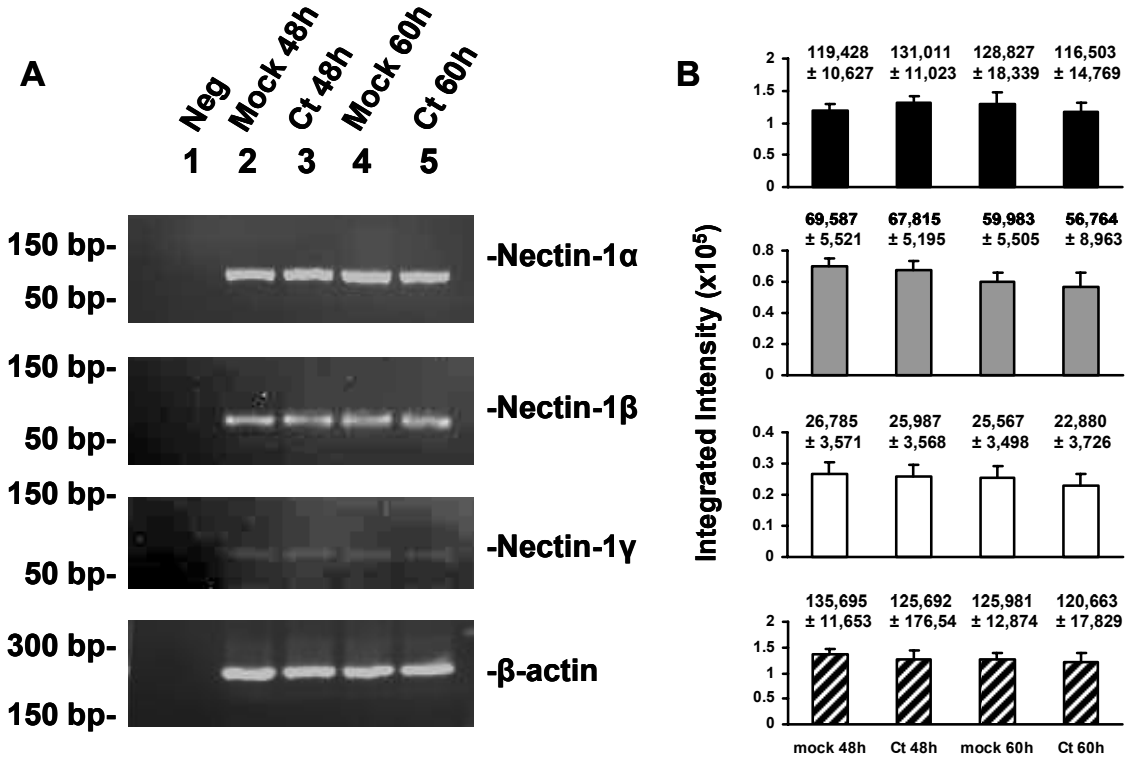

Figure 2.5 *C. trachomatis* infection of HeLa cells does not alter accumulation of nectin-1α, β and γ transcripts. (a) HeLa cells were mock- or *C. trachomatis*-infected at 1 MOI. Total RNA was isolated at 48 or 60 hpi as described and subjected to RT-PCR analyses using transcript-specific oligonucleotide probes. Nectin-1α (117bp), Nectin-1β (105bp), Nectin-1γ (75bp) and β-actin (230bp) amplimers were electrophoresed and photographed. Representative photographs are shown. The position of DNA size markers are shown to the left of each gel image in base pairs (bp). (b) Specific DNA amplimers were quantified as described. Nectin-1α (black bars), Nectin-1β (grey bars), Nectin-1γ (white bars) and β-actin (diagonally striped bars) amplimer quantities were normalized to host genome copy number as ascertained by PCR with human GAPDH-specific primers (data not shown). The values plotted are averages of three independent experiments ± SEM. The value obtained for each independent experiment is the average obtained from three biological replicates.

Discussion

Chlamydiae have evolved various strategies to avoid host defense systems and complete their intracellular growth cycle. Chlamydiae have acquired specific mechanisms to prevent productively and persistently infected cells from undergoing apoptosis (Dean & Powers, 2001; Fan *et al.*, 1998; Fischer *et al.*, 2004; Geng *et al.*, 2000; Pirbhai *et al.*, 2006; Rajalingam *et al.*, 2001), which may allow chlamydiae to maintain long-term survival within infected cells. Chlamydiae also alter host cell:cell contacts and signal transduction (Prozialeck *et al.*, 2002; Xia *et al.*, 2003), which may facilitate chlamydial growth. Finally, chlamydiae also modify the host cell cytoskeleton *(Dong et al., 2004c*; Majeed *et al.*, 1993), which may benefit chlamydial intravacuolar replication. In the present study, we observed a remarkable loss of nectin-1 in *C. trachomatis*-infected HeLa cells. We further demonstrated that active *C. trachomatis* protein synthesis and/or replication and viable *C. trachomatis* EBs are required for this effect. Lastly, we also noted that persistent chlamydial infection, induced by penicillin G, also down-regulates nectin-1 accumulation. Because different persistence inducers act upon the developing chlamydiae by divergent mechanisms, we must examine other stressors to determine whether the nectin-1 decrease is a general characteristic of persistently-infected epithelial cells.

It is currently accepted that nectin-1 plays a critical role in the formation and maintenance of adherens junctions (AJs) and tight junctions (TJs). Disruption of nectin-1 would inevitably affect the integrity of cell-cell junctions, which may be implicated in chlamydial pathophysiologic effects on the host. This notion is consistent with a recent study showing that cervical epithelial cells separate from each other as a consequence of *C. trachomatis* infection (Prozialeck *et al.*, 2002). Interestingly, the important human pathogens *Helicobacter pylori*, *Shigella flexneri*, and *Salmonella typhimurium* also down-regulate essential components of adhesion and tight junctions, including E-cadherin, claudin-1, and ZO-1. This leads to decreased transepithelial electrical resistance and increased paracellular permeability, which facilitates cell-to-cell spreading of these pathogens (Jepson *et al.*, 1995; Sakaguchi *et al.*, 2002; Sears, 2000; Terres *et al.*, 1998).

Collectively, these findings suggest that disruption of adhesion and tight junctions to compromise cell-cell barriers may be an important strategy exploited by intracellular pathogens to manipulate host cells. Although *C. trachomatis* serovar E is not an invasive pathogen, it may diminish nectin-1 expression to aid lateral cell to cell spread. Surprisingly, in contrast to *C. trachomatis* and the pathogens mentioned above, *C. pneumoniae* up-regulates the expression of adherens junction proteins VE-cadherin, N-cadherin, and β-catenin and transiently down-regulates the expression of the tight junction protein occludin to alter junctional complexes, facilitating its transmission in human brain microvascular endothelial cells (MacIntyre *et al.*, 2002). It is currently unknown why *C. trachomatis* and *C. pneumoniae* might use two distinct mechanisms to interact with junctional complexes.

While nectin-1 protein expression was decreased in *C. trachomatis*-infected HeLa cells, accumulation of nectin-1α, β and γ transcripts was unchanged. These data indicate that reduction of nectin-1 was not regulated transcriptionally and, thus, is likely down-regulated at the post-transcriptional level. Recently, several host proteins have been reported to undergo degradation upon *C. trachomatis* infection (Balsara *et al.*, 2006; Dong *et al.*, 2004c; Dong *et al.*, 2005a; Fischer *et al.*, 2004; Pirbhai *et al.*, 2006; Ying *et al.*, 2005; Zhong *et al.*, 1999; Zhong *et al.*, 2000). The chlamydia-secreted protease CPAF (chlamydial protease/proteasome-like activity factor) is responsible for the degradation of many of these host proteins. Given the broad range of cellular proteins cleaved by CPAF, it is possible that CPAF could also degrade nectin-1. Notably, there is little or no difference in CPAF expression between productive and persistent infections (Heuer *et al.*, 2003; Shaw *et al.*, 2002). Thus, the observation that nectin-1 was decreased in both *C. trachomatis* productively- and persistently-infected samples is consistent with the notion that CPAF degrades nectin-1. Alternatively, it is also possible that chlamydial infection induces a host cell derived protease or proteosomal activity which degrades nectin-1. The role of CPAF and other chlamydial or host derived proteases in regulating nectin-1 accumulation in chlamydiae-infected cells requires further investigation.

Although the mechanism by which *C. trachomatis* down-regulates nectin-1 expression is of significant interest, a more important question remains--why does *C. trachomatis* reduce host cell nectin-1 accumulation? There are at least two plausible hypotheses. First, nectin-1 down-regulation, and subsequent disruption of cell:cell adhesions, may facilitate host structural rearrangements required for inclusion enlargement or EB release at the end of the developmental cycle. This prediction is supported by the observation that nectin-1 accumulation starts decreasing at 36 hpi, which is mid-developmental cycle for *C. trachomatis* serovar E. The second, and perhaps more intriguing possibility, is that nectin-1 down-regulation facilitates *C. trachomatis* dissemination within the host genital tract (Supplementary Fig. S3). In this case, weakening of contacts between an infected genital epithelial cell and adjacent, uninfected cells would allow release of the intact, infected cell into the genital tract lumen. The infected cell would then drift away from original infection site in the genital mucus, aiding chlamydial dissemination in at least two ways. First, chlamydial EB within the exfoliated cell would be shielded from neutralization by secretory IgA, cationic anti-microbial peptides (CAMPs) and other anti-bacterial compounds, as long as the host cell remained intact. Second, if the infected cell ruptured in close proximately to the genital mucosa, the local concentration of infectious EB would be very high, increasing the probability that mucosal epithelia in the vicinity would be successfully infected. In contrast, EB released from host cells still within the genital epithelial layer (Supplementary Fig. S3, right) would be both quickly diluted and immediately subject to neutralization by anti-bacterial compounds. Therefore, only host cells in close proximity to the release site would have a high probability of becoming infected. Thus, we envision that the released, inclusion-containing epithelial cell would act like a chlamydial “cluster bomb”, allowing delivery of a concentrated inocula over a relatively long distance and increasing the possibility of a “hit” if the host cell ruptured near the genital epithelial cell layer. Interestingly, release of intact, chlamydiae-infected host cells has been observed from polarized monolayers in culture (Wyrick *et al.*, 1989) as well as from infected epithelium *in vivo* (Doughri *et al.*, 1972; Soloff *et al.*, 1985). Of course, it is always possible that the observed nectin-1 decrease is an indirect side-effect of other host

cytoskeletal or physiologic alterations induced during chlamydial infection. Future studies will be directed toward determining which of these hypotheses are correct.

Finally, it should be noted that nectin also plays a critical role in intracellular signaling. Recent studies have demonstrated that Cdc42 and Rac small G proteins are activated by *trans*-interactions of nectin in a PI3 kinase-independent manner in epithelial cells (Honda *et al.*, 2003; Kawakatsu *et al.*, 2002). Although the precise mechanisms of this activation remain to be elucidated, Cdc42 and Rac activities are required for the formation and maintenance of AJs in epithelial cells (Braga *et al.*, 1997; Braga *et al.*, 1999; Etienne-Manneville & Hall, 2002; Takaishi *et al.*, 1997; Van Aelst & Symons, 2002). It is reasonable to speculate that the disruption of cell-cell junctions observed by Prozialeck *et al* (Prozialeck *et al.*, 2002) upon *C. trachomatis* infection may result from insufficiently activated Cdc42 and Rac in response to reduced levels of nectin-1. Furthermore, activated Cdc42 and Rac stimulate the c-Jun N-terminal kinase (JNK) signaling pathway (Honda *et al.*, 2003), which has been implicated in regulating cell growth, transformation and apoptosis (Johnson & Lapadat, 2002; Lin, 2003). As previously discussed, chlamydial infection strongly inhibits host cell apoptosis and it is possible that reduced nectin-1/JNK signaling may play a role in this inhibition. Given the known interplay between nectin-1 and host cellular signaling pathways, dissecting the effect of *C. trachomatis* infection on nectin-mediated signal transduction events will be a key aspect of future experiments.

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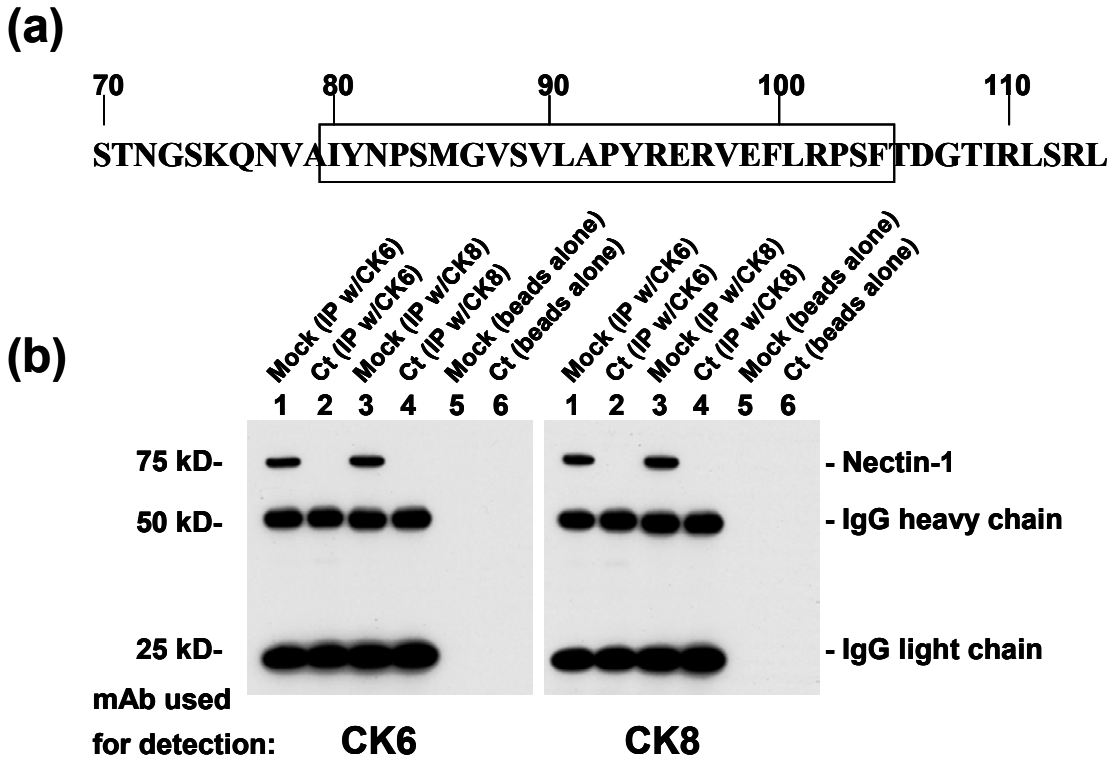
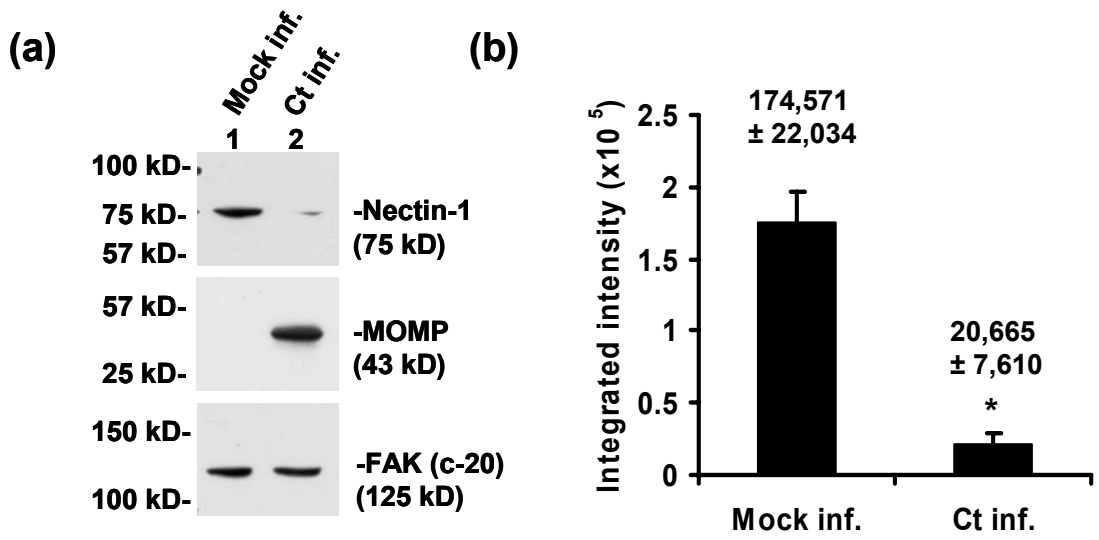
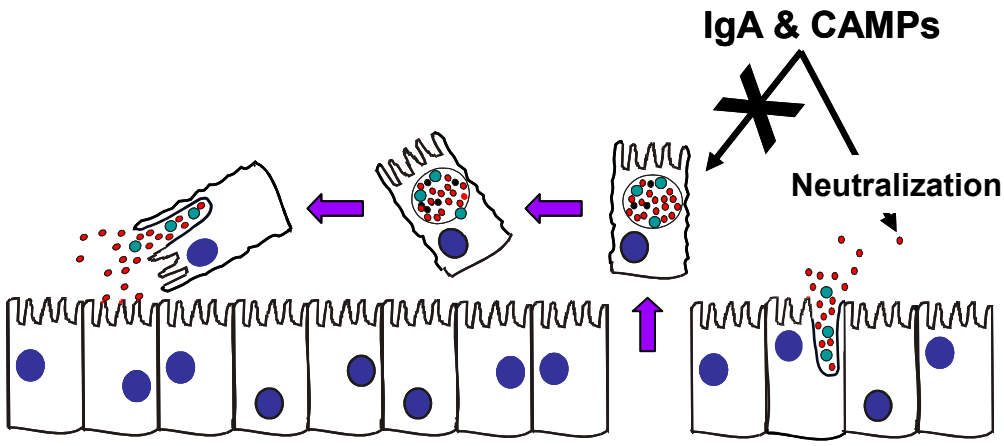
Supplementary Material



Supplementary Figure 2.1 Nectin-1 antibodies CK6 and CK8 detect the same protein. Mock- (lanes 1, 3 and 5) or *C. trachomatis*-infected (lanes 2, 4 and 6) HeLa monolayers were harvested at 48 hpi in 1X IP lysis buffer (Saltarelli *et al.*, 1993). Cell lysates were homogenized by passing them through a 20-gauge needle 10 times and were quantified using a Bradford protein assay. Equivalent amounts of protein from each lysate were immunoprecipitated using either nectin-1-specific mAb CK6 (lanes 1 and 2), nectin-1-specific CK8 mAb (#37-5900, Invitrogen-Zymed; lanes 3 and 4) or protein A beads alone (#17-0974-01, Amersham Pharmacia Biotechnology; lanes 5 and 6). These antibodies recognize two overlapping linear epitopes between nectin-1 amino acids 80 and 104 [Fig. S2.1a, boxed sequence (Krummenacher *et al.*, 2000)]. Immunoprecipitates were then boiled for 10 min, electrophoresed and then Western blotted using either CK6 (Fig. S2.1b, left panel) or CK8 (Fig. S2.1b, right panel) to detect nectin-1. Both CK6 and CK8 mAbs immunoprecipitated identically sized 75kD proteins from mock-infected cells but not from *C. trachomatis*-infected cells (Fig. S2.1b), confirming that this protein was indeed nectin-1. Additionally, little or no nectin-1 is immunoprecipitated from chlamydia-infected cells, which is consistent with previous observations. The 50 and 25 kD bands are heavy and light chains from the immunoprecipitating antisera that are detected by the anti-mouse secondary antibody. The data shown here are representative of three independent experiments.



Supplementary Figure 2.2 Nectin-1 accumulation is reduced in *C. trachomatis*-infected polarized HeLa cells. Polarized HeLa cells were either mock- or *C. trachomatis* serovar E-infected at an MOI of 1. Cell lysates were collected 48 hpi and Western blotted using anti-nectin-1 CK6, anti-*C. trachomatis* MOMP or anti-FAK (C-20). Nectin-1 bands were quantified, normalized to the internal control protein FAK and plotted in (b). Results are expressed as the average of three biological replicates ± SEM. $P \leq 0.05$ was considered significant. Groups significantly different from the mock-infected controls are indicated by asterisks (*). These data are representative of three independent experiments.



Supplementary Figure 2.3 Potential role of nectin-1 down-regulation in *C. trachomatis* dissemination within the host genital tract. See the text for a detailed description of the figure. Red dots represent EBs; green circles represent RBs.

Supplementary Table S2.1. RT-PCR primers and positive control oligonucleotides.

Name	Forward primer	Reverse primer
hNectin-1α	AGCACCAAGAAGCACGTGTA	GGCCTTCTTCTCGTCGTCT
hNectin-1β	CCCAGCAGGCAAAGC TC	CAGGGACAGCTTCTGCAAGT
hNectin-1γ	GCATGGGCTTGATGTCAG	TCATACTGTCCCCGTTTTACA
hNectin-1α positive control oligo	AGCACCAAGAAGCACGTGTATGGCAACGGCTACAGCAAGGCA GGCATCCCCCAGCACCACCCACCAATGGCACAGAACCTGCAG TACCCCGACGACTCAGACGACGAGAAGAAGGCC	
hNectin-1β positive control oligo	CCCAGCAGGCAAAGCTCCCTTGTGCCTGAGGATATCCAGGTTG TCCACCTGGACCCAGGGAGGCAGCAGCAGCAAGAAGAGGAGG ACTTGCAGAAGCTGTCCCTG	
hNectin-1γ positive control oligo	GCATGGGCTTGATGTCAGACAGCTGTGACCCTGGACAGGGCCC CCCCCACCATCTGTAAAACGGGGACAGTATGA	

Supplementary References

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CHAPTER 3

THE HOST ADHERENS JUNCTION MOLECULE NECTIN-1 IS DEGRADED BY CHLAMYDIAL PROTEASE-LIKE ACTIVITY FACTOR (CPAF) IN *CHLAMYDIA TRACHOMATIS*-INFECTED GENITAL EPITHELIAL CELLS

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Running title: Nectin-1 is degraded by CPAF in *C. trachomatis*-infected cells.

Keywords: *Chlamydia trachomatis*; Nectin-1; Adherens junction; CPAF

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Abstract

Nectin-1 is an adhesion protein implicated in the organization of adherens junctions and tight junctions in epithelial cells. Previous studies in our laboratory demonstrated that nectin-1 accumulation was significantly decreased in *Chlamydia trachomatis*-infected HeLa cells. In the present study, Western blot analyses indicated that nectin-1 down-regulation was *C. trachomatis* concentration-dependent. The half life of nectin-1 was also greatly diminished in *C. trachomatis*-infected cells compared to that observed in mock-infected cells, indicating that nectin-1 was likely down-regulated post-translationally. The chlamydia-secreted protease CPAF is known to degrade several important host proteins; CPAF expression within infected cells correlated with the time-dependent cleavage of nectin-1. Notably, CPAF proteolytic activity is inhibited by lactacystin but not by the proteosome inhibitor MG132. In vivo inhibition experiments demonstrated that nectin-1 down-regulation was blocked by lactacystin exposure. In contrast, MG132 had no effect. Finally, cell-free cleavage assays demonstrated that functional recombinant GST-CPAF$_{wt}$ protein degrades nectin-1. This degradation was blocked by lactacystin, as previously observed in vivo. Collectively, these results indicate that nectin-1 is degraded by CPAF in *C. trachomatis*-infected cells, a novel strategy that chlamydiae may use to aid their dissemination.

Introduction

Chlamydia trachomatis is a Gram-negative, obligate intracellular bacterium that is one of the leading causes of sexually transmitted disease and the most common cause of preventable blindness worldwide. Long-term *C. trachomatis* genital tract infections are often chronic and asymptomatic, leading to severe complications including epididymitis and prostatitis in men as well as pelvic inflammatory disease, ectopic pregnancy and infertility in women (Darville, 2000a). *C. trachomatis* alternates between two morphologically distinct forms and has a unique biphasic developmental cycle. This cycle begins when infectious but metabolically inactive elementary bodies (EB) bind to and enter mucosal epithelial cells. Once internalized, the EBs remain within a membrane-bound inclusion and transform into non-infectious but metabolically active reticulate bodies (RB). Using nutrients from host cell, the RBs multiply, divide by binary fission and eventually differentiate back into EBs, which are then released from the host cell (Wyrick, 2000).

To ensure their successful intracellular replication and long-term survival within infected hosts, chlamydiae have developed various strategies to evade host defense systems. Recently, a chlamydia-secreted protein, designated chlamydia protease-like activity factor (CPAF), has been identified. CPAF is hypothesized to play important roles in promoting chlamydial replication and protecting infected cells from host immune detection (Dong *et al.*, 2004c; Kawana *et al.*, 2007; Pirbhai *et al.*, 2006; Zhong *et al.*, 1999; Zhong *et al.*, 2000; Zhong *et al.*, 2001). CPAF is synthesized in chlamydial inclusions and subsequently secreted into the cytoplasm of infected host cells (Fan *et al.*, 2002; Zhong *et al.*, 2001). CPAF is initially produced as an inactive proenzyme of ~70 kDa and rapidly processed into two fragments, a 35 kDa C-terminal portion (CPAFc) and a 29 kDa N-terminal portion (CPAFn), which must form intramolecular dimers to acquire proteolytic activity (Dong *et al.*, 2004a; Dong *et al.*, 2004b; Zhong *et al.*, 2001). Furthermore, the proteolytic activity of CPAF is inhibited by the cytosolic proteasome inhibitor lactacystin but not by other proteasome inhibitors such as MG132 and MG115 or by broad spectrum protease inhibitors such as PMSF and pepstatin A (Zhong *et al.*,

2000; Zhong *et al.*, 2001). Moreover, CPAF is responsible for degrading host transcription factors USF-1 and RFX5, required for host cell major histocompatibility complex (MHC) class I and II antigen activation (Zhong *et al.*, 1999; Zhong *et al.*, 2000). Similarly, CPAF was found to be both necessary and sufficient for cleaving keratin 8 (Dong *et al.*, 2004c), pro-apoptotic BH3-only proteins (Pirbhai *et al.*, 2006), and CD1d glycoprotein (Kawana *et al.*, 2007). Keratin 8 is a key subunit of intermediate filaments and cleavage of keratin 8 may facilitate chlamydial replication by increasing the solubility of intermediate filaments (Dong *et al.*, 2004c). Pro-apoptotic BH3-only proteins are required for apoptosis in response to cytotoxic stimuli or exogenous stress, and they function by neutralizing their pro-survival counterparts or activating pro-apoptotic Bcl-2 family members Bax and Bak (Bouillet & Strasser, 2002). Degradation of BH3-only proteins contributes to chlamydial anti-apoptotic activity which may help chlamydiae evade CTL-mediated host cell apoptosis induction and, thus, benefit long-term survival of chlamydiae in infected hosts (Pirbhai *et al.*, 2006). CD1d is an MHC-like glycoprotein that presents lipid antigen to natural killer T cells. Down-regulation of CD1d may help *C. trachomatis* escape detection by the host innate immune system (Kawana *et al.*, 2007).

Previous studies in our laboratory have demonstrated that accumulation of the host adherens junction molecule nectin-1 is significantly decreased in *C. trachomatis* serovar E-infected cervical epithelial cells at a post-transcriptional level (Sun *et al.*, 2008). Nectin-1, a member of the nectin family, is a Ca^{2+}-independent cell adhesion molecule (CAM) with three extracellular Ig-like loops, a single transmembrane domain and a cytoplasmic region (Takai & Nakanishi, 2003). Nectins are intimately involved in formation of cell-cell adhesions, which subsequently, in cooperation with other CAMs, function in formation and maintenance of adherens junctions (AJs) and tight junctions (TJs) between neighboring epithelial cells. Using the nectin-based adhesion system, AJs stabilize the epithelium and establish apical-basal polarity of epithelial cells. Additionally, nectins regulate activities of Rho small GTPases and subsequently modulate epithelial barrier functions through reorganization of the actin cytoskeleton and the integrity of intercellular junctions (Braga, 2002). Given the broad range of cellular proteins that are

CPAF targets, we hypothesized that CPAF may degrade nectin-1 in *C. trachomatis*-infected genital epithelial cells. Because nectin-1 is very important for formation and maintenance of AJs and TJs in epithelial cell layers, CPAF-mediated nectin-1 degradation could weaken contacts between an infected genital epithelial cell and adjacent, uninfected cells. This would allow release of the intact, infected cells into genital tract lumen, facilitating chlamydial dissemination within genital tract and aiding establishment of ascending genital tract infection.

Materials and methods

Cell culture and chlamydial infection.

HeLa cells, a human cervical adenocarcinoma epithelial cell line (ATCC No. CCL2), were maintained at 37 °C in Minimal Essential Medium (MEM, Gibco) with Earle's salts and L-glutamine supplemented with 10 % fetal calf serum (Atlanta Biologicals) in an atmosphere of 5 % CO_2 and 95 % humidified air. *C. trachomatis* LGV2 (L2) was used to generate purified CPAF for cell-free cleavage assays. *C. trachomatis* E/UW-5/CX was used for the rest of the experiments. Standardized inocula of *C. trachomatis* E were propagated in McCoy cells as previously described (Wyrick *et al.*, 1996; Zhong *et al.*, 1988). *C. trachomatis* was used to infect HeLa cells as described previously (Sun *et al.*, 2008). Unless otherwise indicated, a multiplicity of infection (MOI) of 1 was used.

Indirect immunofluorescence assay.

HeLa cells were cultured on glass coverslips in 24-well plates overnight and then either mock- or *C. trachomatis* E-infected as described (Sun *et al.*, 2008). After various time periods, the samples were fixed with 100% pre-cooled methanol at −20 °C for 10 min, followed by a brief rinse with 1x PBS. Fixed cells were then blocked for 45 min at 25 °C in 1x PBS containing 15 % FBS. After blocking, some fixed samples were incubated with anti-nectin-1 CK6 (1:50 dilution, sc-21722, Santa Cruz Biotechnology) and anti-*C. trachomatis* major outer membrane protein (MOMP; 1:200 dilution, B65266G, Meridian Life Sciences). The other fixed samples were incubated with either

anti-CPAFn (1:10 dilution, recognizing N terminus fragment of CPAF, a kind gift from Dr. G.M. Zhong, University of Texas, San Antonio, TX) or anti-MOMP antibodies. These incubations were carried out for 1 h at 25 °C. Samples were then stained with Alexa fluor 488 donkey anti-mouse IgG (1:1000 dilution for nectin-1 and 1:500 dilution for CPAFn, A21202, Invitrogen) or Texas red-conjugated donkey anti-goat IgG (1:200 dilution, 59820, Jackson ImmunoResearch Laboratories) for 1 h at 25 °C. Between incubations, the samples were washed twice with 1x PBS. After the final washes, the samples were rinsed with distilled H_2O and mounted onto a glass slide using mounting medium (M01, Biomeda Corp). The following controls were included to confirm the specificity of nectin-1 or CPAF staining: i) primary antibody alone and ii) secondary antibody alone. Images were captured at 400 x using a Zeiss Axiovert S100 inverted microscope and Axiocam camera. Alexa fluor 488-labeled cells were photographed using a FITC-band pass filter; Texas red-labeled cells were photographed using a Rhodamine-long pass filter. Color images were converted to grayscale for publication using Adobe Photoshop.

Western blotting.

Monolayers of HeLa cells were lysed and denatured as described by Deka et al. (Deka *et al.*, 2006). The Western blot assays were performed as described previously (Sun *et al.*, 2008). Briefly, samples were quantified and normalized for total protein content by SDS-polyacrylamide gel electrophoresis (PAGE) followed by SYPRO Ruby staining (BioRad) according to the manufacturer's instructions. Samples were diluted to identical concentrations, requantified as above to confirm that the relative concentration were the same and subjected to SDS-PAGE and Western blotting (Deka *et al.*, 2006). Primary antibodies used for Western blotting included mouse monoclonal anti-nectin-1 CK6 (1:200 dilution), mouse monoclonal anti-β-actin (1:5000 dilution, MAB1501, Chemicon), mouse monoclonal anti-CPAFn (1:1000 dilution), mouse monoclonal anti-cytokeratin-8 (1:5000 dilution, C-5301, Sigma), and goat polyclonal anti-MOMP (1:5000 dilution). Primary antibody binding was detected with corresponding secondary antibodies conjugated to horseradish peroxidase. Specific bands were quantified using an FX phosphorimager and Quantity One V2.5.0 software (Bio-Rad). To control for small

variations in cell number and gel loading between sample lanes, nectin-1 quantity in each sample was normalized (Deka *et al.*, 2006; Sun *et al.*, 2008) to the amount of β-actin protein detected in that same lane. Previous studies from our and other laboratories have demonstrated that β-actin protein accumulation is not altered by chlamydial infection and, therefore, makes an ideal internal control (Deka *et al.*, 2006)

In vivo proteasome inhibitor treatment.

Monolayers of HeLa cells were either mock- or *C. trachomatis* E-infected. At 24 h post infection (hpi), either dimethyl sulfoxide (DMSO, diluent control), lactacystin (30 μM in DMSO, no. 426102, Calbiochem) or MG132 (30 μM in DMSO, no. 474790, Calbiochem) was added to the cell cultures as indicated. Cell lysates were collected at 48 hpi and processed for Western blot analyses using anti-nectin-1 and anti-β-actin antibodies. Duplicate cell cultures were assayed using the live/dead assay kit (Molecular Probes) to assure that any decrease in protein expression observed is not due to toxicity of the inhibitors.

Cell-free cleavage assays.

The cell-free cleavage assays were carried out as previously described (Zhong *et al.*, 2001). Briefly, cytosolic extracts (CE) containing target proteins keratin 8 and nectin-1 were prepared and used as substrate. The enzyme sources included *C. trachomatis* L2-infected HeLa cell cytosolic extracts (L2S100) containing natural CPAF, a functional recombinant GST-$CPAF_{wt}$ fusion protein, and the inactive recombinant GST-$CPAF_{D502A}$ mutant at the concentrations listed in the Figure 5 legend (kind gifts from Dr. G. M. Zhong, University of Texas, San Antonio, TX). The cytosolic extracts were made as described (Dong *et al.*, 2004c) by extracting $1x10^7$ mock- or *C. trachomatis*-infected HeLa cells with 1 ml of a buffer containing 1 % NP-40, 0.5 % Triton X-100 and 150 mM NaCl in 50 mM Tris (pH 8.0) plus a protease inhibitor cocktail (Sigma). The enzyme was mixed with the substrate in the presence or absence of inhibitor lactacystin (Calbiochem). All reactions were carried out at 37 °C for 1.5 h and then subjected to Western blot analyses using anti-nectin-1 and anti-keratin 8 antibodies.

Statistical analyses.

All experiments were repeated three times and each repeat contained three biological replicates, with exception of cell-free cleavage assays which were repeated twice. Statistical analyses were performed using Microsoft Excel. A two-sample *t* test for independent samples was used for comparison of means; *p*-values ≤ 0.05 were considered significant.

Results

Nectin-1 down-regulation is C. trachomatis concentration-dependent.

Previous studies in our laboratory demonstrated that host nectin-1 accumulation was decreased by up to 85 % in *C. trachomatis*-infected HeLa cells. Nectin-1 down-regulation also required active *C. trachomatis* protein synthesis and replication (Sun *et al.*, 2008). The obvious next question was: does a chlamydial factor down-regulate nectin-1 in infected cells? Previous infection studies (Sun *et al.*, 2008) were performed at 1 MOI, which infects 80-90% of HeLa cells in monolayer culture. To determine whether nectin-1 was down-regulated in a dose-response manner, triplicate monolayers of HeLa cells were either mock-infected or infected with different concentrations of *C. trachomatis* E as indicated. Cell lysates were collected at 60 hpi and processed for SDS-PAGE analyses (Fig. 3.1A, B). Duplicate gels were either Sypro Ruby stained for total protein quantification (data not shown) or Western blotted (Fig. 3.1A) using anti-nectin-1 CK6 mAb or anti-β-actin mAb, as described previously (Deka *et al.*, 2006). Nectin-1 bands were quantified, normalized to the β-actin internal control protein, and plotted in Fig. 3.1B. As shown in Fig. 3.1A, compared to mock-infected HeLa cell cultures, nectin-1 accumulation was significantly reduced in HeLa cell cultures infected with *C. trachomatis* at an MOI of 1 or 0.5 inclusion forming units/cell. In contrast, infection with lower quantities of *C. trachomatis* (0.25 and 0.125 MOI) did not significantly reduce nectin-1 accumulation. These data clearly demonstrate that nectin-1 down-regulation within infected cells is *C. trachomatis* concentration-dependent.

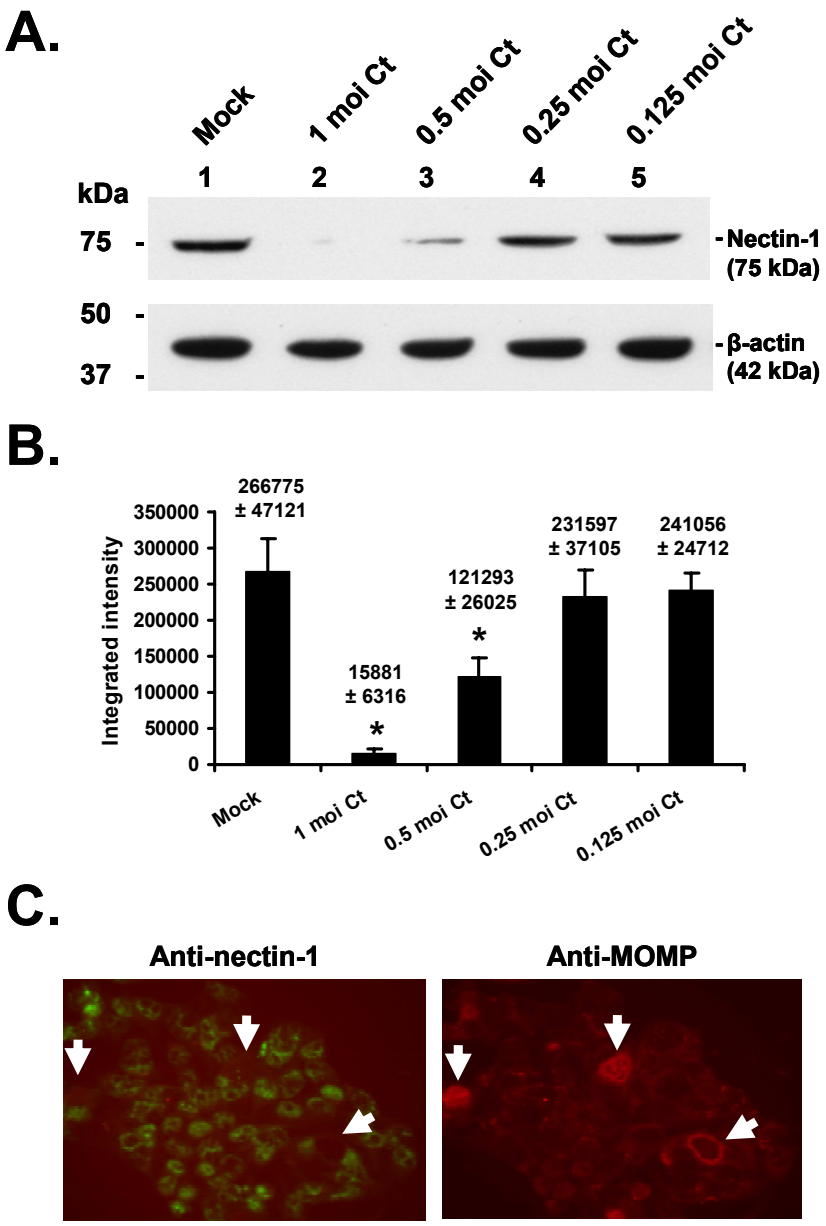

Figure 3.1 Nectin-1 down-regulation is *C. trachomatis* concentration-dependent. (A) HeLa cells were either mock-infected or infected with different concentrations of *C. trachomatis* serovar E. Cell lysates were collected at 60 hpi and Western blotted using anti-nectin-1 (CK6) and anti-β-actin. (B) Nectin-1 bands were quantified and normalized to the β-actin internal control and plotted. Results are expressed as the mean ± SEM of three biological replicates. Those data points significantly different from mock-infected controls are indicated by asterisks ($P \leq 0.05$). (C) HeLa cells were cultured on coverslips and *C. trachomatis* serovar E-infected at 0.25 MOI. After 48 h, cells were fixed and subjected to IFA with nectin-1 and MOMP antibodies. Images were captured at 400 x. All data shown are representative of three independent experiments.

To confirm that nectin-1 was decreased only in *C. trachomatis*-infected cells, we employed indirect immunofluorescence (IFA) methodology. HeLa cells were cultured on coverslips and *C. trachomatis*-infected at an MOI of 0.25 to assure that both uninfected and infected cells would be visible within a single microscopic field. After 48 h, cells were fixed and double-stained with nectin-1 and MOMP specific antibodies. As expected, MOMP was detected in a small number of *C. trachomatis*-infected HeLa cells (Fig. 3.1C, right panel, white arrows). Interestingly, nectin-1 was visible in uninfected cells but was not observed in *C. trachomatis*-infected HeLa cells (Fig. 3.1C, left panel, white arrows). These data indicate that nectin-1 accumulation was only decreased in *C. trachomatis*-infected HeLa cells.

The half-life of nectin-1 in C. trachomatis-infected cells is much shorter than that observed in mock-infected cells.

To determine whether nectin-1 might be subject to *C. trachomatis*-mediated proteolytic degradation, we next determined the half-life of nectin-1 in mock- and *C. trachomatis*-infected HeLa cells. Triplicate monolayers of HeLa cells were either mock- or *C. trachomatis*-infected at an MOI of 1 and then incubated in growth medium plus 2 μg/ml cycloheximide, which halts eukaryotic but not *C. trachomatis* protein synthesis. Pulse chase experiments in which HeLa cells were incubated in ^{35}S-methionone-containing medium demonstrated that host protein synthesis is inhibited by >95% under these conditions {(Deka *et al.*, 2007) and unpublished results}. Previous studies in our laboratory demonstrated that *C. trachomatis* infection first significantly reduced nectin-1 accumulation at 36 hpi. The observed reduction was maximal at 60 hpi (Sun *et al.*, 2008). Therefore, cell lysates were collected for half-life analyses beginning at 36 hpi (mid-development) and extending into the late-developmental cycle (50 and 57 hpi). Samples were Western blotted (Fig. 3.2A) and both nectin-1 and β-actin bands were quantified and plotted. These plots were subsequently used to calculate the half-life of each protein in mock- and *C. trachomatis*-infected cells (Fig. 3.2B, C). The half-life of nectin-1 in *C. trachomatis*-infected cells was much shorter (~ 7 h) than that observed in mock-infected cells (> 62 h; data not shown). These data strongly suggest that nectin-1 is down-regulated by proteolytic degradation in *C. trachomatis*-infected cells.

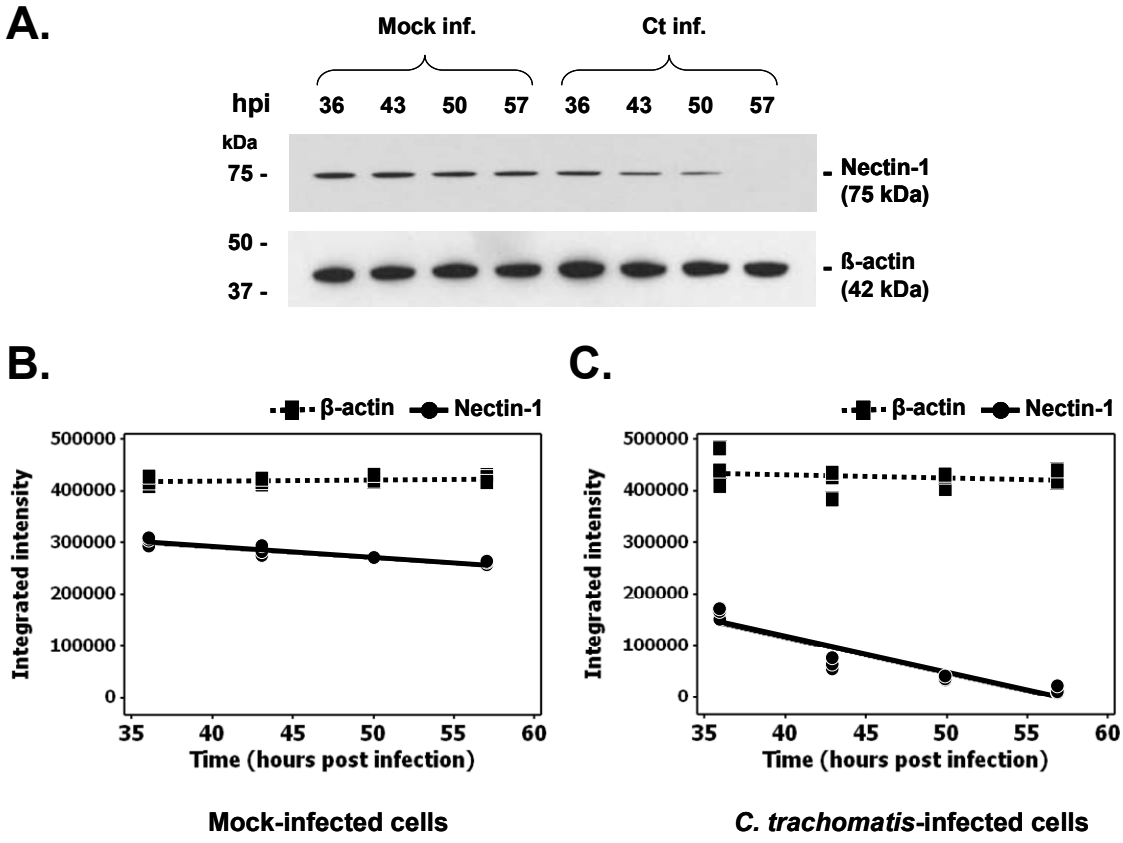

Figure 3.2 The half-life of nectin-1 in *C. trachomatis*-infected cells is much shorter than that observed in mock-infected cells. HeLa cells were mock- or *C. trachomatis*-infected at 1 MOI with addition of 2 μg/ml cycloheximide. Cell lysates were collected at 36, 43, 50 and 57 hpi and processed for Western blot analysis as described (Fig. 3.2A). Nectin-1 and β-actin bands were plotted in Fig. 3.2B and C using Minitab 15 statistical software. The half-life of nectin-1 in mock- or *C. trachomatis*-infected cells was calculated from the plots in Fig. 3.2B and C, respectively. The means of three biologic replicates from three independent experiments are shown.

CPAF expression correlates with C. trachomatis infection time-dependent cleavage of nectin-1.

Published studies and the data presented above suggested that a previously identified chlamydial protease (CPAF) might be responsible for cleaving nectin-1. To test this possibility, we first examined whether CPAF expression kinetics correlate to the timing of nectin-1 cleavage in *C. trachomatis*-infected cells. HeLa cells were either mock- or *C. trachomatis*-infected and duplicate cell cultures were collected at 0 and 24 hpi (early development), 36 hpi {mid-development when significant nectin-1 degradation is first observed (Sun *et al.*, 2008)} as well as at 48 and 60 hpi (late development). Cells were either: i) fixed for IFA (Fig. 3.3A) and stained with anti-CPAFn and anti-MOMP or ii) processed for Western blotting (Fig. 3.3B). Western blots were probed using: i) anti-CPAFn, ii) anti-nectin-1 CK6, iii) anti-MOMP and iv) anti-β-actin. As previously observed (Sun *et al.*, 2008), *C. trachomatis* infection significantly decreased nectin-1 accumulation from 36 to 60 hpi (Fig. 3.3B). CPAF was first detected at 24 hpi by IFA (Fig. 3.3A) and 36 hpi by Western blotting (Fig. 3.3B); with maximal expression at 60 hpi. These data indicate that CPAF expression within infected cells correlates well with the timing of nectin-1 cleavage.

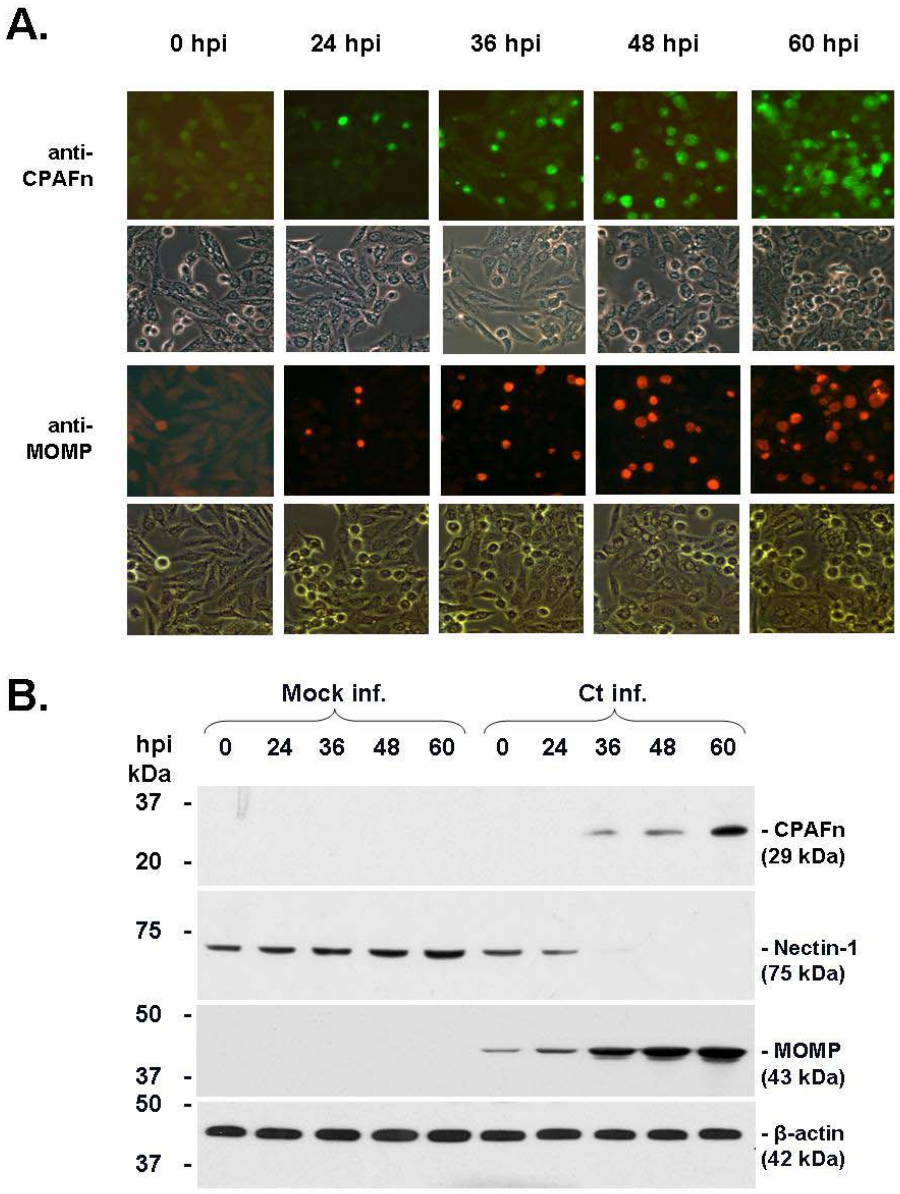

Figure 3.3 CPAF expression correlates with the *C. trachomatis* infection time-dependent cleavage of nectin-1. Duplicate cell cultures were collected at 0, 24, 36, 48 and 60 hpi and either fixed for IFA by staining with anti-CPAFn or anti-MOMP (A) or processed for Western blot analyses using: (i) anti-CPAFn, (ii) anti-nectin-1 (CK6), (iii) anti-MOMP and (iv) anti-β-actin (B). IFA images were captured at 400×. Phase contrast images of stained and non-stained cells are also shown. The data shown are representative of three independent experiments.

Nectin-1 down-regulation in C. trachomatis-infected cells is inhibited by the proteasome inhibitor lactacystin but not by MG132.

The proteolytic activity of CPAF is inhibited by lactacystin, an irreversible proteasome inhibitor, but not by the similar proteosomal inhibitor MG132 (Zhong *et al.*, 2001). Therefore, we examined nectin-1 accumulation in *C. trachomatis*-infected HeLa cells exposed to either lactacystin or MG132. Triplicate monolayers of HeLa cells were either mock- or *C. trachomatis*-infected. At 24 hpi, growth medium plus either DMSO (diluent control), lactacystin (30 μM in DMSO) or MG132 (30 μM in DMSO) was added to each culture as indicated in Fig 3.4A. Cell lysates were collected at 48 hpi and processed for Western analyses (Fig. 3.4A). Nectin-1 bands were quantified, normalized to the β-actin control and plotted in Fig. 3.4B. As expected, nectin-1 accumulation was significantly decreased in *C. trachomatis*-infected and *C. trachomatis* plus DMSO-infected samples. Notably, down-regulation of nectin-1 was blocked by lactacystin-exposure but not by MG132 (Fig. 3.4B), strongly suggesting that CPAF, rather than the host proteosome, mediates the observed down-regulation of nectin-1 in *C. trachomatis*-infected HeLa cells.

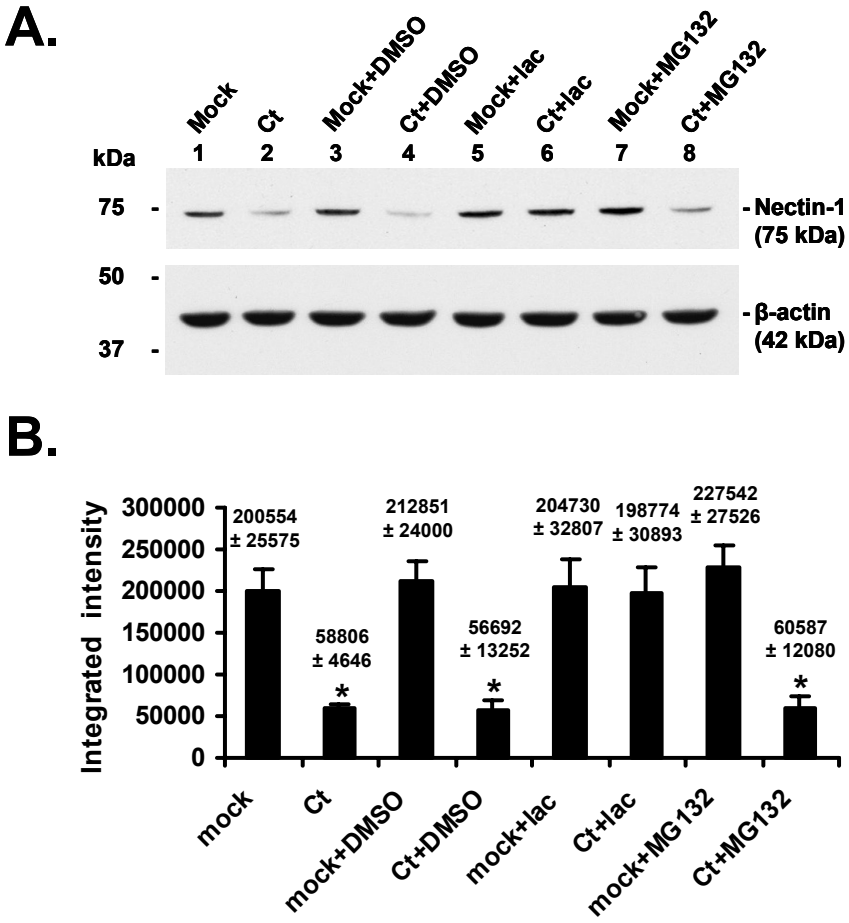

Figure 3.4 Down-regulation of nectin-1 in *C. trachomatis*-infected HeLa cells is inhibited by the cytosolic proteasome inhibitor lactacystin but not by MG132. HeLa cells were either mock- or *C. trachomatis*-infected as described in Fig. 3.2. DMSO (diluent control), lactacystin (30 μM) or MG132 (30 μM) was added to cell cultures at 24 hpi. Cell lysates were collected at 48 hpi and processed for Western blot analyses as described. Nectin-1 bands were quantified, normalized to the β-actin internal control and plotted in Fig. 3.4B. Results are expressed as the mean ± SEM of three biological replicates. Those data significantly different from the mock-infected controls are indicated by asterisks ($P \leq 0.05$). All data are representative of three independent experiments.

CPAF is sufficient for nectin-1 degradation in a cell-free assay.

To evaluate whether CPAF alone is sufficient to degrade nectin-1 in *C. trachomatis*-infected cells, functional recombinant GST-$CPAF_{wt}$ fusion protein purified

from *E. coli* was used to cleave nectin-1 in cell-free, in vitro cleavage assays (Fig. 3.5). As a positive control for CPAF activity, the blots were also probed for keratin 8, which is degraded by both natural CPAF present in *C. trachomatis* L2-infected cell cytosolic extract (L2S100) and the GST-$CPAF_{wt}$ fusion protein (Dong *et al.*, 2004a). Notably, L2S100 (a kind gift from Dr. G. Zhong) degraded both nectin-1 and keratin 8 in these assays (Fig. 3.5, lane 2). As expected, degradation of nectin-1 and keratin 8 by L2S100 extract was reduced when lactacystin was added (Fig. 3.5, lane 3). More importantly, purified recombinant GST-$CPAF_{wt}$ protein (a kind gift from Dr. G. Zhong) also cleaved nectin-1 in a concentration dependent manner (Fig. 3.5, lanes 4-6). Again, lactacystin reduced GST-$CPAF_{wt}$ activity on nectin-1 (Fig. 3.5, lane 7), as previously observed in vivo. Collectively, these observations indicate that nectin-1 degradation is not mediated by the host proteosome or proteolytic enzymes and demonstrate that CPAF activity is sufficient for the nectin-1 degradation observed in *C. trachomatis*-infected HeLa cells.

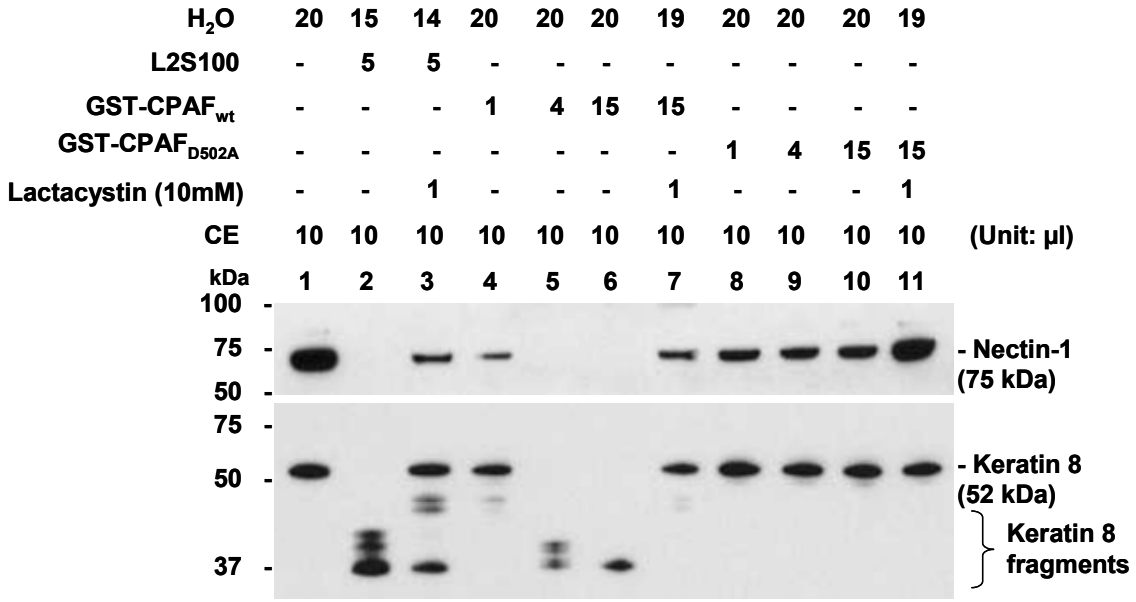

Figure 3.5 CPAF is sufficient for degrading nectin-1. A cell-free cleavage assay was performed using: i) *C. trachomatis* serovar L2-infected cell cytosolic extract (L2S100) which contains natural CPAF (0.0235 µM), ii) a functional recombinant GST-$CAPF_{wt}$ fusion protein expressed in *E. coli* and iii) the *E. coli*-expressed, recombinant inactive GST-$CPAF_{D502A}$ mutant. The components in each reaction, as well as the amount of each component, are listed above each lane in Fig. 3.5. Reactions in lanes 4 and 8 contained

0.33 μmol of either GST-$CAPF_{wt}$ or GST-$CPAF_{D502A}$. Other reactions contained either 1.33 μmol (lanes 5 and 9) or 5 μmol (lanes 6, 7, 10 and 11) respectively. Cytosolic extract (CE) from mock-infected HeLa cells provides the substrates for CPAF-cleavage, including nectin-1 and keratin-8. All reactions were incubated at 37 °C for 1.5 h and then subjected to Western blot analyses using anti-nectin-1 (CK6) and anti-keratin-8. The data are representative of two independent experiments.

Discussion

Chlamydiae are obligate intracellular bacteria that progress through their developmental cycle within an inclusion in the eukaryotic host cell. In order to survive and successfully replicate, chlamydiae have evolved a variety of strategies to cope with the harsh intracellular environment, such as avoiding fusion with lysosomes (Ojcius *et al.*, 1998), prevention of host cell apoptosis (Fan *et al.*, 1998), and altering host cell signal transduction (Xia *et al.*, 2003). A chlamydia-secreted protease, termed CPAF, degrades a number of host cell proteins during chlamydial infection, including transcription factors RFX5 and USF-1, keratin 8, pro-apoptotic BH-3 only proteins, and CD1d glycoprotein. Thus, CPAF activity may contribute to chlamydial anti-apoptotic activity, evasion of host immune detection and modification of host cell cytoskeleton (Dong *et al.*, 2004c; Kawana *et al.*, 2007; Pirbhai *et al.*, 2006; Zhong *et al.*, 2000).

Previous studies in our laboratory demonstrated that nectin-1, a key host cellular adhesion protein, is significantly down-regulated in *C. trachomatis*-infected HeLa cells at a post-transcriptional level (Sun *et al.*, 2008). In the current study, we focused on elucidating the molecular mechanism(s) by which chlamydiae down-regulate nectin-1. Collectively, our observations have led us to conclude that CPAF is both necessary and sufficient for nectin-1 degradation. Interestingly, several studies of other human pathogens suggest that disruption of cell-cell junctions is an important strategy to help intracellular pathogens to spread from cell to cell. *Helicobacter pylori*, a major etiological agent in peptic ulcer disease, decreases host cell E-cadherin, an essential protein for maintenance of AJs (Terres *et al.*, 1998), and alters distribution of ZO-1, a key TJ

scaffolding protein (Krueger *et al.*, 2007). *Shigella flexneri*, an important cause of acute bacillary dysentery in humans, reduces functional components of TJs, including ZO-1, ZO-2, and claudin-1 in infected intestinal epithelial cells (Sakaguchi *et al.*, 2002). These alterations, triggered by pathogen infection, are associated with decreased transepithelial electrical resistance (TER) and increased paracellular permeability, which subsequently result in elongated host cell phenotype and increased migration. As described before, nectin-1 is a key component of AJs and plays critical roles in formation and maintenance of AJs and TJs. Down-regulation of nectin-1 by CPAF would be expected to reduce the integrity and strength of AJs and TJs, leading to loosening of adjacent cell-cell junctions. Relaxation of cell:cell adhesions may allow *C. trachomatis* to rearrange host cell structures required for inclusion enlargement or for EB release at the end of the developmental cycle. On the other hand, disruption of cell-cell junctions may facilitate *C. trachomatis* dissemination within infected host genial tract by allowing release of intact *C. trachomatis*-infected cells from epithelial monolayers. This prediction is consistent with previous observations that cervical epithelial cells separate from each other as a consequence of *C. trachomatis* infection (Prozialeck *et al.*, 2002). Released infected cells could then drift away from original infection sites in the genital mucus and establish infection in new locations, which may help explain the phenomenon of chronic, ascending infection from the lower to the upper human genital tract.

Acknowledgments

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CHAPTER 4

NECTIN-1 IS REQUIRED FOR HERPES SIMPLEX VIRUS (HSV) CO-INFECTION-INDUCED *CHLAMYDIA TRACHOMATIS* PERSISTENCE

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Running title: necint-1 is required for HSV co-infection-induced *C. trachomatis* persistence.

Keywords: *Chlamydia trachomatis*, HSV, Co-infection, Nectin-1.

Abbreviations: EB, elementary body; RB, reticulate body; HSV-1, 2, herpes simplex virus type 1, 2; HVEM, herpes viral entry mediator; IFN, interferon; MOMP, major outer-membrane protein; STD, sexually transmitted disease; 3-O-S-HS, 3-O sulfated heparan sulfate; TNF, tumor necrosis factor; gD, glycoprotein D; IFU, inclusion-forming unit; CPAF, chlamydial protease/proteasome-like activity factor; m.o.i, multiplicity of infection; h.p.i, hours post infection.

Summary

Studies from our laboratory indicate that during *Chlamydia trachomatis*/HSV co-infection, the chlamydiae become persistent. Also, interaction of purified, recombinant HSV-2 glycoprotein D (gD) with the host cell surface mediates this effect in the absence of viral infection. Therefore, we hypothesize that gD/host cell surface receptor interaction initiates a host HeLa cell response that restricts chlamydial development. During viral entry, HSV gD interacts with one of 4 host co-receptors: HVEM (herpes virus entry mediator), nectin-1, nectin-2, and 3-*O*-sulfated heparan sulfate. HVEM and nectin-1 are high-affinity entry receptors for both HSV-1 and HSV-2. Nectin-2 mediates HSV-2 entry with high efficiency but is essentially inactive for HSV-1, while 3-*O*-sulfated heparan sulfate facilitates HSV-1, but not HSV-2, entry. Western blot and RT-PCR analyses demonstrate that HeLa and HEC-1B cells express nectin-1 and nectin-2, but no HVEM. Because both HSV-1 and HSV-2 trigger persistence, these data suggest that, of the co-receptors, nectin-1 is the most likely involved. Interestingly, previously published studies indicate that nectin-1 is strongly down-regulated by 36 hours post-infection (h.p.i) in *C. trachomatis*-infected HeLa cells. Similarly, Western blot analyses demonstrate that nectin-1 is decreased by 68% in *C. muridarum*-infected HeLa cells by 24 h.p.i. Notably, HSV co-infection experiments using either *C. muridarum* or *C. trachomatis* indicate that the chlamydae become resistant to the effects of viral co-infection at 24 and 36 h.p.i, respectively. These data suggest that HSV co-infection can not interfere with chlamydial development in the absence of nectin-1. Co-infection experiments using nectin-1-specific HSV-1 mutants suggest that nectin-1 is, indeed, required for persistence induction. Additional studies in single co-receptor-expressing CHO cells demonstrate that, despite the fact that HSV-1 enters both HVEM and nectin-1-expressing cells, viral co-infection reduces chlamydial infectivity only in the CHO-nectin-1 cell line. These data confirm that virus/nectin-1 interaction is sufficient for chlamydial persistence induction. Although nectin-1 ligation is known to activate the small G protein, Cdc42, pull-down assays indicate that Cdc42 is not activated in co-infected HeLa cells. Thus, we hypothesize that this anti-chlamydial response is mediated through an as yet uncharacterized nectin-1-activated host signaling pathway.

Introduction

According to the Centers for Disease Control and Prevention, sexually transmitted diseases (STDs) are among the most common infections in the United States. Approximately 19 million new STDs cases occur each year in the United States, almost half of them among people ages 15 to 24 (Weinstock *et al.*, 2004). Two of the most commonly reported STD agents in the United States are *Chlamydia trachomatis* (serovars D-K; 4,000,000 new cases/year) and Herpes Simplex Virus (primarily HSV-2; 200,000-500,000 new cases/year) (Bulter, 1997).

C. trachomatis is a Gram-negative, obligate intracellular bacterium that frequently causes chronic, asymptomatic genital infections. Such infections can result in complications such as epididymitis, prostatitis, ectopic pregnancy, salpingitis, and infertility (Darville, 2000a). All chlamydiae share a unique biphasic developmental cycle, alternating between two morphologically distinct forms. The extracellular, infectious but metabolically inert form (the elementary body or EB, 0.3μm diameter) attaches to and enters mucosal epithelial cells via receptor mediated endocytosis (Wyrick, 2000). Following the fusion of EB-containing endosomes, EBs develop into larger (1μm diameter), metabolically active but non-infectious reticulate bodies (RBs). Using ATP and metabolites from the host cell, RBs grow and divide within an enlarged endosomal sac (the inclusion). After 8-12 rounds of replication, the RBs mature into infectious EBs, which are released from the host cell (Wyrick, 2000).

When developing chlamydiae are exposed to unfavorable environmental conditions, they deviate from the normal developmental cycle into a viable but non-infectious state termed persistence (Hogan *et al.*, 2004), where they can remain for months (Galasso & Manire, 1961). Several studies have demonstrated that under appropriate circumstances, chlamydial persistence may occur *in vivo* (Bragina *et al.*, 2001; Dean *et al.*, 2000; Fortenberry *et al.*, 1999; Gerard *et al.*, 2001; Patton *et al.*, 1994). Known inducers include IFN-γ, TNF-α and penicillin-exposure as well as amino acid, glucose and iron deprivation (Beatty *et al.*, 1994a; Darville *et al.*, 2000; Gerard *et al.*,

2001; Raulston, 1997). Persistent chlamydiae continue to synthesize unprocessed 16S rRNA and replicate chromosomes but fail to divide (Gerard *et al.*, 1998; Gerard *et al.*, 2001). At the ultrastructural level, persistent chlamydiae appear as swollen, misshapen, diffuse RBs with increased membrane blebbing. Notably, because they are viable, persistent chlamydiae have ability to re-enter and complete the normal developmental cycle once the persistence "inducer" is removed.

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are members of the viral family *Herpesviridae*. The HSV virion has a large double-stranded DNA genome that is packed within a capsid shell, which is in turn coated with a protein layer (the tegument) and an envelope composed of lipids and more than a dozen viral glycoproteins (Spear & Longnecker, 2003). Though HSV-1 causes some genital infections, HSV-2 is the major causative agent of genital herpes. Initial HSV-2 infection usually occurs on the mucous membranes and skin surrounding the genitals, where painful skin vesicles are formed. The virus subsequently establishes life-long latent infection in the sacral ganglia. Viral replication and symptomatic infection can be periodically reactivated, during which lesions and virions are present (Corey *et al.*, 1983).

Entry of HSV into cells is initiated when the virion binds to cell surface receptor heparan sulfate using the viral envelope proteins gC and/or gB. The viral gD envelope glycoprotein then interacts with one of 4 cellular co-receptors, including HVEM (herpes virus entry mediator), nectin-1, nectin-2 or 3-O sulfated heparan sulfate (3-O-S-HS) (Spear, 2004). HVEM is a member of the tumor necrosis factor (TNF) receptor family which is present on lymphoid and epithelial cells and serves as a co-receptor for both HSV-1 and HSV-2. Nectin-1 and -2 are cell adhesion molecules belonging to the immunoglobulin superfamily which play important roles in the formation of adhesion and tight junctions (Sakisaka & Takai, 2004). Nectin-1 mediates entry of both HSV-1 and HSV-2 equally well, while nectin-2 facilitates HSV-2 entry but is nearly inactive for HSV-1. Conversely, 3-O-S-HS primarily mediates HSV-1 entry (Spear, 2004). The binding of gD to any one of the these co-receptors facilitates the fusion of the viral envelope with the host cell plasma membrane. Once the viral capsid enters the host cell,

replication takes place in the nucleus. New virions are assembled in the nucleus and egress the host cell by vesicular transport (Roizman, 2001a).

Several previous studies from our laboratory demonstrate that HSV-2 super-infection of *C. trachomatis* serovar E-infected genital epithelial cells induces chlamydial persistence (Deka *et al.*, 2006). In addition, induction of *C. trachomatis* persistence by HSV is neither host cell type nor virus strain specific (Deka *et al.*, 2007). *De novo* host/viral protein synthesis and productive HSV replication are not required for chlamydial persistence induction (Deka *et al.*, 2007). Furthermore, HSV co-infection-induced chlamydial persistence is not mediated by any previously identified persistence inducer or anti-chlamydial pathway (Vanover *et al.*, 2008). Unpublished data (Vanover, 2009b) from our laboratory indicate that HSV glycoprotein D (gD) interaction with host cell surface is sufficient to induce chlamydial persistence. Therefore, we performed co-infections using co-receptor-specific HSV mutants and co-receptor expressing CHO host cell lines to test the hypothesis that virus interaction with one of the known co-receptors is sufficient to alter the chlamydial developmental cycle.

Methods

Cells, viruses and chlamydiae.

Chinese hamster ovary (CHO) cell lines including CHO-C8 (containing the pcDNA3 plasmid vector alone), CHO-HVEM, CHO-nectin-1, and CHO-nectin-2 (kind gifts from Dr. Patricia Spear, Northwestern University) were used in co-infection experiments. CHO-C8 cells express cell surface HS but lack all known HSV co-receptors. HSV-1 KOS binds to CHO-C8 cells, but does not enter, even at multiplicities (10-100 m.o.i) that infect HeLa cells with 100% efficiency (Johnson & Spear, 1989). CHO-HVEM, CHO-nectin-1 and CHO-nectin-2 express recombinant HVEM, nectin-1 and nectin-2, respectively. CHO-HVEM and CHO-nectin-1 are permissive for HSV-1 KOS entry (Yoon & Spear, 2004). The other cell lines used in the study were HeLa cervical adenocarcinoma epithelial cells (ATCC No. CCL2) and HEC-1B endometrial epithelial cells (ATCC No. HTB-113). Wild type HSV strains HSV-2 333 and HSV-1 KOS were

obtained from Dr. Mary K. Howett (Drexel University) and Dr. Udayasankar Kumaraguru (East Tennessee State University), respectively. The parental stain HSV-1 KOS/FRT-gD (expressing wild type gD) and the gD amino acid substitution mutants HSV-1 KOS/FRT-gD_{G43P}, HSV-1 KOS/FRT-gD_{Q27P} and HSV-1 KOS/FRT-$gD_{A3C/Y38C}$ were obtained from Dr. Patricia Spear (Northwestern University). The parental strain and all mutants express β-galactosidase (β-gal) activity upon host cell entry (Yoon & Spear, 2004). *C. trachomatis* E/UW-5/CX was originally obtained from Dr. S.P.Wang and Dr. C.C.Kuo (University of Washington) and *C. muridarum* strain Nigg was obtained from Dr. Roger Rank (University of Arkansas Health Sciences Center).

Co-infection experimental design.

Co-infections were performed as previously described by Deka *et al* (Deka *et al.*, 2006). In most experiments, host cells were divided into four groups, $1x10^6$ cells per 60mm culture dish, for mock-infection, chlamydial-infection, HSV-infection, and chlamydia/HSV double infection. Host cells were infected with a dilution of crude EB stock (200 µl) calculated to infect > 80% of the cells and with HSV at an m.o.i of 10 (10 PFU/cell). Mock-infected cells were treated similarly except they were exposed to 200 µl of either 2SPG (0.2 M sucrose, 6 mM NaH_2PO_4, 15 mM Na_2HPO_4, 5 mM L-glutamine, pH 7.2; mock chlamydial infection) or growth medium (mock viral infection).

SDS-PAGE and Western blotting.

Monolayers of host cells were lysed and denatured as previously described (Deka *et al.*, 2006). The Western blot assays were conducted as described by Sun *et al* (Sun *et al.*, 2008). Briefly, samples were quantified using SYPRO Ruby stain (Bio-Rad) according to the manufacturer's instructions. Samples were diluted to identical concentrations, re-quantified to confirm that the relative concentrations were the same and subjected to SDS-PAGE and Western blotting (Deka *et al.*, 2006). Primary antibodies used included mouse monoclonal anti-nectin-1 CK6 (1:200 dilution; sc-21722, Santa Cruz Biotechnology), goat polyclonal anti-chlamydial major outer-membrane protein (MOMP; 1:5000 dilution; B65266G, BioDesign International), mouse monoclonal anti-β-actin (1:5000 dilution; MAB1501, Chemicon), goat polyclonal anti-

nectin-2 (1:1000 dilution; AF2229, R&D systems) and goat polyclonal anti-HVEM (N-19) (1:500 dilution; sc-7766, Santa Cruz Biotechnology). Primary antibody binding was detected with corresponding secondary antibodies conjugated to horseradish peroxidase and visualized using SuperSignal West Pico reagent (Pierce). Bands of interest were quantified using an FX phosphorimager and Quantity One V2.5.0 software (Bio-Rad). To control for small variations in cell number and gel loading between sample lanes, the nectin-1 quantity in each sample was normalized to the amount of β-actin protein detected in that same lane (Sun & Schoborg, 2008).

RNA isolation, reverse transcription and RT-PCR.

Total RNA was isolated from experimental samples using the RNeasy Mini (Qiagen) kit as described previously (Deka *et al.*, 2006). RNA preparations were quantified by measuring OD at 260 and 280 nm. All samples had OD_{260}/OD_{280} ratios > 1.9. Agilent bioanalyses also indicated that all RNAs used had a RIN of >9.8. RNA samples were subjected to reverse transcription as described previously (Deka *et al.*, 2006) and RT-PCR reactions were subsequently carried out using RT(+) and RT(-) cDNA reactions as templates, as appropriate. Experimental template cDNAs were diluted from 1/10 to 1/1000 in double-distilled H_2O and synthetic control DNA targets were diluted from 10 to 0.01pg/ml, ensuring that each reaction was quantified in the linear amplification range. The specific primers and synthetic control DNA targets for HVEM and nectin-2 were designed using Vector NTI Advance V10 (Invitrogen) and are listed in Supplementary Table S1. Nectin-1α, -1β and -1γ specific primers and their oligonucleotide amplification controls were previously described (Sun *et al.*, 2008). Most reactions were performed using the following cycling conditions (unless otherwise indicated): 94°C, 1 min; 60°C, 1 min; 72°C, 1 min for 35 cycles. The resulting PCR products were electrophoresed and quantified as previously described (Sun *et al.*, 2008).

β-galactosidase assay.

This assay was performed as described previously (Montgomery *et al.*, 1996). In this assay, β-gal expression (and virion entry) is indicated by the formation of an insoluble blue reaction product. Six hours after HSV infection, cells were washed with

1x PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 10 min at room temperature. Following fixation, the cells were washed with 1x PBS and permeablized with 2 mM $MgCl_2$/0.01% deoxycholate/0.02% NP-40 in PBS for 10 min at room temperature. The cells were incubated with 0.5 mg/ml X-gal (5-bromo-3-indoyl-β-D-galactopyranoside) in PBS for at least 1 hour at 37°C. Images were obtained using an Epson Perfection 3200 Photo Scanner and Photoshop Elements Software.

Chlamydial titration by subpassage.

Chlamydial titrations were conducted as previously described (Deka *et al.*, 2006) using Pathfinder anti-chlamydial stain (Bio-Rad) to stain chlamydial inclusions formed from subpassaged EBs. The number of inclusion-forming units (IFU) in the undiluted inoculum was derived from triplicate counts and expressed as IFU ml^{-1}.

Transmission electron microscopy.

C. trachomatis-infected or *C. trachomatis*/HSV co-infected HeLa cells were processed for high-contrast TEM as described (Wyrick, 1994). Counter-stained gold thin sections were examined using a Tecnai 10 (FEI) transmission electron microscope operating at 60-80 kV.

HSV-1 plaque assay.

At 20h post HSV-1 infection, cell culture supernatants were collected and centrifuged at 4000*g* for 5 min at 4°C to remove cell debris. Plaque assays were performed on the resulting supernatants as described (Duff & Rapp, 1971). Quadruplicate, infected cultures were incubated with methylcellulose at 37°C for 72h, followed by removing methylcellulose and staining with a solution of 5% formaldehyde and 0.5% crystal violet for 1h. Plates were then washed and plaques were counted. Average plaque counts from each set of plates were used to calculate the pfu ml^{-1} present in the original supernatant.

Statistical analyses.

Statistical analyses were performed using Microsoft Excel. A two-sample *t* test for independent samples was used for comparison of means; *p*-values ≤ 0.05 were considered significant.

Results

HeLa and HEC-1B cells express nectin-1 and nectin-2 but not HVEM.

Previous data from our laboratory have demonstrated that HSV-induced chlamydial persistence occurs in both HeLa and HEC-1B cells (Deka *et al.*, 2007). To aid in elucidating which, if any, host HSV co-receptors are involved, we first determined which co-receptors HeLa and HEC-1B cells express. HeLa or HEC-1B cell lysates were subjected to SDS-PAGE and Western blotted with either HVEM, nectin-1, nectin-2 or β-actin antibodies. CHO-HVEM, CHO-nectin-1 and CHO-nectin-2 cell lysates were used as positive controls, respectively. As shown in Fig. 1(a), both HeLa and HEC-1B cells express nectin-1 and nectin-2, but not HVEM. As expected, HVEM, nectin-1 and nectin-2 are easily detected in the appropriate CHO cell line. These data indicate that the HeLa and HEC-1B cell lines used in our experiments express nectin-1 and 2, but not HVEM.

Because it is possible that HeLa and HEC-1B cells express only a small quantity of HVEM that was below the level of Western blot detection, total RNA from HeLa, HEC-1B, or CHO-HVEM cells was isolated and subjected to RT-PCR using specific primers for HVEM. In each experiment, a four log dilution series of synthetic control DNA was used to generate standard curves for amplification. Experimental samples were only quantified if they fell within the linear range of the PCR. All amplimers were the expected size (Fig. 1b) and the identity of each was confirmed by DNA sequencing (data not shown). Amplification products were not observed in template-negative samples (Fig. 1b, lane 6) or in RT(-) controls (data not shown). As shown in Fig. 1(b), CHO-HVEM cell controls express a large amount of HVEM mRNA (lane 9). However, HVEM transcript was not observed in either HeLa or HEC-1B samples, even after 38 cycles of amplification (lanes 7 and 8). Similarly, total RNA from HeLa, HEC-1B and the

appropriate CHO control cell lines was subjected to RT-PCR using specific primers for nectin-1α, -1β, -1γ and nectin-2 (see supplementary data, Fig. S1a,b). As expected, both HeLa and HEC-1B cells express nectin-1 and nectin-2 mRNAs, although in a lower amount than do the CHO-nectin-1 or CHO-nectin-2 cell lines.

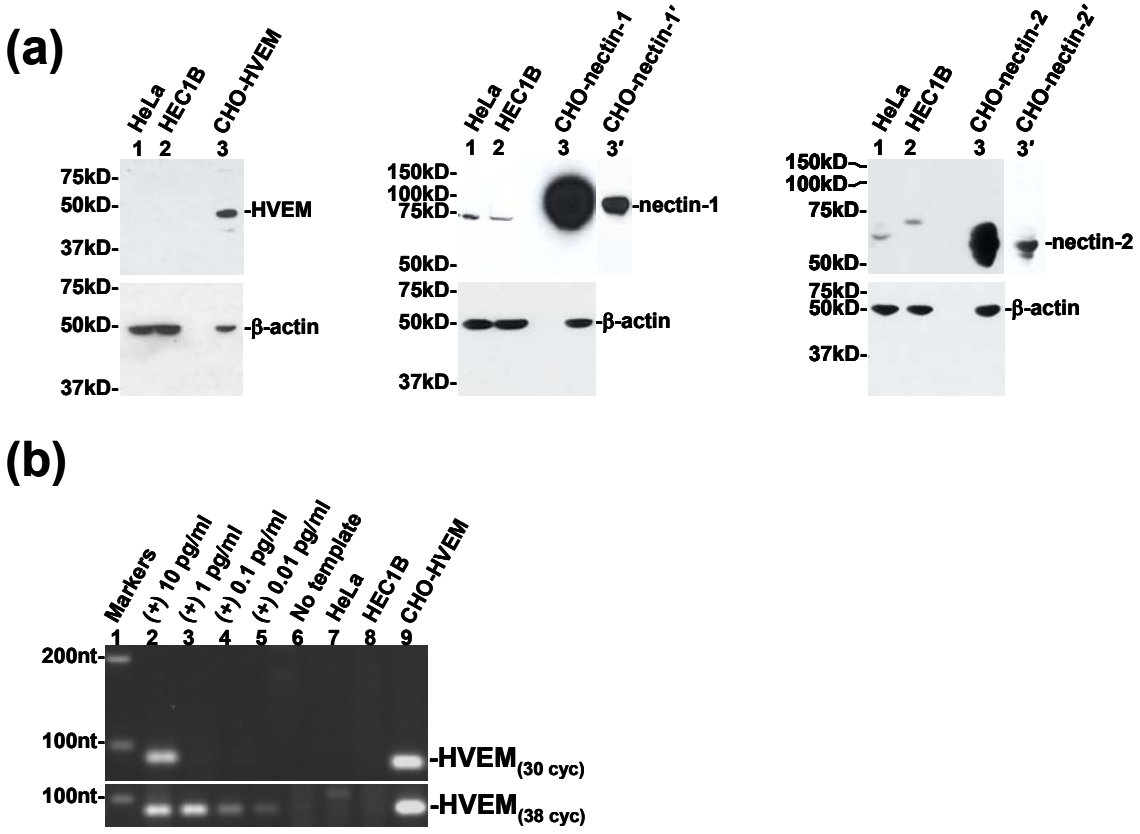

Figure 4.1 HeLa and HEC-1B cells express nectin-1 and nectin-2 but not HVEM. (a) HeLa or HEC-1B cell lysates were subjected to SDS-PAGE and Western blotted with either HVEM, nectin-1, nectin-2 or β-actin antibodies. CHO-HVEM, CHO-nectin-1 and CHO-nectin-2 cell lysates were used as positive controls, respectively. (b) Total RNA from HeLa, HEC-1B or CHO-HVEM cells was isolated and subjected to RT-PCR using specific primers for HVEM. In each experiment, a four log dilution series of synthetic control DNA was used to generate standard curves for amplification. Experimental samples were only quantified if they fell within the linear range of the PCR. The position of DNA size markers are shown to the left of each gel image in base pairs (bp).

Collectively, these above data demonstrate that both HeLa and HEC-1B cells express nectin-1 and nectin-2, but express little or no HVEM. In addition, it has been demonstrated that anti-HVEM antibodies only had marginal effects on the entry of HSV-1 into HeLa cells, suggesting that HVEM is probably not a major receptor mediating HSV entry into HeLa cells (Montgomery *et al.*, 1996). On the other hand, previous studies have demonstrated that both HSV-1 and HSV-2 trigger chlamydial persistence (Deka *et al.*, 2007) and both use HVEM and nectin-1 equally well (Spear, 2004). Moreover, nectin-2 mediates HSV-2 entry with high efficiency but is nearly inactive for HSV-1, while 3-O-S-HS facilitates HSV-1, but not HSV-2 entry (Spear, 2004). Thus, together, these data suggest that, of the co-receptors, nectin-1 is the most likely candidate involved in HSV-induced chlamydial persistence.

HSV co-infection does not affect the chlamydial developmental cycle in the absence of nectin-1.

During the process of co-infection studies on *C. trachomatis*, we examined other chlamydial species to see whether they also enter persistence in HSV co-infected host cells. *C. muridarum*, formerly the mouse pneumonitis biovar of *C. trachomatis*, naturally infects only members of the family Muridae (Everett *et al.*, 1999). HeLa cells were either mock-, HSV-2-, *C. muridarum*-, or co-infected. To determine the effect of HSV co-infection on *C. muridarum* development, HeLa cells were infected with *C. muridarum* for 6h, 12h or 24h, followed by HSV-2 infection. Cells were collected 20h post HSV-2 infection and processed for chlamydial EB titration. As shown in Fig. 2(a), when compared with chlamydia singly-infected controls, production of infectious EBs was significantly decreased when HSV-2 was added at 6h and 12h post-chlamydial infection. In contrast, no significant reduction in chlamydial infectivity was observed when the virus was added at 24h post-chlamydal infection (Fig. 2a). In addition, the effect of HSV-2 on *C. muridarum* infectivity is greater at 6h than at 12h.

Published data from our laboratory demonstrated that nectin-1 is degraded in *C. trachomatis*-infected HeLa cells at 36 to 48 h.p.i (Sun *et al.*, 2008). Based on this information, we examined *C. muridarum*-infected HeLa cells to see whether nectin-1

down-regulation correlates with the time post-infection at which *C. muridarum* becomes insensitive to HSV co-infection. HeLa cells were either mock- or *C. muridarum*-infected. Cell lysates were collected at 0, 6, 12, 24, 36 h.p.i and Western blotted with (i) anti-nectin-1, (ii) anti-MOMP, (iii) anti-β-actin. A representative blot is shown in Fig. 2(b); the quantification of nectin-1 is shown in Fig. 2(c). These data clearly demonstrate that *C. muridarum* also degrades nectin-1 and that significant degradation is first observed at 24 h.p.i. Thus, these results suggest that HSV co-infection can not disrupt the normal chlamydial developmental cycle in the absence of nectin-1.

It is important to note that, in all of our published co-infection experiments using *C. trachomatis*, the virus was added at 24 h post-chlamydia infection. Our data indicate that in *C. trachomatis*-infected HeLa cultures nectin-1 quantity is essentially unaltered at 24 h.p.i (Sun *et al.*, 2008). Therefore, we also examined whether HSV co-infection can influence chlamydial development when HSV was added at 36h and 48h post *C. trachomatis*-infection, at which times significant nectin-1 down-regulation is observed (Sun *et al.*, 2008). HeLa cells were either mock-, HSV-2-, *C. trachomatis*-, or co-infected. For co-infected HeLa cells, cells were co-infected with HSV-2 either 24h, 36h, or 48h after addition of the chlamydiae. Cells were collected at 20h post HSV-2 infection and processed for chlamydial EB titration. As shown in Fig. 2(d), when compared with chlamydia singly-infected controls, a significant decrease in the production of infectious EBs occurred in co-infected cells when HSV-2 was added at 24h and 36h but not when HSV-2 was added at 48h post-chlamydial infection. In addition, the effect of HSV-2 on *C. trachomatis* infectivity is greater at 24h than at 36h. These data are consistent with the *C. muridarum* results and suggest that nectin-1 is required for HSV co-infection-induced disruption of the chlamydial developmental cycle.

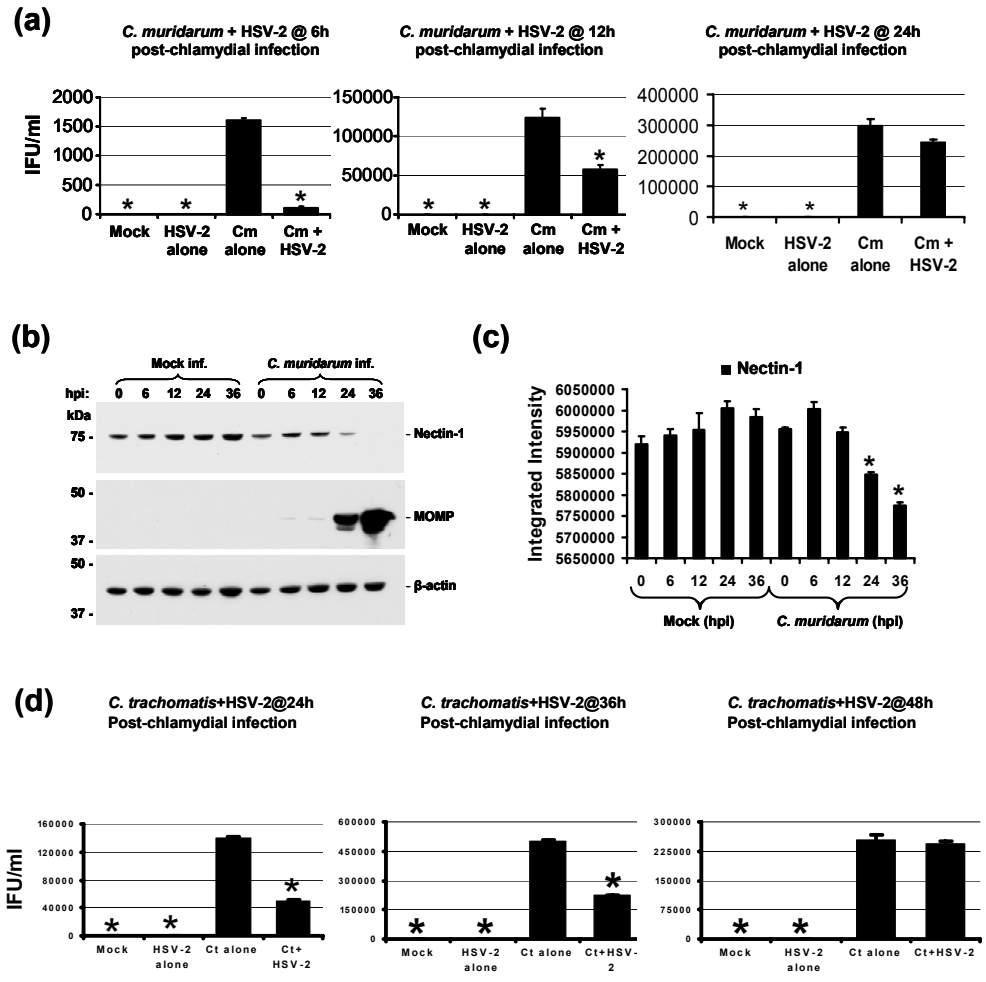

Figure 4.2 HSV co-infection does not affect the chlamydial developmental cycle in the absence of nectin-1. (a) HeLa cells were either mock-, HSV-2-, *C. muridarum*-, or co-infected. For co-infected HeLa cells, cells were infected with *C. muridarum* for 6h, 12h or 24h, followed by HSV-2 infection. Cells were collected 20h post HSV-2 infection and processed for chlamydial EB titration. Asterisks (*) indicate titres that are significantly different compared with those from *C. muridarum* singly-infected cells (P<0.05). (b) HeLa cells were either mock- or *C. muridarum*-infected. Cell lysates were collected at 0, 6, 12, 24, 36 h.p.i and Western blotted with (i) anti-nectin-1, (ii) anti-MOMP, (iii) anti-β-actin. (c) The quantity of nectin-1 detected in panel (b) was normalized to the internal control protein β-actin and plotted. Groups significantly (P<0.05) different from *C. trachomatis*-infected cells at 0 h.p.i are indicated by asterisks (*). (d) HeLa cells were either mock-, HSV-2-, *C. trachomatis*-, or co-infected. For co-infected HeLa cells, cells were co-infected with HSV-2 either 24h, 36h or 48h after addition of the chlamydiae. Cells were collected at 20h post HSV-2 infection and processed for chlamydial EB titration. Asterisks (*) indicate titres that are significantly different compared with those from *C. trachomatis* singly-infected cells (P<0.05). The data shown in this figure are representative of three independent experiments.

HSV-induced chlamydial persistence requires nectin-1.

Our published and unpublished data suggest that HSV gD/co-receptor interaction halts the chlamydial developmental cycle. As a further step in defining if nectin-1 is involved, we have examined if HSV-induced chlamydial persistence requires nectin-1 by co-infection using HSV-1 mutants with altered co-receptor specificities. HeLa cells were either mock-, singly-, or co-infected with *C. trachomatis* and various wild type (HSV-2 and HSV-1 KOS/FRT-gD_{wt}) or mutant viruses (HSV-1 KOS/FRT-gD_{G43P}, HSV-1 KOS/FRT-gD_{Q27P}, and HSV-1 KOS/FRT-$gD_{A3C/Y38C}$). HSV-2 (333) co-infection was used as a positive control for chlamydial persistence induction. The parental stain HSV-1 KOS/FRT-gD expresses wild type gD and enters host cells via HVEM, nectin-1, and 3-O-S-HS with high/moderate efficiency and via nectin-2 with very low efficiency. HSV-1 KOS/FRT-gD_{G43P} can only enter using nectin-1. HSV-1 KOS/FRT-gD_{Q27P} uses both nectin-1 and nectin-2 equally well. Neither G43P nor Q27P use HVEM or 3-O-S-HS, even when infections are performed at an m.o.i of 200 (Yoon & Spear, 2004). HSV-1 KOS/FRT-$gD_{A3C/Y38C}$ uses HVEM and 3-O-S-HS but not the nectins to enter host cells. The parental stain and the three mutants also express β-gal activity upon host cell entry (Yoon & Spear, 2004). Cells were collected at 20h post viral infection and processed for chlamydial EB titration as described in the Methods. As shown in Fig. 3(a), HSV-2, gD_{wt} and all three mutants significantly reduced chlamydial EB production. Because we have found HeLa cells express little or no HVEM and HSV-1 KOS/FRT-$gD_{A3C/Y38C}$ has been reported to be HVEM/3-O-S-HS specific (Yoon & Spear, 2004), it was unexpected to see the double mutant represses chlamydial infectivity.

To find out the reason behind this phenomenon, we performed β-gal assays to check the specific entry phenotypes of HSV-1 KOS/FRT-gD_{wt}, -gD_{G43P}, -gD_{Q27P}, and -$gD_{A3C/Y38C}$ using CHO cell lines expressing single co-receptors: CHO-C8, CHO-HVEM, CHO-nectin-1, and CHO-nectin-2. CHO-C8 cells express cell surface HS but lack any known HSV co-receptors. As shown in Fig 3(b), as expected, no β-gal activity is observed in CHO-C8 cells. For gD_{wt}-infected cells, β-gal activity is strongly expressed in CHO-HVEM and CHO-nectin-1 but is weakly expressed in CHO-nectin-2. These results were expected because nectin-2 supports HSV-2 entry but is nearly inactive for entry of

HSV-1. For gD_{G43P}-infected cells, β-gal activity is only present in the CHO-nectin-1 well. HSV-1 KOS-gD_{Q27P} expresses β-gal activity in both CHO-nectin-1 and CHO-nectin-2 cells, as expected. In $gD_{A3C/Y38C}$-infected cells, β-gal activity is present in CHO-HVEM cells. However, we also observed a low, but reproducible, level of β-gal expression in CHO-nectin-1 cells, which indicates that despite the published reports (Yoon & Spear, 2004), $gD_{A3C/Y38C}$ can attach to and enter nectin-1 expressing CHO cells, albeit at an efficiency much lower than that observed for HVEM-expressing cells. Given that the only co-receptor usage that the three mutants share is nectin-1, these data suggest that virus interaction with this co-receptor is necessary for persistence induction in our system.

We also tested the specific entry phenotypes of HSV-1 KOS/FRT-gD_{wt}, -gD_{G43P}, -gD_{Q27P}, and -$gD_{A3C/Y38C}$ on HeLa cells. As shown in Fig. 3(c), no β-gal activity is present in mock-, *C. trachomatis*-, HSV-2- or *C. trachomatis* /HSV-2-infected HeLa cells. As expected, the wild type (gD_{wt}) and the two mutants (gD_{G43P} and gD_{Q27P}) enter HeLa cells with high efficiency. However, the double mutant $gD_{A3C/Y38C}$ does not show detectable entry activity in this assay, which is not surprising since this assay is relatively insensitive and nectin-1 expression on HeLa cells is much lower than that on CHO-nectin-1 cells, as shown by the data in Fig. 1(a) (middle panel).

Persistent forms of *C. trachomatis* have a characteristic electron microscopic appearance (Beatty *et al.*, 1993; Matsumoto & Manire, 1970). To confirm the results from Fig. 3(a), we therefore conducted transmission electron microscopy as described in the Methods. Electron micrographs demonstrated that in *C. trachomatis* singly-infected HeLa cells, EBs were present and RBs appeared normal (Fig. 3d). In contrast, in HSV-1 KOS/FRT-gD_{wt}, -gD_{G43P}, -gD_{Q27P}, or -$gD_{A3C/Y38C}$ co-infected HeLa cells, EBs were absent from chlamydial inclusions and the RB exhibited an enlarged, abnormal morphology as well as increased membrane blebs, characteristic of persistence (Fig. 3d). Collectively, these data are again consistent with the supposition that nectin-1 is required for HSV co-infection-induced chlamydial persistence.

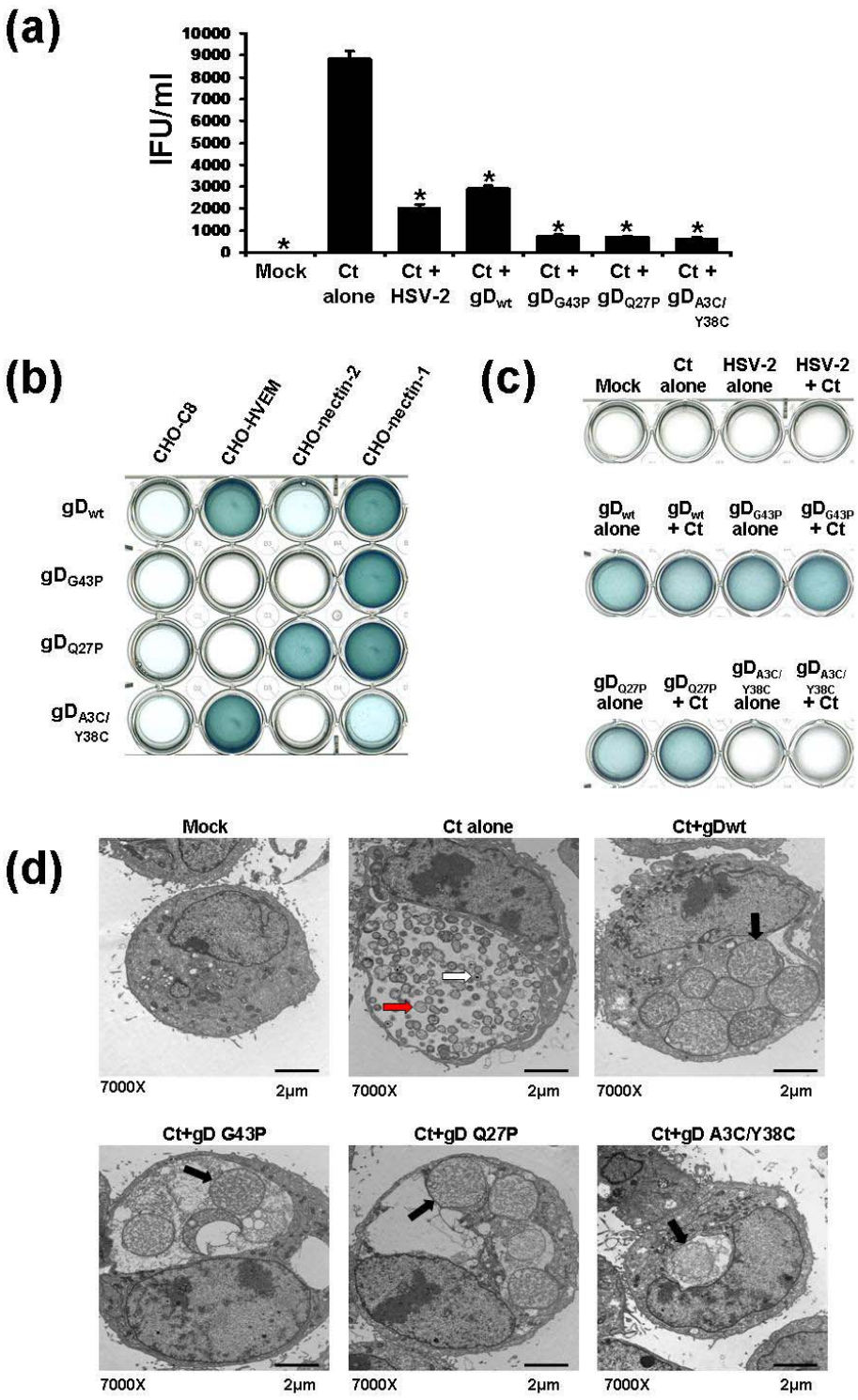

Figure 4.3 HSV-induced chlamydial persistence requires nectin-1. (a) HeLa cells were either mock-, singly-, or co-infected with *C. trachomatis* and various wild type (HSV-2

and HSV-1 KOS/FRT-gD_{wt}) or mutant viruses (HSV-1 KOS/FRT-gD_{G43P}, HSV-1 KOS/FRT-gD_{Q27P} and HSV-1 KOS/FRT-$gD_{A3C/Y38C}$). HSV-2 co-infection was used as a positive control for chlamydial persistence induction. Asterisks (*) indicate titres that are significantly different compared with those from *C. trachomatis* singly-infected cells ($P<0.05$). (b) CHO cell lines expressing single co-receptors, CHO-C8, CHO-HVEM, CHO-nectin-1 and CHO-nectin-2, were infected with HSV-1 KOS/FRT-gD_{wt}, -gD_{G43P}, -gD_{Q27P} and -$gD_{A3C/Y38C}$, respectively, for 6 hours. The infected cells were then processed by β-gal assays as described in the Methods. (c) HeLa cells were either mock-, singly-, or co-infected with *C. trachomatis* and HSV-2, HSV-1 KOS/FRT-gD_{wt}, -gD_{G43P}, -gD_{Q27P} or -$gD_{A3C/Y38C}$, respectively, for 6 hours. The infected cells were then processed by β-gal assays as described in the Methods. (d) HeLa cells were either mock-, *C. trachomatis*-infected or co-infected with *C. trachomatis* and HSV-2, HSV-1 KOS/FRT-gD_{wt}, -gD_{G43P}, -gD_{Q27P} or -$gD_{A3C/Y38C}$, respectively. Cells were harvested for TEM analyses. White arrows on electron micrographs indicate EBs; Red arrow indicates normal RB; black arrows indicate abnormal RBs. The data shown are representative of three independent experiments.

<u>HSV attachment/entry using nectin-1 alone is sufficient to induce chlamydial persistence.</u>

Previous studies indicate that HSV attachment and/or entry into the host cells is sufficient for inducing chlamydial persistence (Deka *et al.*, 2007), suggesting that viral attachment/entry may provide the necessary stimulus to trigger a novel host pathway which restricts chlamydial development (Vanover *et al.*, 2008). To confirm that nectin-1 is, indeed, required for this process, we performed co-infections with *C. trachomatis* and HSV-1 KOS in the previously described single co-receptor expressing CHO cell lines. Cells were first chlamydia infected, and then HSV-1 KOS was added 24 hours later. Cells were collected at 20h post HSV-1 infection and processed for chlamydial EB titration and plaque assays. Chlamydial titer analyses demonstrated that though there are differences in total *C. trachomatis* production from different CHO cell lines, the characteristic HSV-1-induced reduction in chlamydial infectivity is only observed in the positive control co-infected HeLa cells and in co-infected CHO-nectin-1 cells (Fig. 4). As expected, supernatant plaque assays demonstrate that co-infected CHO-HVEM and CHO-nectin-1 cells produce similar amounts of virus, while CHO-C8 cultures produce essentially none (data not shown). These data are consistent with our previous observation that HSV-1 enters CHO-HVEM and CHO-nectin-1 cells with high efficiency while showing no

detectable entry activity in CHO-C8 cells (Fig. 3b). Therefore, these data confirm that HSV-attachment/entry using nectin-1 co-receptor alone is sufficient to induce chlamydial persistence.

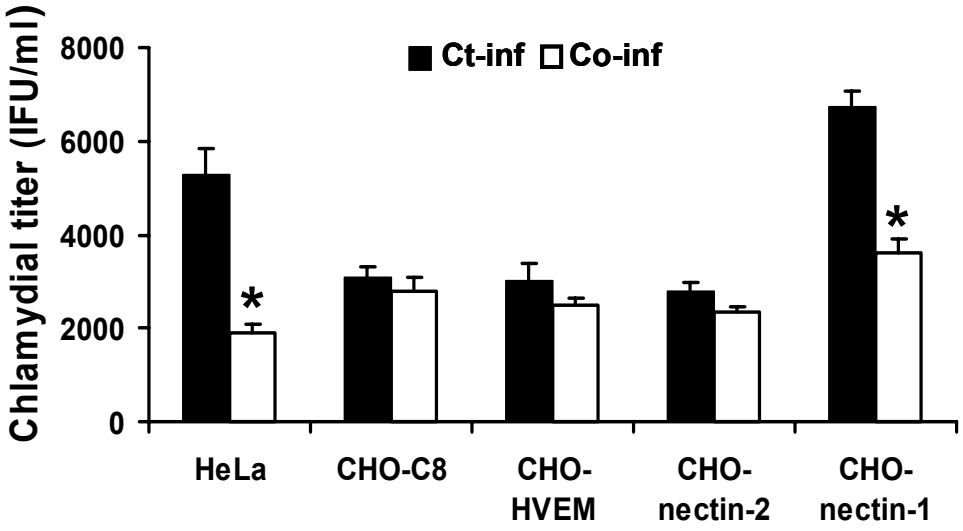

Figure 4.4 HSV attachment/entry using nectin-1 alone is sufficient to induce chlamydial persistence. HeLa, CHO-C8, CHO-HVEM, CHO-nectin-1 and CHO-nectin-2 were either *C. trachomatis*-infected or co-infected with *C. trachomatis* and HSV-1 KOS. For co-infected cells, Cells were first chlamydia infected, and then HSV-1 KOS was added 24 hours later. Cells were collected at 20h post HSV-1 infection and processed for chlamydial EB titration. Asterisk (*) indicates titre that is significantly different compared with that from *C. trachomatis* singly-infected same cells ($P<0.05$). The data shown are representative of three independent experiments.

Discussion

When exposed to certain adverse environmental factors, developing chlamydiae deviate from the normal developmental cycle into a viable but non-replicative persistent state (Hogan *et al.*, 2004) and can remain in this state for weeks or months (Galasso & Manire, 1961). Persistent chlamydial infections increase the likelihood that an individual will experience prolonged inflammation of the genital tract and, thus, increasing the possibilities for development of severe disease sequelae. Several inducers of chlamydial persistence have been well studied, including interferon-γ (IFN-γ), IFN-α, IFN-β, tumor necrosis factor-α (TNF-α), and penicillin G exposure, as well as amino acid, iron, and glucose deprivation (Beatty *et al.*, 1994a; Darville, 2000b; Gerard *et al.*, 2001; Raulston, 1997). Previously, our laboratory has established a tissue culture model of *C. trachomatis*/HSV-2 co-infection system. Data from this model indicate that viral co-infection stimulates chlamydiae to become persistent (Deka *et al.*, 2006), as evidenced by numerous observations: i) fewer or no EBs and enlarged, abnormal RB morphology; ii) a significant decrease in infectious chlamydial EB production; iii) decreased accumulation of chlamydial major outer membrane protein (MOMP) and increased accumulation of chlamydial heat-shock protein HSP-1 (HSP60-1); iv) continued accumulation of chlamydial genomic DNA and constant expression of chlamydial 16S rRNA transcripts.

Subsequently, Deka *et al* have demonstrated that viral co-infection-induced chlamydial persistence is not host cell type-specific because it occurs in both HeLa and HEC-1B cell lines (Deka *et al.*, 2007). HSV induction of chlamydial persistence is also not virus-specific due to both HSV-1 and HSV-2 co-infection can interfere with normal chlamydial development (Deka *et al.*, 2007). Additionally, our laboratory has demonstrated that co-infection-stimulated chlamydial persistence is not mediated by any currently characterized persistence inducer or anti-chlamydial pathway (Vanover *et al.*, 2008), suggesting a novel host pathway may be triggered by viral co-infection to restrict chlamydial development. Furthermore, co-infection with UV-inactivated, replication-incompetent virus or replication-competent HSV-2 in the presence of cyclohexamide drives chlamydiae to become persistent, indicating productive viral replication is not

required for this effect and suggesting that events during HSV attachment/entry are sufficient to stimulate co-infection-induced persistence (Deka *et al.*, 2007). Finally, unpublished data from our laboratory demonstrate that exposure of *C. trachomatis*-infected cells to cross-linked, soluble HSV envelope glycoprotein D:Fc fusion proteins decreases chlamydial infectivity, indicating that interaction of HSV gD with the host cell surface is sufficient to stimulate chlamydial persistence (Vanover, 2009a).

HSV gD is the main determinant of cell recognition for viral entry. During the initial stages of viral invasion, gD binds to one of its co-receptors: nectin-1, nectin-2, 3-O-S-HS, and HVEM, followed by fusion of viral envelope and host cell membrane. Nectin-1 and nectin-2 are important cell adhesion molecules of the immunoglobulin super-family and are distributed on fibroblasts and epithelial cells. They mainly are involved in formation and maintenance of adherens and tight junctions (Sakisaka & Takai, 2004). While nectin-1 mediates entry of both HSV-1 and HSV-2, nectin-2 mediates entry of primarily HSV-2 (Spear, 2004). 3-O-S-HS is widely expressed on human cells and facilitates HSV-1, but not HSV-2, entry (Spear, 2004). HVEM is a member of the TNF receptor family. Its natural ligands include LIGHT, B and T lymphocyte attenuator (BTLA) and lymphotoxin-α (Gonzalez *et al.*, 2005; Sedy *et al.*, 2005). HVEM is present on lymphoid and epithelial cells and serves as a high-affinity entry co-receptor for both HSV-1 and HSV-2 (Spear, 2004). However, Montgomery *et al.* have shown that HVEM is probably not the primary receptor for HSV entry into HeLa cells, even HVEM cDNA was originally cloned from a HeLa cell library, because anti-HVEM serum only had marginal effects on the entry of HSV-1 into HeLa cells (Montgomery *et al.*, 1996). On the other hand, unpublished data by Vanover *et al.* from our laboratory have shown that non-crosslinked soluble HSV-2 gD:Fc fusion proteins interaction with HeLa cells did not stimulate chlamydial persistence. However, when chlamydiae-infected HeLa cells exposed to immunoglobulin cross-linked gD:Fc fusion proteins, the developing chlamydiae became persistent (Vanover, 2009a). Interestingly, the dimerizing of nectins is required for nectin *trans*-interaction induced downstream cell signaling cascades (Ogita & Takai, 2006b). Finally, our studies have shown that both HSV-1 and HSV-2 can drive developing chlamydiae to enter persistence state (Deka *et al.*, 2007). Thus, collectively,

these observations suggest that, of the four known co-receptors, nectin-1 is the most potential candidate for stimulating HSV co-infection-induced chlamydial persistence in this system.

In the present study, the goal was to test the hypothesis that nectin-1 is required for HSV co-infection-induced chlamydial persistence. We first examined which HSV co-receptors were expressed in HeLa and HEC-1B cells based on the fact that HSV co-infection-induced persistence occurs in both HeLa and HEC-1B cells (Deka *et al.*, 2007). Notably, both Western blot and RT-PCR analyses demonstrate that HeLa and HEC-1B cells express nectin-1 and nectin-2, but not HVEM. These observations are consistent with the supposition that nectin-1 is the most likely candidate for stimulation of co-infection-induced persistence. Secondly, co-infection studies in *C. muridarum* revealed that a significant decrease in chlamydial infectivity occurs in co-infected cells when HSV-2 is added at 6h and 12h but not at 24h post-chlamydial infection. The effect caused by HSV-2 on *C. muridarum* infectivity is greater at 6h than at 12h. Additional studies demonstrate that there is a nearly 68% reduction in host cellular nectin-1 by 24 hours post-*C. muridarum* infection. These data suggest that HSV co-infection does not interfere with chlamydial development in the absence of nectin-1, which supports the hypothesis that nectin-1 interaction with HSV is probably required for stimulating a novel host pathway to induce chlamydial persistence. Thirdly, using HSV-1 mutants with altered co-receptor specificities, we examined whether HSV co-infection-induced chlamydial persistence requires nectin-1 by chlamydial titration assay and TEM. Surprisingly, we found that co-infection in HeLa cells with the parental strain HSV-1 KOS/FRT-gD_{wt} or the three HSV-1 mutants HSV-1 KOS/FRT -gD_{G43P}, -gD_{Q27P}, and -$gD_{A3C/Y38C}$ drove the chlamydiae to become persistent. These results were unexpected because HeLa cells do not express HVEM (Fig. 1a) and -$gD_{A3C/Y38C}$ is only HVEM/3-O-S-HS specific (Yoon & Spear, 2004). However, we observed later by β-gal assays that despite the reported results that $gD_{A3C/Y38C}$ enters cells via HVEM and 3-O-S-HS (Yoon & Spear, 2004), $gD_{A3C/Y38C}$ has lower ability to attach to and enter nectin-1-expressing CHO cells. Therefore, the only co-receptor usage that the parental strain and the three mutants share is nectin-1. Because β-gal assay is relatively insensitive and nectin-1 expression on HeLa cells is

much lower than that on CHO-nectin-1 cells (Fig. 1a), it is not surprising to see no detectable β-gal activity of $gD_{A3C/Y38C}$ in HeLa cells. Finally, we determined whether HSV attachment/entry using nectin-1 co-receptor alone is sufficient to stimulate chlamyidal persistence using co-receptor-deficient CHO cell lines. The results demonstrated that HSV-1-induced reduction in chlamydial infectivity is only observed in positive control HeLa co-infection and in CHO-nectin-1 cells, but not in CHO-C8, CHO-HVEM, and CHO-nectin-2 cells. Although Western blotting and RT-PCR analyses demonstrated that HeLa cells and HEC-1B cells do not express HVEM, it is possible that the expression of HVEM is below our detection level. However, the fact that CHO-nectin-1 cells support persistence during co-infection while CHO-HVEM cells do not provides very strong evidence that HSV interaction with nectin-1 triggers viral co-infection-induced persistence. It is especially important to point out that HSV-1 enters and produces progeny virions in both CHO-nectin-1 and CHO-HVEM cells, but chlamydial infectivity is decreased only when nectin-1 is present. These data suggest that co-infection induced persistence specifically results from HSV gD/nectin-1 interaction with nectin-1, rather than from events that occur during or subsequent to virion entry. Overall, these observations confirmed that nectin-1 is indeed required for HSV co-infection-induced chlamydial persistence.

As mentioned before, HSV co-infection-induced chlamydial persistence is not mediated by any known persistence inducer or anti-chlamydial pathway (Vanover *et al.*, 2008), suggesting a novel host signaling pathway activated by interaction of HSV gD with nectin-1 could have a negative effect on chlamydial development. Studies have shown that the *trans*-interactions of nectins induce activation of cellular signaling molecule Cdc42 small G proteins through c-Src. The activation of Cdc42 and c-Src furthermore cause the activation of Rac small G proteins. Activated Cdc42 and Rac selectively induce activation of c-Jun N-terminal kinase, but not p38 MAP kinase or extracellular signal-regulated kinase (ERK) (Takai *et al.*, 2003). Eventually, activated Cdc42 and Rac are involved in regulation of cell-cell adhesion, gene expression and cell polarization (Takai *et al.*, 2003). Interestingly, temporarily activated Cdc42 and Rac1 have been observed at 15 and 30 min after HSV-1 infection of MDCK canine kidney

cells (Hoppe *et al.*, 2006). It is very intriguing to envision that HSV/nectin-1 interaction-activated chlamydial persistence response is transmitted through the Cdc42/Rac1 signaling pathway. However, active GTPase pull-down assays indicate that Cdc42 is not activated in co-infected HeLa cells (see supplementary data, Fig. S4.2), suggesting this anti-chlamydial response is likely mediated through another, as yet uncharacterized, nectin-1-activated host signaling pathway. Identification of this uncovered nectin-1-activated cellular signal transduction pathway will be conducted in future in that dissection of this pathway will increase our understanding of the host immune response to chlamydial infection and provide new and valuable information of chlamydia/host cell interaction.

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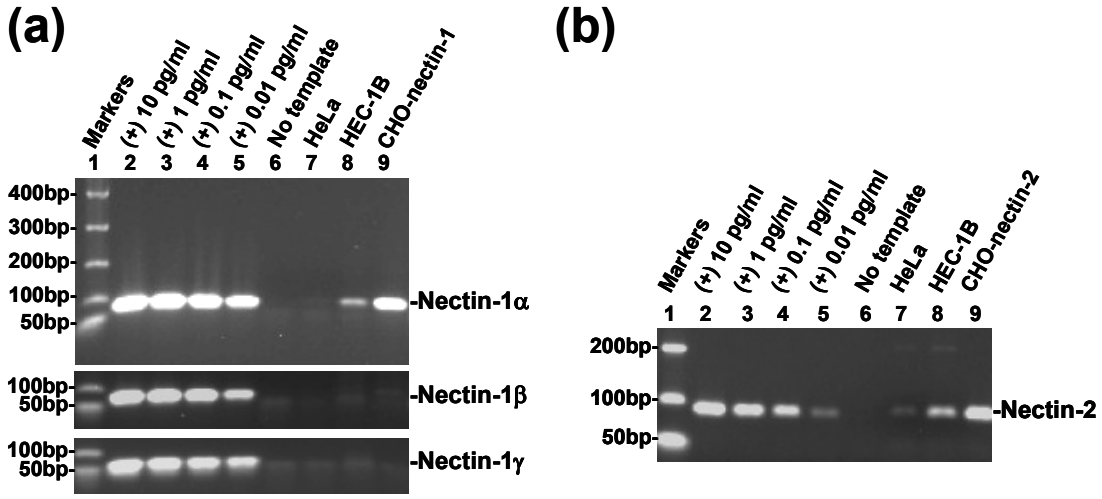

Supplementary Figure 4.1 (a) and (b) HeLa and HEC-1B cells express nectin-1 and nectin-2. Total RNA from HeLa, HEC-1B and the appropriate CHO control cell lines was isolated and subjected to RT-PCR using specific primers for nectin-1α, -1β, -1γ and nectin-2. In each experiment, a four log dilution series of synthetic control DNA was used to generate standard curves for amplification. Experimental samples were only quantified if they fell within the linear range of the PCR. The position of DNA size markers are shown to the left of each gel image in base pairs (bp).

Supplementary Figure 4.2 Endogenous Cdc42 activities upon HSV-1 infection. HeLa cells at low density were infected with HSV-1. The levels of GTP-bound Cdc42 were determined in uninfected cells (mock) and in cells at 15, 30, 60, 120 min post-infection. Pull-down experiments were performed according to the manufacturer's instructions (#89857, Active Cdc42 Pull-Down and Detection Kit, Thermo Scientific). Equal amounts of samples were resolved on SDS-PAGE gels and subjected to Western blotting using mouse anti-Cdc42 antibodies. *In vitro* GTPγS and GDP control treatments were performed to ensure the pull-down procedures were working properly.

Supplementary Table S4.1. RT-PCR primers and positive control oligonucleotides.

Name	Forward primer	Reverse primer
Nectin-2	TCGGAGCACAGCCCACTCAA	TTGTGGCTCCAGGGTGCA
HVEM	TGTGTGAACCCTGCCCTCCA	GCGTTCTCTGTCCTGGAGCA
Nectin-2 positive control oligonucleotide	CCTCGGAGCACAGCCCACTCAAGACCCCCTACTTTGATGCTGGCGCCTCATGCACTGAGCAGGAAATGCCTCGATACCATGAGCTGCCCACCTTGGAAGAACGGTCAGGACCCTTGCACCCTGGAGCCACAAGCCTGGGG	
HVEM positive control oligonucleotide	CACAGTGTGTGAACCCTGCCCTCCAGGCACCTACATTGCCCACCTCAATGGCCTAAGCAAGTGTCTGCAGTGCCAAATGTGTGACCCAGCCATGGGCCTGCGCGCGAGCCGGAACTGCTCCAGGACAGAGAACGCCGTGT	

CHAPTER 5

CONCLUSIONS

Over the years, extensive research efforts have been put into uncovering the pathogenic mechanisms by which *C. trachomatis* causes disease. Numerous investigations have revealed that *C. trachomatis* has evolved a variety of strategies to be able to productively replicate and persistently survive within the eukaryotic host cell. For example, upon infection, *C. trachomatis* prevents infected cells from undergoing apoptosis, which allows the chlamydiae to maintain long-term infection within host cells. *C. trachomatis* can also alter host cell-cell contacts and signal transduction pathways, facilitating chlamydial growth. Finally, *C. trachomatis* also modifies the host cell cytoskeleton, benefiting chlamydial intravacuolar replication.

In our study, we first showed that nectin-1, an important component of host cell adhesion junctions, is significantly decreased in *C. trachomatis*-infected genital epithelial cells (Sun *et al.*, 2008). We further demonstrated that CPAF, a chlamydia-secreted protease, is responsible for nectin-1 degradation (Sun, 2009). Recently, several investigators have reported data that suggest that CPAF has important roles in chlamydial replication, growth, and dissemination. Zhong *et al*. reported that CPAF cleaves RFX5, USF-1, pro-apoptotic BH-3 only proteins and keratin-8. RFX5 and USF-1 are transcription factors, which are required for MHC class I and II antigen expression (Zhong *et al.*, 1999; Zhong *et al.*, 2000). Cleaving RFX5 and USF-1 as well as subquent suppressing MHC class I and II antigen expression may help infected cells escape immune detection. Therefore, the chlamydiae would have sufficient time to complete their replication in infected hosts. Pro-apoptotic BH-3 only proteins are required for apoptosis in response to cytotoxic stimuli or exogenous stress, and they function by neutralizing their pro-survival counterparts or activating pro-apoptotic Bcl-2 family members Bax and Bak (Pirbhai *et al.*, 2006). Down-regulation of BH-3 only proteins contributes to chlamydial anti-apoptotic activity which may help chlamydiae evade CTL-mediated host cell apoptosis induction and, thus, benefit chlamyidal long-term survival

within infected host cells (Pirbhai *et al.*, 2006). Keratin-8 is a component of intermediate filaments and is also a major plasminogen receptor in select epithelial and carcinoma cells (Dong *et al.*, 2004c). Cleavage of keratin-8 would increase the solubility of the host cell cytoskeleton, thus facilitating the expansion of chlamydia-laden inclusion, which is essential for chlamydial intracellular replication. Kawana *et al.* also found that CPAF can effectively degrade CD1d glycoprotein, an MHC-like protein that presents lipid antigen to natural killer T cells (Kawana *et al.*, 2007). Degradation of CD1d protein may prevent recognition or elimination of chlamydiae from the host innate immune system (Kawana *et al.*, 2007). Balsara *et al.* demonstrated that CPAF activity induces proteolysis of mitotic cyclin B1, a protein involved in cell cycle, resulting in a slowed progression of infected cells through the later stages of the cell cycle (Balsara *et al.*, 2006). Unfortunately, the lack of a genetic system in chlamydiae has made it difficult to directly determine whether CPAF function is necessary for chlamydial development or disease. However, several indirect observations suggest that CPAF is, indeed, vitally important for development and/or establishment of infection. First, all chlamydial genomes yet sequenced contain a CPAF homologue (Read *et al.*, 2000; Read *et al.*, 2003; Stephens *et al.*, 1998). It seems unlikely that the gene would be evolutionarily maintained if it were not required for progression of the developmental cycle or successful infection by the organism. Second, immunization of naive mice with purified CPAF protein induces an immune response that protects against subsequent vaginal challenge with *C. muridarum* (Li *et al.*, 2007; Li *et al.*, 2008), indirectly indicating that CPAF is likely to be important for maintenance of infection *in vivo*. Given these data, CPAF likely has critical roles in both protecting infected cells from host immune detection and facilitating chlamydial intracellular growth and survival. Thus, it appears that chlamydiae have evolved a single molecule (CPAF) for multiple purposes.

What are the possible biological consequences of nectin-1 degradation? As mentioned before, nectin-1 is an adhesion molecule that is critically important for the formation and maintenance of AJs and TJs. As a result, we would predict that degradation of nectin-1 would inevitably affect the integrity of host cell-cell junctions. This notion is supported by recent data showing that cervical epithelial cells separate

from each other as a consequence of *C. trachomatis* infection (Prozialeck *et al.*, 2002). Numerous studies have shown that some important human pathogens, such as *Helicobacter pylori*, *Shigella flexneri* and *Salmonella typhimurium*, down-regulate essential components of adhesion and tight junctions including E-cadherin, claudin-1, and ZO-1. This down-regulation leads to decreased transepithelial electrical resistance and increased paracellular permeability, which facilitates cell-to-cell spreading of these pathogens (Jepson *et al.*, 1995; Sakaguchi *et al.*, 2002; Sears, 2000; Terres *et al.*, 1998). These findings suggest that disruption of adhesion and tight junctions to compromise cell-cell barriers may be an important strategy exploited by intracellular pathogens to ensure their dissemination. Although *C. trachomatis* serovar E is not an invasive pathogen, it may diminish nectin-1 expression and subsequently disrupt cell-cell adhesions, aiding lateral cell-to-cell spread. Alternatively, nectin-1 down-regulation might facilitate host structural rearrangements required for inclusion enlargement or EB release at the end of the developmental cycle. This prediction is supported by the observation that nectin-1 accumulation starts to decrease at 36 hpi, which is mid-developmental cycle for *C. trachomatis* serovar E (Sun *et al.*, 2008).

Another intriguing possibility is that CPAF-mediated down-regulation of nectin-1 might facilitate long distance *C. trachomatis* dissemination within the host genital tract (Fig. S2.3). Weakening of contacts by CPAF between an infected host genital epithelial cell and adjacent, uninfected cells could allow release of the intact, infected cell into genital tract lumen. The infected cell would then drift away via fluid dynamics or tissue movement from the original infection site to a new tissue site, aiding establishment of ascending genital tract infection. Notably, release of intact, chlamydiae-infected host cells from polarized monolayers in culture (Wyrick *et al.*, 1989) as well as from infected epithelium *in vivo* has been observed (Doughri *et al.*, 1972; Rank *et al.*, 2008; Soloff *et al.*, 1985). Soloff *et al.* (Soloff *et al.*, 1985) and Doughri *et al.* (Doughri *et al.*, 1972) observed that PMNs are in direct contact with epithelial cells in guinea-pig cervical cells infected with *C. caviae* and bovine intestinal epithelial cells infected with *C. psittaci*, respectively. Recently, Rank *et al.* (Rank *et al.*, 2008) have observed a similar phenomenon in *in vivo* using a guinea-pig conjunctival model of *C. caviae* infection.

They also presented ultrastructural evidence that, in contrast to lysis of infected cells, the majority of infected cells are pushed off the epithelium. Most of the detached, infected cells appear to be healthy with intact microvilli, nuclei, and mitochondria. Rank *et al.* further proposed that PMNs responding to chlamydial infection participated in the detachment of chlamydia-infected cells from the mucosal surface, resulting in the release of intact, infected cells. As PMN infiltration intensifies, they also observed breaches in the epithelial monolayer, resulted from weakened and disrupted epithelial tight junctions, desmosomes and junctional complexes. These data are consistent with our observation that nectin-1 is degraded by CPAF in *C. trachomatis*-infected genital epithelial cells. Thus, it appears reasonable to hypothesize that the PMNs literally push intact, infected cells out of the mucosal epithelial layer. Given our data, we would assert that the chlamydia-mediated down-regulation of nectin-1 facilitates this process by loosening the junctions between adjacent host epithelial cells (Fig. 5.1). If nectin-1 degradation does indeed facilitate release of intact, infected cells from the genial mucosa, the question remains - how might this process benefit the chlamydiae? We would propose that release of infected cells provides at least two beneficial functions. First, the detached infected cells provide a protected niche for chlamydial EBs from neutralization by secretory IgA, cationic anti-microbial peptides and other anti-bacterial compounds in surrounding environment. Second, the inflammatory reaction is very intense at the local site of original infection because of increased local production of IFN-gamma and other anti-chlamydial cytokines; it is therefore likely that few susceptible cells would be available. The dislodging of infected cells may allow the chlamydial EBs to be delivered to new, uninfected tissue sites in the genital tract. Once the infected cells ruptured, the local concentration of infectious EB would be very high, increasing the possibility that mucosal epithelia would be successfully infected. In contrast, EBs liberated from host cells still within the genital epithelial layer would be both quickly diluted and immediately subject to neutralization by anti-bacterial compounds. An *in vitro* time-lapse cinematographic study by Neeper *et al* showed the dramatic bursting of an infected cell to release EBs into the surrounding milieu (Neeper *et al.*, 1990). Thus, based on the available information, we envision that the detached, inclusion-containing epithelial cell would act like a chlamydial “cluster bomb”, allowing delivery of a concentrated

inoculum over a relatively long distance and increasing the possibility of a "hit" if the host cell ruptured near the genital epithelial cell layer. More importantly, this mechanism may also help to explain how the chlamydiae ascend the genital tract from the initial site of infection in the cervix to the fallopian tubes in the female patients.

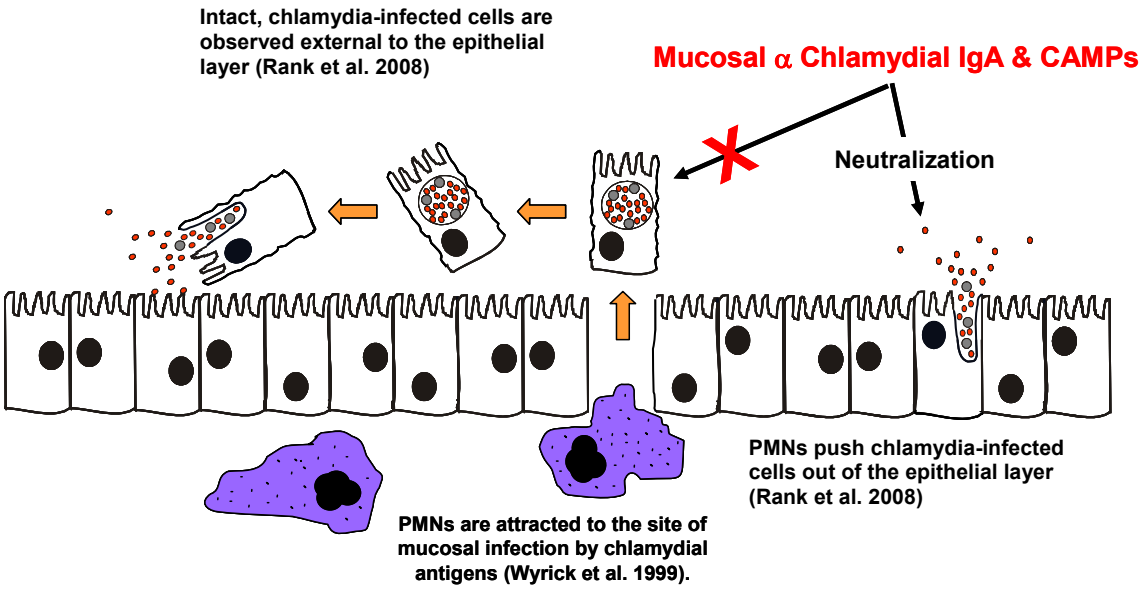

Figure 5.1 Potential roles of nectin-1 down-regulation and PMNs in *C. trachomatis* dissemination within the host genital tract. Red dots represent EBs; grey circles represent RBs.

Another interesting possibility is that nectin-1 may facilitate chlamydial entry and that down-regulation of nectin-1 may be used by the chlamydiae to reduce the possibility of subsequent infection of the same cell by other chlamydiae. This would be analogous to viral interference, a phenomenon defined as protection of host cells against subsequent or re-infection by similar viruses, conferred as a result of prior infection with a different virus. One common mechanism of viral interference occurs when a virus down-regulates its receptor after infecting a host cell. The possibility that chlamydiae use nectin-1 down-regulation to reduce subsequent infection of the same cell with other chlamydiae is supported by our and others' observations. In our study, more infectious chlamydiae are

produced from *C. trachomatis*-infected CHO-necin-1 cells than from the other CHO cell lines, even in the absence of HSV co-infection (Fig. 4.4). One possible explanation for this observation is that chalmydiae enter cells that have nectin-1 (like HeLa and CHO-nectin-1) more efficiently than they enter cells that don't have nectin-1. The chlamydiae might then down-regulate nectin-1 to reduce the efficiency with which other chlamydiae enter. Moulder *et al.* have found that mouse fibroblasts (L cells) infected with *C. psittaci* (strain 6BC) are resistant to superinfection with the same strain (Moulder *et al.*, 1980). Later, they observed that exogenous ^{14}C-labeled *C. psittaci* does not attach to *C. psittaci*-infected L cells at measurable rates. However, when exogeneous *C. psittaci* chlamydiae are centrifuged onto host cell monolayers or added to monolayers pretreated with the polycation diethylaminoethyl (DEAE)-dextran, they enter *C. psittaci*-infected L cells and multiply normally (Moulder *et al.*, 1981). Polyacrylamide gel electrophoresis of L cell proteins surface labeled with ^{125}I by lactoperosidase-catalyzed iodination revealed widespread differences in surface labeling between wild-type and *C. psittaci*-infected L cells. Particularly, the most prominent change is the generalized reduction in intensity of labeling of proteins migrating in the molecular weight range from 60kDa to 100kDa. These observations suggest that resistance to superinfection is caused by chlamydia-indcued changes in the structure or cell surface expression of the putative host cell *C. psittaci* receptor(s) (Moulder *et al.*, 1982). As we mentioned in Chapter 2, the molecular weight of nectin-1 is about 75kDa, which falls in the range of 60-100kDa. Also, nectin-1 accumulation starts to significantly decrease in *C. trachomatis*-infected HeLa cells at 36 hpi, which is mid-developmental cycle for *C. trachomatis* serovar E. Although Ridderhof *et al* have shown that prior infection does not influence superinfection with another serovar of *C. trachomatis* during the first 24h following the initial infection (Ridderhof & Barnes, 1989), they did not investigate times after 24 hpi. It is, therefore, possible that superinfecting chlamydae added after 36 hpi are less able to enter the same cell when nectin-1 is significantly degraded. This possibility assumes that nectin-1 is actually required for efficient chlamydial entry. Because there are several other possible explanations for our observation, defining any role of nectin-1 in chlamydial entry will await future experiments.

In Chapter 4, we focused specifically on identifying the co-receptor that HSV exploits to drive *C. trachomatis* to enter a persistent state. Previous data from our laboratory and observations from other investigators strongly suggest that nectin-1 has the most potential for triggering HSV co-infection-induced chlamydial persistence. First, both HVEM and nectin-1 serve equally well as co-receptors for HSV-1 and HSV-2 entry (Spear, 2004). In contrast, nectin-2 primarily mediates entry of HSV-2 and 3-O-S-HS only facilitates HSV-1 entry (Spear, 2004). Therefore, our previously published observation that both HSV-1 and HSV-2 are capable of inducing chlamydial persistence (Deka *et al.*, 2007) greatly diminish the possibility that either nectin-2 or 3-O-S-HS deliver the persistence signal. Thus, HVEM and nectin-1 are the strongest candidates for triggering persistence. Second, although HVEM cDNA was originally isolated from a HeLa cell library, Montgomery *et al* have shown that HVEM is probably not the primary receptor for HSV entry into HeLa cells in that anti-HVEM serum only had marginal effects on the entry of HSV-1 into HeLa cells (Montgomery *et al.*, 1996). Last, unpublished data by Vanover *et al.* from our laboratory have shown that non-crosslinked HSV-2 gD:Fc fusion proteins interaction with HeLa cells did not stimulate chlamydial persistence. However, when chlamydiae-infected HeLa cells were exposed to immunoglobulin cross-linked gD:Fc fusion proteins, the developing chlamydiae became persistent (Vanover, 2009b). It has been reported that the dimerizing of nectins is required for nectin *trans*-interaction induced downstream cell signaling cascades (Ogita & Takai, 2006a). Perhaps cross-linking gD:Fc fusion proteins increases receptor aggregation and thus, increases the efficiency of downstream cell signaling. Collectively, these data suggest that, of the 4 known co-receptors, nectin-1 is the most likely candidate for stimulating persistence in this system.

The goal of the experiments presented in Chapter 4 was to test the hypothesis that nectin-1 is required for HSV co-infection-induced chlamydial persistence. First, because previous data from our laboratory indicated that HSV co-infection triggers chlamydial persistence in both HeLa and HEC-1B cells (Deka *et al.*, 2007), we ascertained which HSV co-receptors were expressed in these two cell types. Importantly, both Western blotting and RT-PCR analyses demonstrated that HeLa and HEC-1B cells express nectin-

1 and nectin-2, but not HVEM (Fig. 4.1a,b). Second, co-infection studies in *C. muridarum* revealed that a significant decrease in chlamydial infectivity occurs in co-infected cells when HSV-2 is added at 6h and 12h, but not at 24h post-chlamydial infection. Additional studies demonstrated that there is a nearly 68% reduction in host cellular nectin-1 by 24 hours post-*C. muridarum* infection (Fig. 4.2b,c). These data suggest that HSV co-infection does not interfere with chlamydial development in the absence of nectin-1. Third, co-infection studies in HeLa cells with *C. trachomatis* and co-receptor-specific HSV-1 mutants indicated that parental strain HSV-1 KOS/FRT-gD_{wt} and the three HSV-1 mutants HSV-1 KOS/FRT -gD_{G43P}, -gD_{Q27P}, and -$gD_{A3C/Y38C}$ all drive developing chlamydiae to become persistent (Fig. 4.3a,d). β-gal assays further demonstrated that the only co-receptor usage that the parental strain and the three mutants share is nectin-1 (Fig. 4.3b,c). Last, we determined that HSV attachment/entry using the nectin-1 co-receptor alone is sufficient to stimulate *C. trachomatis* to enter persistence using co-receptor-deficient CHO cell lines (Fig. 4.4). The results demonstrated that HSV-1-induced reduction in infectious EB production is observed in HeLa cell controls and CHO-nectin-1 cells, but not in CHO-C8, CHO-HVEM and CHO-nectin-2 cells. Although our results demonstrated that HeLa cells and HEC-1B cells do not express HVEM, it is possible that the expression of HVEM is below our detection level. However, the fact that CHO-nectin-1 cells support persistence during co-infection while CHO-HVEM cells do not provides very strong evidence that HSV interaction with nectin-1 triggers viral co-infection-induced persistence. It is especially important to point out that HSV-1 enters and produces progeny virions in both CHO-nectin-1 and CHO-HVEM cells, but chlamydial infectivity is decreased only when nectin-1 is present. These data suggest that co-infection induced persistence specifically results from HSV gD/nectin-1 interaction with nectin-1, rather than from events that occur during or subsequent to virion entry. Overall, these observations confirmed that nectin-1 is indeed required for HSV co-infection-induced chlamydial persistence.

Studies by Vanover *et al.* demonstrated that HSV-2 co-infection-induced chlamydial persistence is not mediated by any currently known persistence inducer or anti-chlamydial pathway (Vanover *et al.*, 2008), suggesting a novel anti-chlamydial

pathway might be activated during HSV co-infection. As mentioned before, co-infection of HeLa cells with either replication incompetent HSV-2_{uv} or with replication-competent HSV-2 in the presence of cyclohexamide continues to drive *C. trachomatis* to enter a persistent state, suggesting that productive HSV replication is not required and that events during HSV attachment and/or entry are sufficient to stimulate chlamydial persistence (Deka *et al.*, 2007). Further, co-incubation of either fixed, HSV-2-infected inducer cells or recombinant HSV gD protein with viable, *C. trachomatis*-infected responder cells suppresses infectious EB production and stimulates the formation of abnormal RBs. These data suggest the events that occur during viral attachment, instead of viral entry, are sufficient to provide stimulus to alter *C. trachomatis* development (Vanover, 2009b). HSV attachment to host cell occurs in several consecutive steps and involves viral glycoproteins binding to their cognate host cell receptors/co-receptors (Spear, 2004). It is known that HSV/host cell surface interactions activate several host cellular signaling cascades. Therefore, it is possible that HSV binding to host cell receptors/co-receptors could stimulate chlamydial persistence by activating cellular signaling pathways that have negative effects on chlamydial development.

A number of studies have demonstrated that HSV triggers anti-viral pathways via Toll-like receptors (TLRs), important critical mediators in generating innate immune responses against viral pathogens (Aravalli *et al.*, 2005; Kurt-Jones *et al.*, 2005; Pyles *et al.*, 2002). This information raises the possibility that stimulation of anti-viral signals during co-infection could have anti-chlamydial effects. HSV gB is reported to be the major glycoprotein responsible for inducing TLR-linked anti-viral transduction cascades in host cells (Aravalli *et al.*, 2007; Kurt-Jones *et al.*, 2005). However, unpublished data from our laboratory indicate that specific antibody neutralization of HSV gB, gC, or gH is not able to restore chlamydial infectivity during co-infection. Further studies by Vanover *et al.* demonstrate that co-incubation of recombinant HSV gD protein with *C. trachomatis*-infected HeLa cells induces persistence, demonstrating that HSV gD interaction with the host cell surface is sufficient to alter the chlamydial developmental cycle (Vanover, 2009b). Notably, IFN-β is one of the first genes activated by HSV-induced TLR signaling (Schneider *et al.*, 2004). However, semiquantitative RT-PCR

studies demonstrate that IFN-β mRNA is not expressed during co-infection (Vanover *et al.*, 2008). Finally, the data presented in Chapter 4 demonstrate that HSV co-infection inhibits the chlamydial developmental cycle in nectin-1-expressing but not in control CHO-C8 cells. Since the CHO control cells and CHO-nectin-1 cells are identical, except for the presence or absence of nectin-1, it seems unlikely that the observed effect is TLR mediated. Therefore, collectively, these observations indicate that HSV interaction with TLRs is not the mechanism underlying the phenomenon of co-infection-induced persistence.

Currently, our data indicate that nectin-1, a co-receptor that interacts with the HSV envelope gD, is required for triggering chlamydial persistence during co-infection. As a member of Immunoglobulin superfamily and an important component of AJs, when stimulated, nectin-1 activates the cell signaling molecule Cdc42 through c-Src. The activation of Cdc42 and c-Src further cause the activation of the small G protein, Rac. Activated Cdc42 and Rac then selectively induce activation of c-Jun N-terminal kinase (JNK), but not p38 MAP kinase or extracellular signal-regulated kinase (ERK) (Takai & Nakanishi, 2003). The JNK pathway is involved in important cellular events such as apoptosis and cell growth (Sakisaka & Takai, 2004). Eventually, activated Cdc42 and Rac are involved in regulation of cell-cell adhesion, gene expression and cell polarization (Takai & Nakanishi, 2003). Interestingly, temporarily activated Cdc42 and Rac1 have been observed at 15 and 30 min after HSV-1 infection of MDCK canine kidney cells (Hoppe *et al.*, 2006). It is very tempting to envision that the HSV/nectin-1 interaction-activated chlamydial persistence response is transmitted through the Cdc42/Rac1 signaling pathway. However, active GTPase pull-down assays indicate that Cdc42 is not activated in co-infected HeLa cells (Fig. S4.2), suggesting this anti-chlamydial response is likely mediated through another, as yet uncharacterized, nectin-1-activated host signaling pathway. In addition, nectin-1 has at least one natural endogenous ligand: nectin-3. It is, therefore, possible that interactions between nectin-1 and nectin-3 could also trigger anti-chlamydial pathways causing persistence in the absence of co-infection. Thus, investigating the interactions between nectin-1 and nectin-3 might provide a clue

regarding which signaling pathway is stimulated and if it activates an anti-chlamydial response.

Several studies demonstrate that HIV or human cytomegalovirus (HCMV) binding of viral glycoproteins to the host cell surface is sufficient to induce a Ca^{2+} influx into the cells (Alfano *et al.*, 1999; Keay *et al.*, 1995; Keay & Baldwin, 1996; Liu *et al.*, 2000). Cheshenko *et al.* recently reported that the calcium-signaling pathway is also rapidly activated, with an increased [Ca^{2+}] in the cytosol, after exposure to HSV (Cheshenko *et al.*, 2003). They further proposed that a signal is transduced after interactions between heparan sulfate proteoglycans and gC or gB, as well as engagement of HSV co-receptors by gD. The transduced signal in turn activates the inositol-1,4,5-triphosphate (IP_3) pathway, leading to a rapid increase in [Ca^{2+}]. The increased [Ca^{2+}] may further promote phosphorylation pathways, facilitating viral penetration and/or the transport of incoming viral capsids to the nucleus (Cheshenko *et al.*, 2003). Calcium signaling has been shown to be important for *C. trachomatis* intracellular survival. For example, type III secretion systems (T3SSs) are unique mechanisms that Gram-negative bacteria use to modulate the host cell environment by delivering bacterial effector proteins into the host cell. Studies show that low calcium enhances the induction of T3SSs in *C. trachomatis* (Jamison & Hackstadt, 2008). Shainkin-kestenbaum *et al.* also reported that the growth of *C. trachomatis* is inhibited by addition of calcium antagonist verapamil (Shainkin-Kestenbaum *et al.*, 1989). It is possible that fluctuation of host cellular calcium levels stimulated by HSV gD/nectin-1 interaction may play a role in *C. trachomatis* intracellular life. Therefore, development of chlamydial persistence by a mechanism that acts via triggering calcium-signaling pathways during co-infection could be a possibility.

Other possible signaling pathways involved in induction of persistence in *C. trachomatis*/HSV co-infected cells include the NF-kB and the JAK/STAT signaling pathways. Both HSV-1 gD and UV-inactivated virions are able to stimulate NF-kB activity from 1-3 hpi (Amici *et al.*, 2006; Teresa Sciortino *et al.*, 2007). HSV infection can cause down-regulation of the JAK/STAT signaling pathway by inducing host cells to

express SOCS3 (Yokota *et al.*, 2005). However, further experimental examination is necessary to determine whether these signaling pathways are required for co-infection-induced persistence. Of course, it is always possible that some unknown signaling pathways are activated by attachment of HSV that in turn cause the induction of chlamydial persistence. It is also possible that several activated host signaling pathways may work together to negatively affect developing chlamydiae. In the future, we plan to screen HSV-infected as well as gD-co-incubated HeLa cells using commercially available phospho-protein-specific antibody microarrays. Once we have a more global view of the host signalling pathways activated by HSV and gD-host cell interaction, specific pharmacologic, dominant-negative and RNAi inhibitors of those pathways will be used to determine which might be involved in this response. Thus, we hope ultimately to determine which signaling pathway is responsible for viral co-infection-induced chlamydial persistence.

As mentioned before, HSV and *C. trachomatis* are two of the most common STD agents in the United States and co-infection with HSV and *C. trachomatis* has been observed clinically. Published data from our laboratory demonstrate that HSV-2 super-infection of *C. trachomatis* serovar E-infected human genital epithelial cells profoundly alters the chlamydial developmental cycle, driving developing chlamydiae to enter persistence. Induction of chlamydial persistence by HSV-2 co-infection has significant implications for pathogenesis. Persistent chlamydiae are refractory to killing by anti-chlamydial drugs, enhancing the likelihood that an individual will experience prolonged inflammation of the genital tract and thus increasing the possibility for development of severe disease sequelae such as pelvic inflammatory disease and ectopic pregnancy. Therefore, it is possible that the severity of the inflammatory response caused by HSV-2/*C. trachomatis* co-infection might be even higher than that in an individual infected with *C. trachomatis* alone. Most of the morbidity associated with chlamydial infections is due to complications arising from the infection, which are thought to be due, at least in part, to release of inflammatory mediators from infected cells. Thus, any stimulus that increases release of these compounds might enhance disease pathology. Numerous membrane blebs have been observed in HSV/*C. trachomatis* co-infected cells. The

presence of membrane blebs has been associated with release of chlamydial LPS from persistently infected cells (Karimi *et al.*, 1989; Wyrick *et al.*, 1999). Although chlamydial LPS is less potent than that of other Gram-negative bacteria, it still has the capacity to enhance the host inflammatory response (Ingalls *et al.*, 1995). HSV-2 co-infection also increases the accumulation of chlamydial immuno-stimulatory antigen cHSP60 (Deka *et al.*, 2006). Increased cHSP60 production would be predicted to result in an increase in inflammation that in turn will lead to an increase in tissue damage. An increase in tissue damage implies an increase in the complications resulting from chlamydial infection.

Given that productive HSV replication generally kills the host cell, co-infection induced chlamydial persistence may seem to have little *in vivo* relevance. However, data from our laboratory demonstrate that productive viral replication is not required for the induction of chlamydial persistence (Deka *et al.*, 2007). It is estimated that about 50-200 defective virions are released for every replication competent virion produced (Roizman, 2001b). Thus, there may be numerous non-viable viral particles able to attach and enter *in vivo*. Because these defective virus particles are incapable of productive replication, lysis of the host cell does not occur. Because only HSV gD is required for triggering chlamydial persistence (Vanover, 2009b), attachment of defective virions or soluble gD protein to *C. trachomatis*-infected cells could stimulate persistence without killing the host cells. This implies that the persistent chlamydiae could survive within co-infected host cells and contribute to pathogenesis *in vivo*. As a result, persistence induced by defective virions is likely of higher *in vivo* relevance in co-infected individuals than that induced by replication-competent virions. More importantly, these exposure conditions are more likely to accurately mimic stimulation of the anti-chlamydial pathway by existing endogenous host ligands than does co-infection with replication-competent HSV. If nectin-1 binding to its endogenous ligands, like nectin-3, can indeed induce chlamydial persistence, this response is likely to have tremendous *in vivo* significance because it would be expected to occur in all chlamydia-infected individuals, not just those co-infected with HSV.

The possibility that nectin-1 interaction with endogenous host nectin-1 ligands also opens up a fascinating alternative explanation for the nectin-1 down-regulation observed in both *C. trachomatis*- and *C. muridarum*-infected cultures. Previously, we hypothesized that nectin-1 diminution might facilitate chlamydial dissemination in the genital tract or serve as a mechanism by which the chlamydiae interfere with re-infection of the host cell. Although both alternatives are still quite possible, another hypothesis suggested by the data in Chapter 4 is that the chlamydiae degrade nectin-1 to evade nectin-1-mediated inhibition of the developmental cycle. This opens up the even more exciting possibility that nectin-1 and other endogenous host factors might tightly regulate chlamydial development in the host cell. Although this is an almost entirely unexplored facet of chlamydia-host interaction, it is not without precedent in the STD field. The replication cycle of Human Papilloma Virus (HPV) is closely regulated by interactions between viral oncoproteins and host cell factors. During HPV infection, viral genomes are coordinately replicated along with the cellular DNA. Upon cell division, viral DNA is partitioned to two daughter cells. One daughter cell will remain undifferentitated, serving as a reservoir for continued infection. The other daughter cell will detach from the basal layer and differentiate in the suprabasal epithelium (Longworth & Laimins, 2004). In normal uninfected epithelia, cells exit the cell cycle once they leave the basal layer. However, in infected cells, expression of the early viral oncoproteins E6 and E7 enables these differentiating cells to remain active in the cell cycle. The E6 mainly interacts with the tumor suppressor p53, resulting in ubiquitination and subsequent degradation of p53 through the recruitment of the ubiquitin ligase E6-AP (Huibregtse *et al.*, 1991). p53 regulates the expression of proteins involved in cell cycle control, including the cyclin kinase inhibitor p21 (Ko & Prives, 1996). When DNA damage occurs, p53 becomes activated and induces expression of p21, leading to cell cycle arrest and apoptosis (Ko & Prives, 1996). Via binding E6 to p53, HPV overcomes the proapoptotic activites of p53 and allows for cell cycle progression (Huibregtse *et al.*, 1991). E7 also inactivates and induces the degradation of retinoblastoma protein (pRB), a cell cycle regulatory protein required for the G_1/S transition and DNA synthesis (Cheng *et al.*, 1995). The abrogation of pRB allows productive viral replication in differentiated suprabasal cells (Chellappan *et al.*, 1992). In this manner, HPV maintains a productive, disseminating infection in the

differentiated epithelial cells and establishes a reservoir of infection in the undifferentiated basal cells. Like HPV, *C. trachomatis* may use nectin-1-induced signaling to regulate productive and chronic genital infection. Investigating the relationships between nectin-1 and other endogenous host factors may reveal unknown, compelling aspects of chlamydia-host cell interaction.

At the present time, the major problem of the chlamydial research field is that there is no workable genetic system for transformation and mutagenesis of the chlamydial genome. Thus, it is not feasible to apply genetic approaches that are currently used for other organisms. In addition, the obligate intracellular nature of these organisms makes research on them even more challenging. However, previous persistence studies have led to important findings in chlamydial biology and pathogenesis. For example, experiments examining the effect of IFN-γ on chlamydial development have led to the identification of an important underlying mechanism that helps control chlamydal infection *in vivo*. Similarly, data from our tissue culture model of HSV-2/*C. trachomatis* co-infection have potentially uncovered a novel host cellular response that limits chlamydial development. Future investigations on the underlying mechanisms that drive developing chlamydiae to enter persistence will provide detailed information of this anti-chlamydial pathway and will allow us to evaluate its contribution to chlamydial pathogenesis and host defense *in vivo*. Such investigations will also reveal new and compelling facets of chlamydia-host cell interaction and will hasten development of *in vivo* models of polymicrobial sexually transmitted infections.

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Pulbications:

1. **Sun, J**., Kintner, J., Whittimore, J., Schoborg, R. The host adherens junction molecule nectin-1 is required for Herpes simplex virus (HSV) co-infection-induced *Chlamydia trachomatis* persistence. (*Manuscript in preparation*)
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Presentations:

1. **Sun, J**., Kintner, J., Whittimore, J., Schoborg, R. The host adherens junction molecule nectin-1 is required for Herpes simplex virus (HSV) co-infection-induced *Chlamydia trachomatis* serovar E persistence. Chlamydial Basic Research Society, 4th Biennial Conference, Little Rock, Arkansas, March 20-23, 2009. (oral presentation)
2. **Sun, J**., Schoborg, R. The host adherens junction molecule nectin-1 is degraded by Chlamydial Protease-like Activity Factor (CPAF) in *Chlamydia trachomatis*-infected genital epithelial cells. The 24th Annual ETSU Student Research Forum, April 2-3, 2008. Poster presentation.
3. **Sun, J**., Kintner, J., Schoborg, R. The host adherens junction molecule nectin-1 is down-regulated in *Chlamydia trachomatis*-infected genital epithelial cells.

The 23rd Annual ETSU Student Research Forum, March 29, 2007. Oral presentation.

4. **Sun, J**., Kintner, J., Schoborg, R. The host adherens junction molecule nectin-1 is down-regulated in *Chlamydia trachomatis*-infected genital epithelial cells. Chlamydial Basic Research Society, 3rd Biennial Conference, Louisville, Kentucky, March 23-27, 2007. Poster presentation.
5. **Sun, J.**, Lajoie, D., Stead, C., Shell, S., Vanover, J., Kintner, J., Whittimore, J., Schoborg, R. Partial characterization of a *Chlamydia trachomatis* elementary body-associated protease: a cautionary tale for protein expression analyses. Chlamydial Basic Research Society, 3rd Biennial Conference, Louisville, Kentucky, March 23-27, 2007. Poster presentation.
6. **Sun, J.**, Lajoie, D., Stead, C., Shell, S., Vanover, J., Kintner, J., Schoborg, R. ChEAP: a novel elementary body-associated chlamydial protease. The 22nd Annual ETSU Student Research Forum, March 31, 2006. Poster presentation.

Seminar:

1. Partial characterization of a *Chlamydia trachomatis* elementary body-associated protease: a cautionary tale for protein expression analysis. 2. The host adherens junction molecule nectin-1 is down-regulated in *Chlamydia trachomatis*-infected genital epithelial cells. September 2007; James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee.

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