The host adherens junction molecule nectin-1 is downregulated in *Chlamydia trachomatis*-infected genital epithelial cells

Jingru Sun, Jennifer Kintner and Robert V. Schoborg

Correspondence
Robert V. Schoborg
schoborg@etsu.edu

Department of Microbiology, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA

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. Interestingly, 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.

Received 16 November 2007

Revised 29 January 2008

Accepted 5 February 2008

INTRODUCTION

The obligate intracellular bacterium *Chlamydia trachomatis* is a significant Gram-negative human pathogen, with four 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, 2000). 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 an 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 signalling 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

Abbreviations: AJ, adherens junction; CPAF, chlamydial protease/ proteasome-like activity factor; DS, desmosome; EB, elementary body; FAK, focal adhesion kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h.p.i., hours post-infection; MOMP, major outer-membrane protein; RB, reticulate body; TJ, tight junction.

Three supplementary figures (with references) showing the protein detected by nectin-1 antibodies CK6 and CK8, changes in nectin-1 accumulation in *C. trachomatis*-infected polarized HeLa cells and the potential role of nectin-1 downregulation in *C. trachomatis* dissemination within the host genital tract, and a supplementary table listing RT-PCR primers and positive control oligonucleotides, are available with the online version of this paper.

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 *in vivo* (Doughri *et al.*, 1972; Soloff *et al.*, 1985). Doughri *et al.* (1972) further suggested that release of chlamydiae within intact host cells might protect the organisms from anti-chlamydial antibodies. In both studies, the authors hypothesize 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). 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 contribute directly to the release observed *in vivo* of infected cells from the infected mucosa.

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 epithelial 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-1 γ) and a cytoplasmic tail. The nectin family currently contains four members (nectins 1–4) (Sakisaka & Takai, 2004; Takai & Nakanishi, 2003). Afadin is a nectin- and F-actin-binding protein that connects the transmembrane 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 downregulation 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 shown previously 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 [American Type Culture Collection (ATCC) no. CCL2], were cultured as described previously (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 either were grown on standard tissue culture plates or were polarized on 3.0 μm , collagen IV-coated chamber inserts (Biocoat 4544, Becton Dickinson), as described elsewhere (Wyrick *et al.*, 1989). Host cells were infected at an m.o.i. of 1 using crude EB stock, which infects ~90 % of the HeLa cells, for 1 h 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 that they were exposed to 200 μl 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 re-fed with growth medium and incubated at 35 °C for the indicated times. Chlamydial titrations were performed as described previously (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 XL-1500, 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 4 °C 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 and 60 hours post infection (h.p.i.) using the method described previously by Deka *et al.* (2006). Total RNA and DNA preparations were quantified by measuring OD at 260 and 280 nm. All samples had OD₂₆₀/OD₂₈₀ ratios >1.9. The quality and concentration of each RNA sample were further confirmed by analysis on a 2100 Bioanalyser instrument (Agilent) using the RNA 6000 Nano LabChip kit. All samples had RNA integrity numbers (RINs) between 9.0 and 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 described previously (Deka *et al.*, 2006), except that 2 µg total RNA was used. RT-PCR reactions were then 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 double-distilled H₂O (ddH₂O)], such that each reaction was in the linear amplification range. The amplification conditions were based on those described previously (Deka *et al.*, 2006). Published primer sets included those for 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. These are listed in Supplementary Table S1. Most reactions were performed using the following cycling conditions: 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 for 30 cycles; 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/Tris-buffered EDTA (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 amplicons.

SDS-PAGE and Western blotting. Monolayers were lysed and denatured as described previously (Deka *et al.*, 2006). Samples were then normalized for total protein content using SYPRO Ruby stain (Bio-Rad) according to the manufacturer's instructions. Samples were diluted to identical concentrations, subjected to SDS-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-chlamydial major outer-membrane protein (MOMP; 1:5000 dilution; B65266G, BioDesign International). Bound primary antibodies were detected using the corresponding secondary antibodies conjugated to horseradish peroxidase and visualized using SuperSignal West 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, the quantity of nectin-1 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 have 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 an m.o.i. of 1. Cell lysates were collected 48 h.p.i. and processed for SDS-PAGE analyses. Duplicate gels were either stained with SYPRO Ruby (data not shown) or Western blotted (Fig. 1a) using anti-nectin-1 CK6 mAb, anti-*C. trachomatis* MOMP or anti-FAK, as described previously (Deka *et al.*, 2006). Nectin-1 bands were quantified, normalized to FAK and plotted in Fig. 1(b). FAK was chosen as an internal control protein because its accumulation is not altered by chlamydial infection (Deka *et al.*, 2006). As expected, MOMP expression was detected in *C. trachomatis*-infected cells alone. Nectin-1 accumulation was decreased by up to 85 % in *C. trachomatis*-infected HeLa cells compared with that in mock-infected controls. Sequential immunoprecipitation and blotting experiments with a different nectin-1-specific mAb (Supplementary Fig. S1b) confirmed that the protein detected on the blots in Fig. 1 was indeed nectin-1. Additionally, little or no nectin-1 was immunoprecipitated by either antibody from chlamydia-infected cells harvested at 48 h.p.i., 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.

Fig. 1. Nectin-1 accumulation is reduced in *C. trachomatis*-infected HeLa cells. HeLa cells were either mock infected (Mock inf.) or *C. trachomatis* serovar E infected (Ct inf.) at an m.o.i. of 1. Cell lysates were collected 48 h.p.i. 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 mean ± SEM of three biological replicates. Significant ($P \leq 0.05$) difference from the mock-infected controls is indicated by an asterisk. The data are representative of three independent experiments.

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. S2a) showed that nectin-1 was also downregulated in *C. trachomatis*-infected polarized cells. At 48 h.p.i., nectin-1 accumulation was decreased by as much as 88% in *C. trachomatis*-infected polarized HeLa cells compared with that in mock-infected controls (Supplementary Fig. S2b). Because similar results were obtained from both non-polarized and polarized HeLa cells, non-polarized cells were used in subsequent experiments.

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

To more precisely define when downregulation of nectin-1 occurs during the *C. trachomatis* developmental cycle, an infection time-course ranging from 0 to 60 h.p.i. was conducted. HeLa cells were either mock- or *C. trachomatis*-infected. Cell lysates were collected at 0, 24, 36, 48 and 60 h.p.i. 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(a); the quantification of this blot is shown in Fig. 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

Fig. 2. Nectin-1 accumulation is reduced in *C. trachomatis*-infected HeLa cells from 36 to 60 h.p.i. (a) Cell lysates from mock-infected (Mock inf.) or *C. trachomatis*-infected (Ct inf.) HeLa cells were collected at 0, 24, 36, 48 and 60 h.p.i. 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) The quantity of Nectin-1 observed in *C. trachomatis*-infected cells is expressed as a percentage of that observed in mock-infected control cells at each time interval. Results are expressed as the mean $\pm$ SEM of three biological replicates. Groups significantly ($P \leq 0.05$) different from *C. trachomatis*-infected cells at 0 h.p.i. are indicated by asterisks. The data are representative of three independent experiments.

plotted (Fig. 2c). These data demonstrate that *C. trachomatis* infection significantly reduced accumulation of nectin-1, starting at 36 h.p.i., and that the reduction was maximal at 60 h.p.i.

Nectin-1 downregulation 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 downregulation. Nectin-1 expression in persistently infected cells was also examined. HeLa cells were either mock- or *C. trachomatis*-infected and re-fed

immediately after infection with growth medium plus ddH₂O, medium plus 60 µg chloramphenicol ml⁻¹, which specifically inhibits the function of bacterial 50S ribosomal subunits, or medium plus 20 U penicillin G ml⁻¹, a known inducer of chlamydial persistence. Cell lysates were harvested at 48 h.p.i. for Western blot analyses (Fig. 3a, b). The quantity of nectin-1 observed in *C. trachomatis*-infected cells from three independent experiments was expressed as a percentage of that observed in mock-infected control cells under the same experimental conditions (Fig. 3c). As a control to confirm that each antibiotic was working as expected, duplicate cultures from each drug-exposure group were subjected to chlamydial subpassage titration analyses.

Fig. 3. *C. trachomatis* protein synthesis and/or replication is/are required for nectin-1 downregulation. (a) Cells were re-fed immediately after *C. trachomatis* infection with growth medium + ddH₂O, 60 µg chloramphenicol ml⁻¹ (camp) or 20 units penicillin G ml⁻¹ (penG). Cell lysates were harvested at 48 h.p.i. and Western blotted using anti-nectin-1 CK6, anti-*C. trachomatis* MOMP or anti-FAK (C-20). (b) The quantities of nectin-1 detected on the blot in panel (a) were normalized to FAK and plotted. (c) The quantity of nectin-1 observed in *C. trachomatis*-infected cells is expressed as a percentage of that observed in mock-infected control cells under that set of experimental conditions. The mean ± SEM from three biological replicates is shown. Groups significantly ($P \leq 0.05$) different from chloramphenicol-exposed, *C. trachomatis*-infected cells are indicated by asterisks. (d) Duplicate cell cultures from each drug-exposure group were subjected to chlamydial subpassage titration. Results are expressed as the mean ± SEM of three biological replicates. Groups significantly ($P \leq 0.05$) different from non-drug-exposed, *C. trachomatis*-infected cells are indicated by asterisks. All data shown here are representative of three independent experiments.

The numbers of inclusion-forming units (IFU) in the undiluted inocula were then calculated and expressed as IFU ml⁻¹ (Fig. 3d). Nectin-1 downregulation in *C. trachomatis*-infected cells was prevented by chloramphenicol exposure (Fig. 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. 3a, lanes 7 and 8), although to a lesser degree than that observed in diluent-exposed, chlamydiae-infected monolayers (Fig. 3b, c). These data indicate that nectin-1 accumulation can also be reduced by penicillin-induced, persistent *C. trachomatis* infection. Chlamydial subpassage experiments demonstrated that both chloramphenicol and penicillin G exposure significantly reduced recovery of infectious EBs, as expected (Fig. 3d).

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

To determine whether viable *C. trachomatis* EBs are required for downregulation 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 h.p.i. and processed for Western blot analyses (Fig. 4a). Average nectin-1 accumulation was quantified, normalized and plotted (Fig. 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 downregulated only if cells are infected with viable EBs.

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

Nectin-1 has three splice variants: nectin-1 α , nectin-1 β and nectin-1 γ . 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-1 α and nectin-1 β proteins both contain the CK6 and CK8-reactive epitopes (Supplementary Fig. S1a), the observed molecular mass (75 kDa) is most consistent with the reported size for nectin-1 α (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 downregulation 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-1 α , nectin-1 β and nectin-1 γ transcripts. Parallel total DNA samples were amplified using GAPDH-specific primers to determine host genome copy number. All amplicons were the expected size (Fig. 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 standard curves for amplification. 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. 5a, lane 1) or in RT(-) controls (data not shown). The quantity of nectin-1 α , β and γ products was similar in mock-compared with *C. trachomatis*-infected samples at both 48 and 60 h.p.i. (Fig. 5a, lanes 2–5). All amplicons were quantified, normalized to host genome copy number, as ascertained by PCR with human GAPDH-specific primers, and plotted in Fig. 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. 5b). These data indicate that expression of nectin-1 is not regulated at the transcriptional level and thus is probably regulated post-transcriptionally.

DISCUSSION

Chlamydiae have evolved various strategies to avoid host defence systems and complete their intracellular growth

Fig. 4. Viable *C. trachomatis* EBs are required for nectin-1 downregulation. (a) HeLa cells were mock (Mock), *C. trachomatis* (Ct) or *C. trachomatis*-UV (Ct-UV) infected. Cells were collected at 48 h.p.i. and proteins were 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 the internal control protein FAK and plotted. Results are expressed as the mean $\pm$ SEM of three biological replicates. Groups significantly ($P \leq 0.05$) different from the mock-infected controls are indicated by asterisks. The data shown are representative of three independent experiments.

Fig. 5. *C. trachomatis* infection of HeLa cells does not alter accumulation of nectin-1 α , β and γ transcripts. (a) HeLa cells were mock (Mock) or *C. trachomatis* (Ct) infected at an m.o.i. of 1. Total RNA was isolated at 48 or 60 h.p.i., as described in Methods, and subjected to RT-PCR analyses using transcript-specific oligonucleotide probes. Nectin-1 α (117 bp), nectin-1 β (105 bp), nectin-1 γ (75 bp) and β -actin (230 bp) amplicons were electrophoresed and photographed. Representative photographs are shown. The positions of DNA size markers are shown to the left of each gel image in bp. Neg, control PCR containing no DNA template. (b) Specific DNA amplicons were quantified as described in Methods. Nectin-1 α (black bars), nectin-1 β (grey bars), nectin-1 γ (white bars) and β -actin (diagonally striped bars) amplicon quantities were normalized to host genome copy number, as ascertained by PCR with human GAPDH-specific primers (data not shown). The values plotted are means $\pm$ SEM of three independent experiments. The value obtained for each independent experiment is the mean obtained from three biological replicates.

cycle. They have acquired specific mechanisms to prevent productively and persistently infected cells from undergoing apoptosis (Dean & Powers, 2001; Dong *et al.*, 2005; 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.*, 2004; 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 downregulates 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 AJs and TJs. Disruption of nectin-1 would inevitably affect the integrity of cell–cell junctions, which may be implicated in chlamydial pathophysiological 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, *Chlamydia pneumoniae* upregulates the expression of adherens junction proteins VE-cadherin, N-cadherin and β -catenin and transiently downregulates the expression of the TJ protein occludin to alter junctional complexes, facilitating its transmission in human brain microvascular endothelial cells (MacIntyre *et al.*, 2002). Why *C. trachomatis* and *C. pneumoniae* might use two distinct mechanisms to interact with junctional complexes is currently unknown.

While nectin-1 protein expression was decreased in *C. trachomatis*-infected HeLa cells, accumulation of nectin-1 α , β and γ transcripts was unchanged. These data indicate that reduction of nectin-1 was not regulated transcriptionally, and thus is likely to be downregulated 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.*, 2004, 2005; Fischer *et al.*, 2004; Pirbhai *et al.*, 2006; Ying *et al.*, 2005; Zhong *et al.*, 1999, 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 also degrades 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 that 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* downregulates 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 downregulation, 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 to decrease at 36 h.p.i., which is mid-developmental cycle for *C. trachomatis* serovar E. The second, and perhaps more intriguing possibility, is that nectin-1 downregulation 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 the 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 and other anti-bacterial compounds, as long as the host cell remained intact. Second, if the infected cell ruptured in close proximity 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 inoculum 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 from polarized monolayers in culture (Wyrick *et al.*, 1989) as well as from infected epithelium *in vivo* has been observed (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 physiological alterations induced during chlamydial infection. Future studies will be directed toward determining which of these hypotheses is correct.

Finally, it should be noted that nectin also plays a critical role in intracellular signalling. 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, 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.* (2002) upon *C. trachomatis* infection results from insufficient activation of Cdc42 and Rac in response to reduced levels of nectin-1. Furthermore, activated Cdc42 and Rac stimulate the c-Jun N-terminal kinase (JNK) signalling 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 signalling may play a role in this inhibition. Given the known interplay between nectin-1 and host cellular signalling pathways, dissecting the effect of *C. trachomatis*

infection on nectin-mediated signal transduction events will be a key aspect of future experiments.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Priscilla B. Wyrick and Dr Dennis M. Defoe for helpful discussion of these experiments and reviewing the manuscript. The authors also thank Dr John Laffan, Dr Sophie Dessus-Babus and Jennifer Vanover for their assistance on this project. This work was supported by NIH Public Health Service grant R21 AI59563 to R.V.S., East Tennessee State University (ETSU) research development committee (RDC) grant 04-024M to R.V.S., the James H. Quillen College of Medicine and the Department of Microbiology.

REFERENCES

- Arno, J. N., Ricker, V. A., Batteiger, B. E., Katz, B. P., Caine, V. A. & Jones, R. B. (1990). Interferon-gamma in endocervical secretions of women infected with *Chlamydia trachomatis*. *J Infect Dis* **162**, 1385–1389.
- Balsara, Z. R., Misaghi, S., Lafave, J. N. & Starnbach, M. N. (2006). *Chlamydia trachomatis* infection induces cleavage of the mitotic cyclin B1. *Infect Immun* **74**, 5602–5608.
- Braga, V. M., Machesky, L. M., Hall, A. & Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell–cell contacts. *J Cell Biol* **137**, 1421–1431.
- Braga, V. M., Del Maschio, A., Machesky, L. & Dejana, E. (1999). Regulation of cadherin function by Rho and Rac: modulation by junction maturation and cellular context. *Mol Biol Cell* **10**, 9–22.
- Darville, T. (2000). *Chlamydia* spp. In *Persistent Bacterial Infections*, pp. 229–261. Edited by J. P. Nataro, M. J. Blazer and S. Cunningham-Rundles. Washington, DC: American Society for Microbiology.
- Dean, D. & Powers, V. C. (2001). Persistent *Chlamydia trachomatis* infections resist apoptotic stimuli. *Infect Immun* **69**, 2442–2447.
- Deka, S., Vanover, J., Dessus-Babus, S., Whittimore, J., Howett, M. K., Wyrick, P. B. & Schoborg, R. V. (2006). *Chlamydia trachomatis* enters a viable but non-cultivable (persistent) state within herpes simplex virus type 2 (HSV-2) co-infected host cells. *Cell Microbiol* **8**, 149–162.
- Deka, S., Vanover, J., Sun, J., Kintner, J., Whittimore, J. & Schoborg, R. V. (2007). An early event in the herpes simplex virus type-2 replication cycle is sufficient to induce *Chlamydia trachomatis* persistence. *Cell Microbiol* **9**, 725–737.
- Dong, F., Su, H., Huang, Y., Zhong, Y. & Zhong, G. (2004). Cleavage of host keratin 8 by a chlamydia-secreted protease. *Infect Immun* **72**, 3863–3868.
- Dong, F., Pirbhai, M., Xiao, Y., Zhong, Y., Wu, Y. & Zhong, G. (2005). Degradation of the proapoptotic proteins Bik, Puma, and Bim with Bcl-2 domain 3 homology in *Chlamydia trachomatis*-infected cells. *Infect Immun* **73**, 1861–1864.
- Doughri, A. M., Storz, J. & Altera, K. P. (1972). Mode of entry and release of chlamydiae in infections of intestinal epithelial cells. *J Infect Dis* **126**, 652–657.
- Eissenberg, L. G., Wyrick, P. B., Davis, C. H. & Rump, J. W. (1983). *Chlamydia psittaci* elementary body envelopes: ingestion and inhibition of phagolysosome fusion. *Infect Immun* **40**, 741–751.
- Etienne-Manneville, S. & Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629–635.
- Fan, T., Lu, H., Hu, H., Shi, L., McClarty, G. A., Nance, D. M., Greenberg, A. H. & Zhong, G. (1998). Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *J Exp Med* **187**, 487–496.
- Farquhar, M. G. & Palade, G. E. (1963). Junctional complexes in various epithelia. *J Cell Biol* **17**, 375–412.
- Fischer, S. F., Vier, J., Kirschnek, S., Klos, A., Hess, S., Ying, S. & Hacker, G. (2004). *Chlamydia* inhibit host cell apoptosis by degradation of proapoptotic BH3-only proteins. *J Exp Med* **200**, 905–916.
- Geng, Y., Shane, R. B., Berencsi, K., Gonczol, E., Zaki, M. H., Margolis, D. J., Trinchieri, G. & Rook, A. H. (2000). *Chlamydia pneumoniae* inhibits apoptosis in human peripheral blood mononuclear cells through induction of IL-10. *J Immunol* **164**, 5522–5529.
- Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345–357.
- Hackstadt, T. (1999). Cell biology. In *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity*, pp. 101–138. Edited by R. S. Stephens. Washington, DC: American Society for Microbiology.
- Hatch, T. (1999). Developmental biology. In *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity*, pp. 29–67. Edited by R. S. Stephens. Washington, DC: American Society for Microbiology.
- Heuer, D., Brinkmann, V., Meyer, T. F. & Szczepek, A. J. (2003). Expression and translocation of chlamydial protease during acute and persistent infection of the epithelial HEp-2 cells with *Chlamydia pneumoniae*. *Cell Microbiol* **5**, 315–322.
- Hogan, R. J., Mathews, S. A., Mukhopadhyay, S., Summersgill, J. T. & Timms, P. (2004). Chlamydial persistence: beyond the biphasic paradigm. *Infect Immun* **72**, 1843–1855.
- Honda, T., Shimizu, K., Kawakatsu, T., Fukuhara, A., Irie, K., Nakamura, T., Matsuda, M. & Takai, Y. (2003). Cdc42 and Rac small G proteins activated by *trans*-interactions of nectins are involved in activation of c-Jun N-terminal kinase, but not in association of nectins and cadherin to form adherens junctions, in fibroblasts. *Genes Cells* **8**, 481–491.
- Jepson, M. A., Collares-Buzato, C. B., Clark, M. A., Hirst, B. H. & Simmons, N. L. (1995). Rapid disruption of epithelial barrier function by *Salmonella typhimurium* is associated with structural modification of intercellular junctions. *Infect Immun* **63**, 356–359.
- Johnson, G. L. & Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298**, 1911–1912.
- Kane, C. D. & Byrne, G. I. (1998). Differential effects of gamma interferon on *Chlamydia trachomatis* growth in polarized and nonpolarized human epithelial cells in culture. *Infect Immun* **66**, 2349–2351.
- Kawakatsu, T., Shimizu, K., Honda, T., Fukuhara, T., Hoshino, T. & Takai, Y. (2002). *Trans*-interactions of nectins induce formation of filopodia and lamellipodia through the respective activation of Cdc42 and Rac small G proteins. *J Biol Chem* **277**, 50749–50755.
- Lauffenburger, D. A. & Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359–369.
- Lin, A. (2003). Activation of the JNK signaling pathway: breaking the brake on apoptosis. *Bioessays* **25**, 17–24.
- MacIntyre, A., Hammond, C. J., Little, C. S., Appelt, D. M. & Balin, B. J. (2002). *Chlamydia pneumoniae* infection alters the junctional complex proteins of human brain microvascular endothelial cells. *FEMS Microbiol Lett* **217**, 167–172.
- Majeed, M., Gustafsson, M., Kihlstrom, E. & Stendahl, O. (1993). Roles of Ca²⁺ and F-actin in intracellular aggregation of *Chlamydia trachomatis* in eucaryotic cells. *Infect Immun* **61**, 1406–1414.
- Nelson, D. E., Virok, D. P., Wood, H., Roshick, C., Johnson, R. M., Whitmire, W. M., Crane, D. D., Steele-Mortimer, O., Kari, L. & other

- authors (2005).** Chlamydial IFN- γ immune evasion is linked to host infection tropism. *Proc Natl Acad Sci U S A* **102**, 10658–10663.
- Pirbhai, M., Dong, F., Zhong, Y., Pan, K. Z. & Zhong, G. (2006).** The secreted protease factor CPAF is responsible for degrading pro-apoptotic BH3-only proteins in *Chlamydia trachomatis*-infected cells. *J Biol Chem* **281**, 31495–31501.
- Prozialeck, W. C., Fay, M. J., Lamar, P. C., Pearson, C. A., Sigar, I. & Ramsey, K. H. (2002).** *Chlamydia trachomatis* disrupts N-cadherin-dependent cell-cell junctions and sequesters β -catenin in human cervical epithelial cells. *Infect Immun* **70**, 2605–2613.
- Rajalingam, K., Al-Younes, H., Muller, A., Meyer, T. F., Szczepek, A. J. & Rudel, T. (2001).** Epithelial cells infected with *Chlamydophila pneumoniae* (*Chlamydia pneumoniae*) are resistant to apoptosis. *Infect Immun* **69**, 7880–7888.
- Sakaguchi, T., Kohler, H., Gu, X., McCormick, B. A. & Reinecker, H. C. (2002).** *Shigella flexneri* regulates tight junction-associated proteins in human intestinal epithelial cells. *Cell Microbiol* **4**, 367–381.
- Sakisaka, T. & Takai, Y. (2004).** Biology and pathology of nectins and nectin-like molecules. *Curr Opin Cell Biol* **16**, 513–521.
- Sears, C. L. (2000).** Molecular physiology and pathophysiology of tight junctions V. Assault of the tight junction by enteric pathogens. *Am J Physiol Gastrointest Liver Physiol* **279**, G1129–G1134.
- Shaw, A. C., Vandahl, B. B., Larsen, M. R., Roepstorff, P., Gevaert, K., Vandekerckhove, J., Christiansen, G. & Birkelund, S. (2002).** Characterization of a secreted *Chlamydia* protease. *Cell Microbiol* **4**, 411–424.
- Soloff, B. L., Rank, R. G. & Barron, A. L. (1985).** Electron microscopic observations concerning the in vivo uptake and release of the agent of guinea-pig inclusion conjunctivitis (*Chlamydia psittaci*) in guinea-pig exocervix. *J Comp Pathol* **95**, 335–344.
- Struyf, F., Plate, A. E. & Spear, P. G. (2005).** Deletion of the second immunoglobulin-like domain of nectin-1 alters its intracellular processing and localization and ability to mediate entry of herpes simplex virus. *J Virol* **79**, 3841–3845.
- Takai, Y. & Nakanishi, H. (2003).** Nectin and afadin: novel organizers of intercellular junctions. *J Cell Sci* **116**, 17–27.
- Takai, Y., Irie, K., Shimizu, K., Sakisaka, T. & Ikeda, W. (2003).** Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization. *Cancer Sci* **94**, 655–667.
- Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H. & Takai, Y. (1997).** Regulation of cell-cell adhesion by Rac and Rho small G proteins in MDCK cells. *J Cell Biol* **139**, 1047–1059.
- Takeichi, M. (1995).** Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* **7**, 619–627.
- Tam, J. E., Knight, S. T., Davis, C. H. & Wyrick, P. B. (1992).** Eukaryotic cells grown on microcarrier beads offer a cost-efficient way to propagate *Chlamydia trachomatis*. *Biotechniques* **13**, 374–378.
- Terres, A. M., Pajares, J. M., O'Toole, D., Ahern, S. & Kelleher, D. (1998).** *H. pylori* infection is associated with downregulation of E-cadherin, a molecule involved in epithelial cell adhesion and proliferation control. *J Clin Pathol* **51**, 410–412.
- Tsukita, S. & Furuse, M. (1999).** Occludin and claudins in tight-junction strands: leading or supporting players? *Trends Cell Biol* **9**, 268–273.
- Van Aelst, L. & Symons, M. (2002).** Role of Rho family GTPases in epithelial morphogenesis. *Genes Dev* **16**, 1032–1054.
- Wyrick, P. B. (2000).** Intracellular survival by *Chlamydia*. *Cell Microbiol* **2**, 275–282.
- Wyrick, P. B., Choong, J., Davis, C. H., Knight, S. T., Royal, M. O., Maslow, A. S. & Bagnell, C. R. (1989).** Entry of genital *Chlamydia trachomatis* into polarized human epithelial cells. *Infect Immun* **57**, 2378–2389.
- Wyrick, P. B., Gerbig, D. G., Jr, Knight, S. T. & Raulston, J. E. (1996).** Accelerated development of genital *Chlamydia trachomatis* serovar E in McCoy cells grown on microcarrier beads. *Microb Pathog* **20**, 31–40.
- Xia, M., Bumgarner, R. E., Lampe, M. F. & Stamm, W. E. (2003).** *Chlamydia trachomatis* infection alters host cell transcription in diverse cellular pathways. *J Infect Dis* **187**, 424–434.
- Ying, S., Seiffert, B. M., Hacker, G. & Fischer, S. F. (2005).** Broad degradation of proapoptotic proteins with the conserved Bcl-2 homology domain 3 during infection with *Chlamydia trachomatis*. *Infect Immun* **73**, 1399–1403.
- Zhong, G., Fan, T. & Liu, L. (1999).** Chlamydia inhibits interferon γ -inducible major histocompatibility complex class II expression by degradation of upstream stimulatory factor 1. *J Exp Med* **189**, 1931–1938.
- Zhong, G., Liu, L., Fan, T., Fan, P. & Ji, H. (2000).** Degradation of transcription factor RFX5 during the inhibition of both constitutive and interferon γ -inducible major histocompatibility complex class I expression in chlamydia-infected cells. *J Exp Med* **191**, 1525–1534.

Edited by: T. P. Hatch