

The novel chlamydial adhesin CPn0473 mediates the lipid raft-dependent uptake of *Chlamydia pneumoniae*

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Summary

***Chlamydiae* are Gram-negative, obligate intracellular pathogens that pose a serious threat to public health worldwide. Chlamydial surface molecules are essential for host cell invasion. The first interaction with the host cell is thereby accomplished by the Outer membrane complex protein B (OmcB) binding to heparan sulfate moieties on the host cell surface, followed by the interaction of the chlamydial polymorphic membrane proteins (Pmps) with host cell receptors. Specifically, the interaction of the Pmp21 adhesin and invasins with its human interaction partner, the epidermal growth factor receptor, results in receptor activation, down-stream signalling and finally internalization of the bacteria. Blocking both, the OmcB and Pmp21 adhesion pathways, did not completely abolish infection, suggesting the presence of additional factors relevant for host cell invasion. Here, we show that the novel surface protein CPn0473 of *Chlamydia pneumoniae* contributes to the binding and invasion of infectious chlamydial particles. CPn0473 is expressed late in the infection cycle and located on the infectious chlamydial cell surface. Soluble recombinant CPn0473 as well as rCPn0473-coupled fluorescent latex beads adhere to human epithelial HEp-2 cells. Interestingly, in classical infection blocking experiments pre-treatment of HEp-2 cells with rCPn0473 does not attenuate adhesion but promotes dose-dependently internalization by *C. pneumoniae* suggesting an unusual mode of action for this adhesin. This CPn0473-dependent promotion of infection by *C. pneumoniae* depends on two**

different domains within the protein and requires intact lipid rafts. Thus, inhibition of the interaction of CPn0473 with the host cell could provide a way to reduce the virulence of *C. pneumoniae*.

Introduction

Chlamydia pneumoniae and *C. trachomatis* are Gram-negative, obligate intracellular human pathogens. *C. pneumoniae* infects the respiratory tract, and virtually everybody is infected at least once during a lifetime (Grayston *et al.*, 1990). *C. trachomatis* causes ocular (serovars A–C) and urogenital-tract infections (serovars D–K and LGV) and is the most common cause of preventable blindness and a leading cause of sexually transmitted diseases worldwide (Bebear and de Barbeyrac, 2009; Hu *et al.*, 2010). The chlamydial life cycle is unique, involving alternation between two developmental forms. The infectious but metabolically inert elementary body (EB) adheres to and invades host cells, and differentiates into the metabolically active reticulate body (RB) in a membrane-bounded vacuole or inclusion. The RB then replicates several times before differentiating into the infectious form and exiting the host cell [reviewed in Abdelrahman and Belland (2005).

EB adhesion is essential for invasion and infection of host cells, but little is known about the proteins involved on either side. Attachment of *C. pneumoniae* is mediated by binding of the conserved adhesin OmcB to heparan sulfate-like proteoglycans (GAG) on human cells (Stephens *et al.*, 2001; Fadel and Eley, 2007; Moelleken and Hegemann, 2008). Interestingly, the GAG specificity of *C. trachomatis* OmcB reflects biovar-specific differences, which might in part account for tissue tropism and the spread of the pathogen (Moelleken and Hegemann, 2008; Fechtner *et al.*, 2013). However, blockade of the OmcB-GAG interaction by various means inhibits infection only moderately, implying that additional adhesin-receptor interactions occur (Wuppermann *et al.*, 2001; Moelleken and Hegemann, 2008; Fechtner *et al.*, 2013). Indeed, members of the *C. pneumoniae* polymorphic membrane protein (Pmp) family have subsequently been identified as adhesins that recognize receptors on human HEp-2 cells (Moelleken *et al.*, 2010; Hegemann and Moelleken, 2012), and it has been demonstrated that Pmp21 also promotes

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internalization by interacting with and activating the epidermal growth factor receptor (EGFR) (Molleken *et al.*, 2013). Blocking experiments with a recombinant Pmp protein in combination with OmcB showed additive effects reducing infection by approximately 70%, indicative of separate adhesion pathways and suggesting that attachment is a multistep process involving at least two distinct pathways (Becker and Hegemann, 2014). Thus, the involvement of other chlamydial and host cell factors in the adhesion of *C. pneumoniae* has been suggested [summarized in Hegemann and Moelleken (2012)]. Adhesion is followed by internalization of the chlamydial EBs, and this is dependent on cholesterol- and sphingolipid-rich lipid-raft domains, as disruption of lipid rafts by depletion of cholesterol with methyl-beta-cyclodextrin (M β CD) or the sequestering agents nystatin and filipin strongly inhibit internalization and infection (Stuart *et al.*, 2003; Korhonen *et al.*, 2012).

Because adhesion and internalization are essential for infection by obligate intracellular pathogens, we suspected that *C. pneumoniae* uptake requires a variety of chlamydial protein–host molecule interactions. Here, we show that the EB surface protein CPn0473 adheres to human epithelial cells and is essential for very early steps

in infection. Moreover, CPn0473 promotes EB uptake dose-dependently in a lipid raft-dependent manner. Thus, we have identified a surface localized protein for the promotion of target-cell invasion by the chlamydial pathogen.

Results

CPn0473 binds to human epithelial cells

In a screen for new chlamydial proteins involved in adhesion/internalization, we employed the yeast display system successfully used previously to identify and characterize the chlamydial adhesins OmcB and Pmp21 (Moelleken and Hegemann, 2008; Moelleken *et al.*, 2010). Among several preselected chlamydial proteins tested, we found that yeast cells presenting the predicted chlamydial protein CPn0473 adhered to HEp-2 cells almost as well as a strain bearing invasin, a known adhesin from *Yersinia pseudotuberculosis* (Fig. 1A). To confirm this, we performed diverse adhesion assays with the purified His-tagged recombinant full length protein, rCPn0473 (508 aa). Fluorescently labelled latex beads coated with rCPn0473 (100 $\mu\text{g ml}^{-1}$), unlike beads coated with either BSA or GST, adhered as strongly to HEp-2 cells ($151 \pm 34.4\%$)

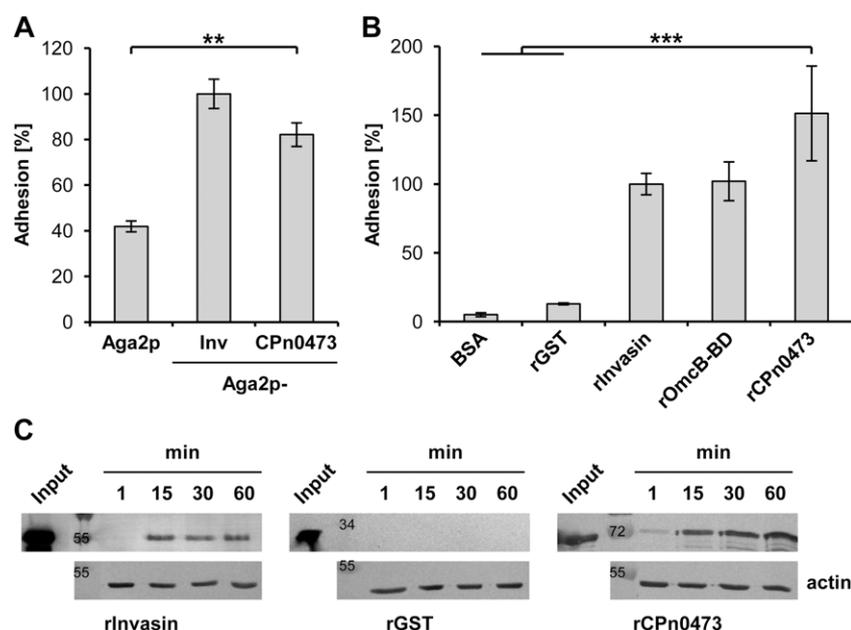


Fig. 1. CPn0473 binds to human epithelial cells

A. Adhesion of chlamydial protein-presenting yeast cells to human cells. HEp-2 cells were incubated with a two-fold excess of yeast cells displaying Aga2, Aga2-Inv or Aga2-CPn0473, and adhesion was quantified by counting the numbers of human cells and attached yeast cells. Binding of Aga2-Inv was set to 100% ($n = 3$).

B. Adhesion of protein-coated, fluorescent latex beads to HEp-2 cells. HEp-2 cells were incubated with a tenfold excess of beads coated with 100 $\mu\text{g ml}^{-1}$ BSA, rGST, rInvasin, rOmcB-BD or rCPn0473. Adhesion was quantified by flow cytometric measurement of the overall fluorescence of HEp-2 cells bearing attached beads (triplicates). The level of binding seen with beads coated with rInvasin was set to 100% ($n = 3$).

C. Adhesion of His-tagged soluble recombinant proteins to HEp-2 cells. HEp-2 cells were incubated with the specified soluble recombinant protein for the indicated times. Unbound protein was washed off and adherent protein quantified on Western blots with a monoclonal anti-His antibody. Positions of MW markers are marked. Shown is a typical experimental result.

Statistical significance of differences is denoted by *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$). n.s., not significant.

asbeads bearing invasins or the binding domain of the chlamydial adhesin OmcB (Fig. 1B). Interestingly, however, no evidence for the uptake of the rCPn0473-coated beads into the human cells was obtained (data not shown). We also tested the soluble His-tagged recombinant CPn0473 itself and found that while binding of invasins was first detectable after 15 min of incubation, attachment of rCPn0473 occurred within 1 min, and amounts of bound rCPn0473 increased with time (Fig. 1C). To further characterize the adhesion properties of CPn0473 we created successive deletion variants and tested their adhesion to human cells when coupled to fluorescently labelled beads and as soluble proteins (Fig. S1A). These experiments determined a 50 amino acid region (aa 307–356) as being essential for CPn0473 adhesion to the host cell. To confirm the impact of these amino acids on binding, we tested a rCPn0473 deletion variant lacking only the amino acids 307–356. This mutant version (rCPn0473 Δ BD) showed a complete loss of binding (Fig. S1B), indicating that this domain is essential for CPn0473-mediated adhesion to human cells.

CPn0473 is expressed late in the infection cycle and localizes on the surface of EBs

In order to determine the expression profile of CPn0473 during a *C. pneumoniae* infection we raised a polyclonal antibody against the full-length rCPn0473 (Fig. S2A, B) and performed western blots over the infection cycle. The CPn0473 protein could first be detected at 48 h post infection (hpi) and the signal stayed until 96 hpi confirming that CPn0473 is a late expressed protein (Fig. 2A). To study the subcellular localization of CPn0473, infected HEp-2 cells were fixed either with methanol to stain all chlamydial proteins or with paraformaldehyde (PFA) followed by differential permeabilization to prevent staining of intrachlamydial proteins (Moelleken and Hegemann, 2008; Wuppermann *et al.*, 2008; Moelleken *et al.*, 2010). As in the western blot analysis, we were able to first detect CPn0473 at 48 hpi inside the chlamydial particles (Fig. 2B). From 72 hpi on CPn0473 was detectable on the surface of the newly synthesized infectious EBs (Fig. 2C). The staining pattern of the intrachlamydial DnaK confirmed the integrity of the bacteria after differential permeabilization. Within cells fixed with PFA and permeabilized with 2% saponin, which disrupts the integrity of the bacteria, DnaK could still be recognized by the antibody, proving the latter's functionality in PFA-fixed cells (Fig. S2C).

For CPn0473 we observed ring-like signals surrounding the EBs, mimicking the pattern observed for the major outer membrane protein MOMP and chlamydial LPS (shown here) and the Pmp21 adhesin described previously (Moelleken *et al.*, 2010) (Fig. 3A, arrowheads; Fig. 3B). Moreover, the CPn0473 signal was also detectable early

during the next round of infection, as it could be visualized on the surface of adherent EBs at 15 min pi (Fig. 3C).

To confirm the surface localization of CPn0473, intact infectious EBs were biotinylated. After lysis, biotinylated proteins were affinity-enriched and analysed via immunoblotting using specific antibodies. While intrachlamydial proteins like EFTu or DnaK could not be detected, surface-localized proteins like Pmp21 and also CPn0473 could be purified after lysis (Fig. 3D). In contrast, neither intrachlamydial nor extra-chlamydial proteins could be detected in whole protein extracts from unbiotinylated chlamydial EBs (Fig. 3D).

To characterize the mode of localization of CPn0473, EBs were treated with ionic and non-ionic detergents. In agreement with previous reports (Wuppermann *et al.*, 2008), PBS does not extract the intracellular proteins S1 and EF-Tu (although it does leach out GroEL1) nor did it remove MOMP or CPn0473 from the EB surface (Fig. 3E). However, most of the CPn0473 (and significant amounts of GroEL1), but no MOMP, S1 or EF-Tu, were found in the supernatant fraction after exposing EBs to 1% Triton X-100. Interestingly, while the portion of CPn0473 remaining in the pellet was intact, almost the entire fraction extracted by Triton X-100 consisted of protein fragments (Fig. 3E, asterisks). Treatment with 2% Sarkosyl extracted almost all the full-length CPn0473 (as well as some S1 and EF-Tu), while MOMP, as a member of the Sarkosyl-insoluble chlamydial outer membrane complex (cOMC) (Caldwell *et al.*, 1981), was retained in the pellet. Thus, these results indicate that CPn0473 is accessible on the EB surface, but is not part of the cOMC.

To explore the possible role of CPn0473 in infection, we performed antibody neutralization experiments. Treatment of EBs with antiserum against EF-Tu had no effect on infection, but preincubation with anti-CPn0473 reduced *C. pneumoniae* infection by about 30% (relative to exposure to the pre-immune serum), an effect comparable to that of anti-OmcB (Fig. 3F). Hence, CPn0473 possesses the characteristics of an adhesin and is important for infection.

rCPn0473 boosts C. pneumoniae infection by enhancing internalization of EBs

To further assess the impact of CPn0473 on infection, we preincubated HEp-2 cells with rCPn0473 prior to infection. Pretreatment of EBs with heparin to block OmcB-mediated adhesion (Moelleken and Hegemann, 2008) reduced infectivity by tenfold, to $9 \pm 1.3\%$ of values for PBS and BSA controls (Fig. 4A). In contrast, preincubation with rCPn0473 for 2 h prior to exposure to *C. pneumoniae* EBs enhanced infection in a dose-dependent manner, and by nearly threefold ($283 \pm 21.6\%$) at an input concentration of $200 \mu\text{g ml}^{-1}$ (Fig. 4A). Indeed, when added together with the EBs, the boosting effect was equal to that seen after a 2 h preincubation (Fig. 4B), while addition at 2 or 6 hpi, i.e. after internalization is complete, did not enhance infectivity any

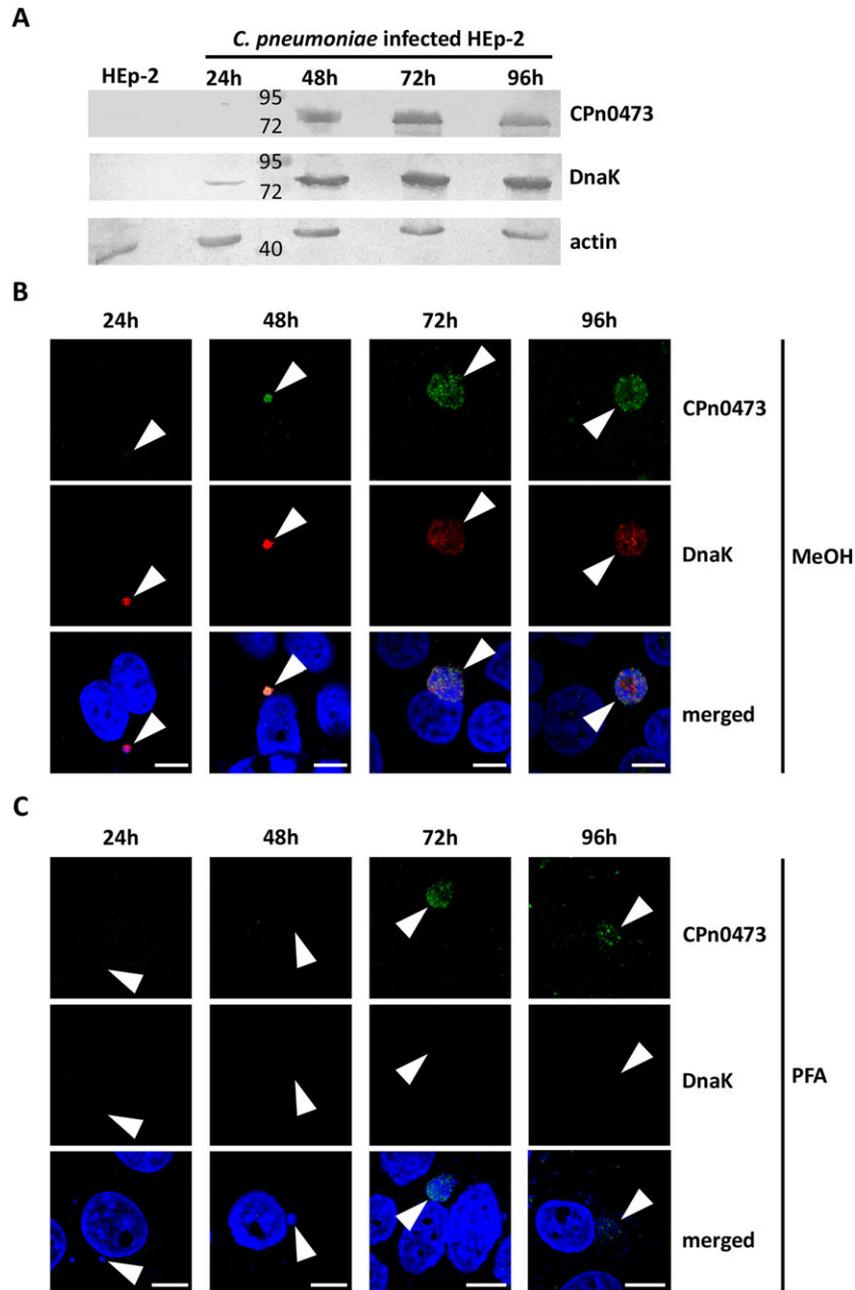


Fig. 2. CPn0473 is expressed late in the infection cycle and located on the surface of infectious EBs

A. Detection of CPn0473 during the course of infection via Western blots (representative blot). HEp-2 cells infected with *C. pneumoniae* (MOI = 10) were collected at indicated time points. The chlamydial chaperone protein DnaK was used to reveal the amount of chlamydia in every probe. CPn0473 expression was analysed via Western blots using anti-CPn0473, anti-DnaK and anti-actin antibodies.

B and C. Expression profile of CPn0473 and the surface localization during a *C. pneumoniae* infection (MOI = 1) via confocal microscopy. HEp-2 cells infected with *C. pneumoniae* were fixed at indicated time points either with MeOH and permeabilized with 0.05% saponin to stain extra- and intra-chlamydial proteins (B) or paraformaldehyde (PFA), followed by differential permeabilization using 0.05% saponin to stain surface localized chlamydial proteins only (C). Anti-DnaK antibodies were used to stain intra-chlamydial proteins. CPn0473 was stained using anti-CPn0473 antibodies. DAPI was used to stain DNA and is only shown in the merge picture. The arrowheads mark the *C. pneumoniae* inclusion. Scale bar: 10 μ m.

more (Fig. 4B). Unlike rOmcB and rPmp21, which mask their interaction partner on the host cell, thus making them inaccessible to the endogenous protein on the EB surface (Moelleken and Hegemann, 2008; Moelleken *et al.*, 2010),

these results indicate that rCPn0473 stimulates adhesion and/or internalization of EBs. In order to dissect protein function in more detail, we tested the adhesion-mediating C-terminus of rCPn0473 (aa 256–508) for its ability to boost

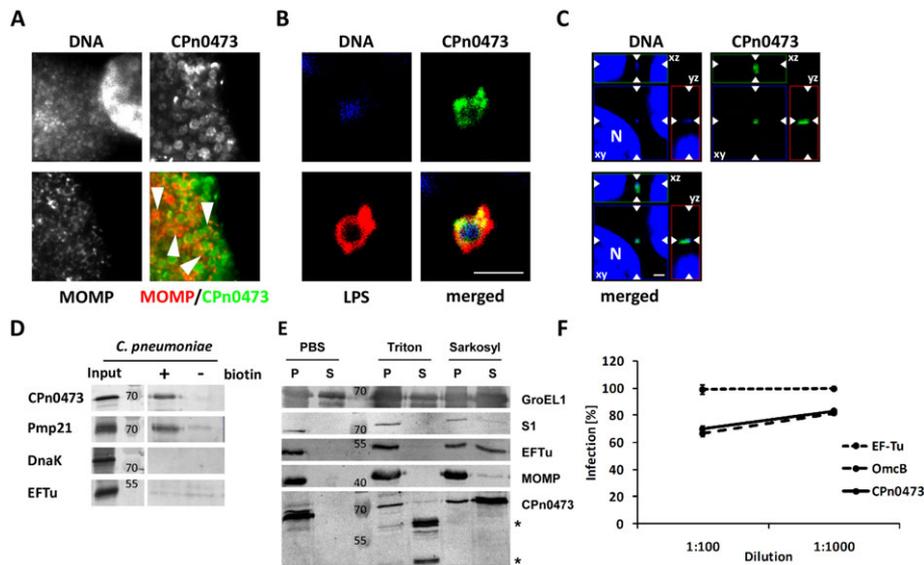


Fig. 3. CPn0473 is expressed on the chlamydial cell surface and contributes to infection by *C. pneumoniae*. A and B. Detection of CPn0473 on the surface of infectious EBs. HEp-2 cells infected with *C. pneumoniae* (MOI = 1) were fixed with PFA at 72 hpi (A) or 96 hpi (B) and permeabilized with 0.1% Triton allowing staining of extrachlamydial proteins only (representative picture). DNA was stained with DAPI, chlamydial MOMP, LPS and CPn0473 with specific polyclonal antibodies. (A) The arrowheads point to EBs in which colocalization of CPn0473 and MOMP can clearly be seen. Scale bar: 2 μ m. (B) The image shows an individual EB released from an inclusion. Scale bar: 1 μ m. C. Localization of CPn0473 on infectious EB by confocal microscopy at 15 min pi shown in all three projections (labelled xy, xz, yz). DNA was stained with DAPI (blue) and CPn0473 with specific polyclonal antibodies (green) (representative picture). N = nucleus. Scale bar: 2 μ m. D. Biotinylation of surface localized CPn0473 on infectious EBs. About 5×10^7 purified EBs were or were not incubated with 5 mM Biotin. After lysis and centrifugation biotinylated as well as non-biotinylated protein solutions were enriched using Streptavidin-Agarose and analysed on Western blots using polyclonal anti-EF-Tu, anti-DnaK, anti-Pmp21 or anti-CPn0473 antibodies. As an input control 1×10^7 purified EBs were loaded (representative blot) ($n = 3$). E. Intact CPn0473 can be extracted from EBs with 2% Sarkosyl. Purified EBs were treated with PBS, 1% Triton X-100 or 2% Sarkosyl and pelleted. The pellet (P) and supernatant (S) fractions were analysed on Western blots using monoclonal anti-S1, and polyclonal anti-GroEL1, anti-EF-Tu, anti-MOMP or anti-CPn0473 antibodies (representative blot). Asterisk indicates CPn0473 fragments. F. Effects on infectivity upon pretreatment of *C. pneumoniae* EBs with various polyclonal antibodies. EBs were treated with pre-immune serum or antiserum at the indicated dilutions prior to incubation with HEp-2 cells (MOI = 20). Infectivity was quantified 50 hpi by counting the numbers of inclusions in ten microscopic fields (duplicates). Infectivity of EBs pretreated with the pre-immune serum at each dilution was set to 100% ($n = 3$).

the infection. Even though the protein variant was able to bind human cells, it did not boost the infection. The N-terminus (aa 1–255) on the other hand was neither able to bind to human cells nor to boost the infection (Fig. S3C). This implies that the N-terminus of CPn0473 (aa 1–255) is required for the boost. Using additional smaller N-terminal deletion variants we could show that the first 150 aa of CPn0473 is essential to boost the infection, as a deletion variant lacking aa 151–255 still boosted infection, while a deletion variant lacking the first 171 amino acids did not show this phenotype anymore (Fig. S3). However, both protein variants were able to bind to HEp-2 cells to the same extent (41% respectively 42% of full-length CPn0473) (Fig. S1C). Thus, the capacities for adhesion and boost formation are located in different regions of CPn0473.

Next we tested whether pretreatment with rCPn0473 stimulated EB adhesion to host cells. In adhesion assays with fluorescence-labelled carboxyfluorescein succinimidyl ester (CFSE) EBs, binding to HEp-2 cells was unaffected by pretreatment with rCPn0473 ($100 \mu\text{g ml}^{-1}$), regardless of the number of inclusion-forming units (IFUs) employed

(Fig. 4C). Indeed, at high molecules of infection (MOI), adhesion of CFSE-labelled EBs was slightly but significantly reduced upon rCPn0473 pretreatment ($85 \pm 5\%$), while the control – pretreatment with heparin, which is known to block adhesion – reduced EB binding to HEp-2 cells to $8.3 \pm 1\%$ (Fig. 4C) (Moelleken and Hegemann, 2008; Molleken *et al.*, 2010). Adhesion was linearly dependent on IFU concentration, indicating that the target-cell surface was not saturated with EBs. Thus, we addressed the role of rCPn0473 in EB internalization using real-time PCR. Pretreatment of EBs with heparin abrogated OmcB-mediated adhesion, reducing relative internalization to $0.58 \pm 0.22\%$ in comparison to PBS and BSA controls ($1.90 \pm 0.56\%$ and $1.16 \pm 0.42\%$ respectively) (Fig. 4D). In contrast, pretreatment of HEp-2 cells with rCPn0473 ($100 \mu\text{g ml}^{-1}$) markedly increased relative internalization to $14.26 \pm 2.67\%$ (Fig. 4D). Again, we tested different deletion variants to analyse the effects of CPn0473 on the chlamydial internalization in more detail. Neither the binding-deficient variant CPn0473 Δ BD nor the deletion variant lacking the first 170 aa (CPn0473 Δ 1-170)

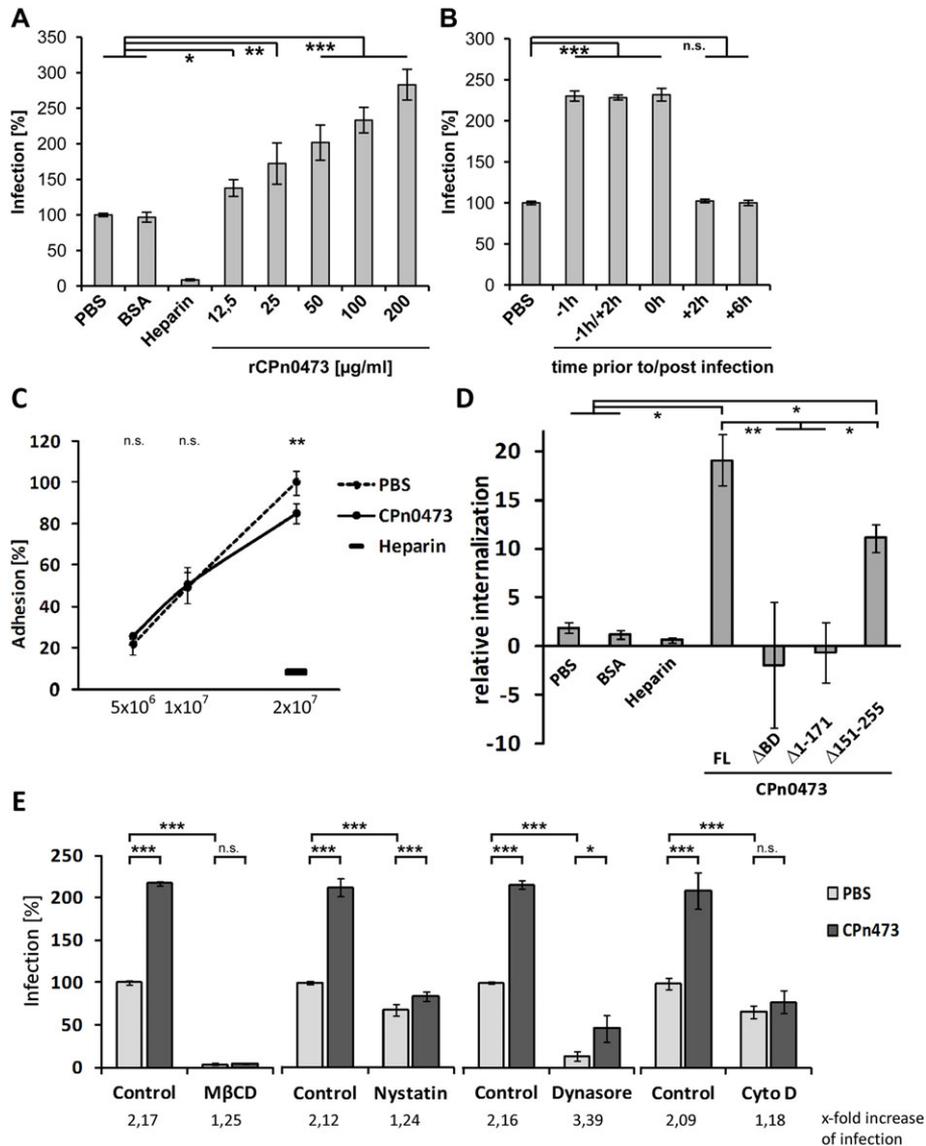


Fig. 4. Recombinant CPn0473 specifically boosts internalization of *C. pneumoniae* EBs

A. Impact of soluble rCPn0473 on the efficiency of infection by *C. pneumoniae*. HEp-2 cells were pretreated with PBS, BSA (200 µg ml⁻¹) or rCPn0473 (at the indicated concentrations) for 2 h prior to infection with *C. pneumoniae* (MOI = 20). As a control, EBs were also pretreated with heparin (500 µg ml⁻¹) ($n = 3$). Infectivity was quantified as in Fig. 2E (duplicates). Infectivity of PBS-treated cells was set to 100%. Statistical significance of differences is denoted as in Fig. 1.

B. Influence of the time of addition of rCPn0473 on infectivity. PBS or rCPn0473 (100 µg ml⁻¹) was added to HEp-2 cells at the indicated time points prior to (–), together with (0 h), after (+), or 1 h before and again 2 h after (–1/+2) infection with *C. pneumoniae* (MOI = 20). At 2 hpi the medium was replaced by chlamydial growth medium either with (in case of –1/+2, +2, +6) or without rCPn0473 ($n = 3$). Statistical significance of differences is denoted as in Fig. 1.

C. Impact of soluble rCPn0473 on adhesion of fluorescence-labelled *C. pneumoniae* EBs to HEp-2 cells. HEp-2 cells were pretreated with PBS or rCPn0473 (100 µg ml⁻¹) for 2 h prior to exposure to the indicated numbers (IFUs) of CFSE-labelled EBs for 1 h at 4°C to allow adhesion but not internalization. Adhesion was quantified by flow cytometric measurement of the overall fluorescence of HEp-2 cells bearing attached CFSE-labelled EBs (triplicates). The fluorescence level associated with binding of 2 × 10⁷ IFUs of CFSE-labelled EBs to cells pretreated with PBS was set to 100% adhesion ($n = 3$). As a control, 2 × 10⁷ IFUs of CFSE-labelled EBs were also pretreated with heparin (500 µg ml⁻¹) and tested for adhesion. Statistical significance of differences is denoted as in Fig. 1.

D. Effect of soluble full-length rCPn0473 (FL) and rCPn0473 deletion variants on internalization of *C. pneumoniae* EBs. HEp-2 cells were pretreated with PBS, BSA or rCPn0473 variants (100 µg ml⁻¹) for 1 h prior to infection with *C. pneumoniae*. As a control, EBs were also pretreated with heparin as in A. At 2 hpi non-internalized EBs were removed by treatment with trypsin. Internalization was then quantified by determining the ratio of chlamydial to human DNA in the cells by real-time PCR using 16S rRNA and GAPDH primers (triplicates) ($n = 3$). Statistical significance of differences is denoted as in Fig. 1.

E. Impact of chemical inhibitors of endocytosis on the infectivity of *C. pneumoniae* and its enhancement by rCPn0473. HEp-2 cells were treated with dynasore (0.1 mM, 30 min), MβCD (5 mM, 60 min), nystatin (30 µg ml⁻¹, 30 min), cytochalasin D (CytoD) (20 µM, 60 min) or the corresponding solvent (DMSO). The medium was then replaced by medium containing the specific inhibitor or the corresponding solvent, rCPn0473 (100 µg ml⁻¹) or PBS and EBs (MOI = 20). At 2 hpi the medium was replaced by chlamydial growth medium. Infection was quantified as in Fig. 3E (duplicates). The infectivity of solvent- and PBS-treated cells was set to 100%. The rCPn0473-induced boosting factor is given above each experiment ($n = 3$). Statistical significance of differences is denoted as in Fig. 1.

boosted internalization. Interestingly, CPn0473 Δ 151-255 was still able to enhance the relative internalization rate of *C. pneumoniae*, albeit to a lesser extent than the full-length rCPn0473 (Fig. 4D).

Next we wanted to test whether pre-loading of rCPn0473 onto infectious EBs would modulate their infectivity in a subsequent infection. Chlamydiae were pretreated with rCPn0473, PBS, BSA or rPmp21 prior to infection of HEp-2 cells. The infectivity of rCPn0473-coated EBs was significantly increased ($177 \pm 37\%$), compared with BSA-pretreated EBs ($104 \pm 14\%$). Interestingly, infectivity of Pmp21-coated EBs was also slightly increased ($125 \pm 46\%$) (Fig. S4A). These data indicate that while EB attachment is unaffected, prior exposure to rCPn0473 specifically increases the efficiency of uptake of EBs into host cells. Interestingly, we found that rCPn0473 also promoted infection by *C. trachomatis* E (to $174 \pm 8.3\%$ in comparison to controls), while the infectivity of serovar L2 was not significantly affected (Fig. S4C). Moreover, rCPn0473 had no effect on the adhesion and invasion properties of beads coated with rPmp21, the only known chlamydial adhesin and invasin (Molleken *et al.*, 2013), suggesting that rCPn0473 does not act as a general booster of internalization (data not shown).

rCPn0473-dependent promotion of infection by C. pneumoniae requires intact lipid rafts

Because internalization of *C. pneumoniae* EBs is dependent on cholesterol- and sphingolipid-rich lipid-raft domains we asked whether such domains are required for promotion of *C. pneumoniae* infection by rCPn0473 (Stuart *et al.*, 2003; Korhonen *et al.*, 2012). Indeed, disruption of lipid rafts with 5 mM M β CD resulted in a strong decrease in adhesion ($59 \pm 13\%$) and in infectivity ($4 \pm 0.8\%$) (Fig. S4B and 4E). Under these conditions, addition of rCPn0473 did not enhance the infection any more ($5 \pm 0.8\%$; 1.25 \times fold increase) (Fig. 4E). To confirm this result, we pretreated HEp-2 cells with the cholesterol-sequestering agent nystatin, which decreased infection to $68 \pm 4.4\%$ of control values. Again, the addition of rCPn0473 enhanced infection of nystatin-treated cells by *C. pneumoniae* (Fig. 4E) by only 1.24-fold, identical to the boost observed in M β CD-treated cells. Thus, while the promotion of an infection by rCPn0473 is largely dependent on intact lipid rafts, a lipid-raft-independent role of CPn0473 cannot be completely excluded.

The establishment and maintenance of lipid raft microdomains in the human membrane is dependent on the cortical actin cytoskeleton (Goswami *et al.*, 2008), [reviewed in (Chichili and Rodgers (2009))]. Therefore, we pretreated HEp-2 cells with the actin-destabilizing agent cytochalasin D, which results in a drop of the infectivity of *C. pneumoniae* ($66 \pm 3.4\%$), as well as the CPn0473-dependent boost of infection ($78 \pm 10\%$; 1.18 \times fold increase) (Fig. 4E). To probe whether rCPn0473 acts during formation of the endocytic

vesicle or after the pinching-off of the vesicle, we inhibited dynamin, a key player in many endocytotic pathways. Inhibition of dynamin with dynasore reduced the level of infection by *C. pneumoniae* to $14 \pm 5.5\%$ of control values (Fig. 4E). Interestingly, addition of rCPn0473 to such cells markedly increased infectivity to $46 \pm 16.2\%$ (3.39 \times fold increase) (Fig. 4E), strongly suggesting that the boosting effect of rCPn0473 is mediated at a level prior to the dynamin-mediated step.

C. pneumoniae enters host cells via lipid-raft marker-positive domains

Having demonstrated that the boosting effect is dependent on cholesterol we also asked if invasion by *C. pneumoniae* EBs might occur via the cholesterol-rich membrane domains known as lipid rafts. To study this issue, infected HEp-2 cells were fixed with PFA at 15 min pi and stained with fluorescence-labelled cholera toxin B-subunit (CTxB), which recognizes the ganglioside GM1 specific for lipid rafts. $97 \pm 2\%$ of the invading EBs were indeed found to be associated with CTxB-positive domains (Fig. 5A, top row) at this early time point. By 60 min pi the entire inclusion membrane surrounding each internalized EB was highly enriched for the lipid-raft marker ($98 \pm 0.04\%$) (Fig. 5A, middle and lower rows).

It has been demonstrated recently that the EGFR, to which the *C. pneumoniae* invasin Pmp21 binds, also accumulates in the plasma membrane of the human cell immediately subjacent to invading bacteria and in the early inclusion membrane (Molleken *et al.*, 2013). When cells were double-stained for lipid rafts (with FITC-labelled CTxB) and for EGFR with a monoclonal antibody early in the infection phase (60 min pi), $82 \pm 10\%$ of the EGFR signals were found to colocalize with CTxB-positive microdomains and the associated bacteria (Fig. 5B, upper row). In contrast, the distribution of human transferrin receptor, which is not involved in the uptake of chlamydial EBs and does not localize to lipid-raft domains, was not correlated with that of the invading bacteria ($5 \pm 1\%$ colocalization) (Fig. 5B, lower row). These data demonstrate that *C. pneumoniae* entry sites coincide with lipid rafts, and EGFR accumulates at these sites, and this may play a positive role in EB uptake.

rCPn0473 binds to lipid raft domains

Interestingly, when analyzing the binding of rCPn0473 to HEp-2 cells microscopically, bound rCPn0473 was distributed in a discontinuous pattern of dots. Thus, we asked if these coincided with lipid rafts by incubating HEp-2 cells with rCPn0473 and CTxB for 15 min at 4°C. We observed similar staining patterns for rCPn0473 and CTxB, with rCPn0473 often accumulating in regions of the PM that were strongly labelled by the lipid-raft indicator (Fig. 5C, upper row). This pattern was more obvious in a rare subpopulation of cells that showed a distinct separation between CTxB-positive

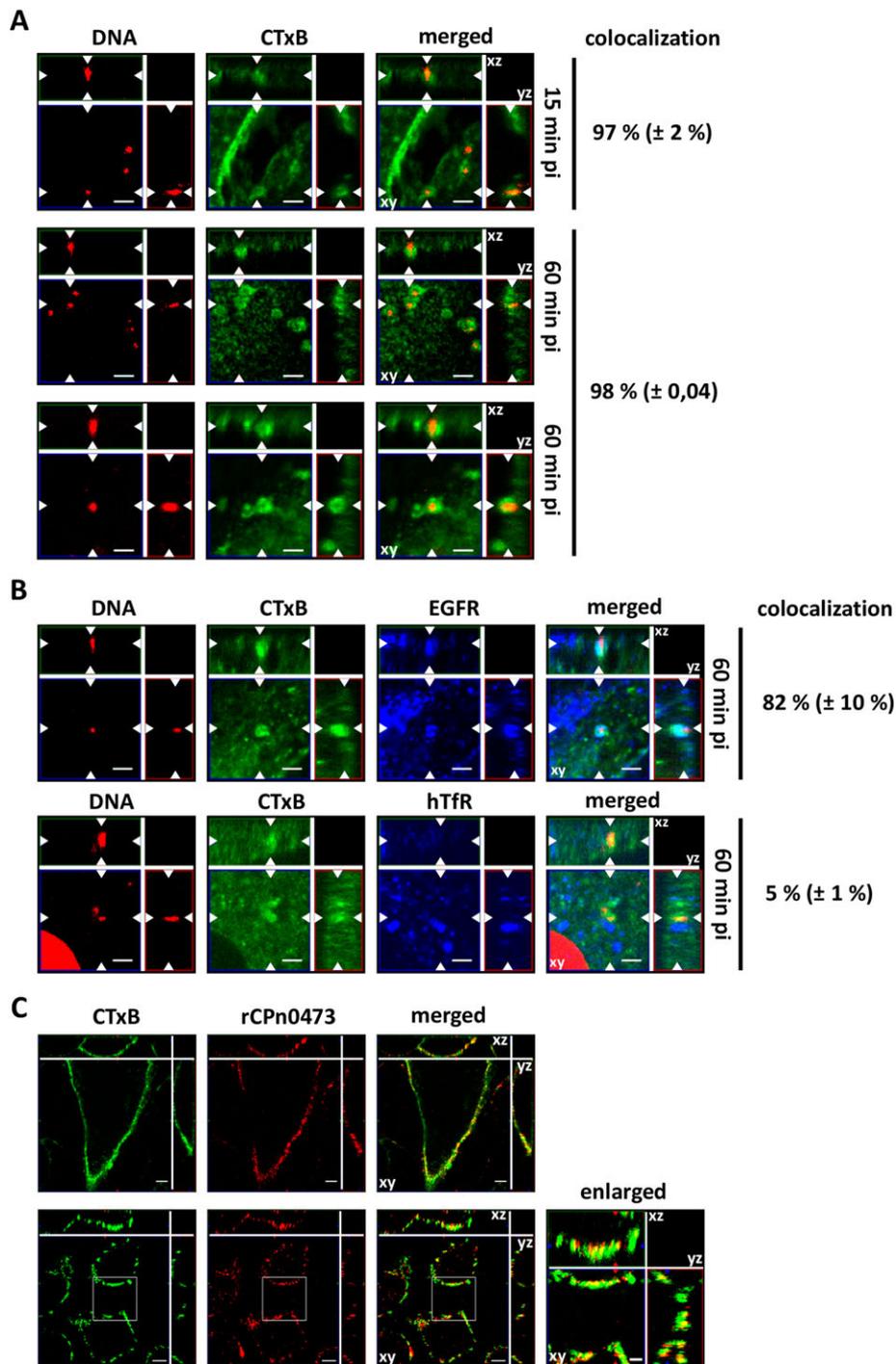


Fig. 5. *C. pneumoniae* induces accumulation of lipid-raft domains

Representative confocal microscopy pictures of early events in infection by *C. pneumoniae* shown in all three projections (labelled xy, xz, yz) (MOI = 20). **C:** Localization of CPn0473 on infectious EB by confocal microscopy at 15 min pi

A. Cells were fixed with PFA at indicated time points and permeabilized with 2% saponin. DNA was stained with DAPI, lipid rafts with FITC-labelled CTxB. Scale bar: 2 μ m. The frequency of colocalization of adhered EBs (at least 120) and CTxB is given in percent ($n = 3$).

B. Cells were fixed with PFA and DNA and lipid rafts were stained as in **A**. The epidermal growth factor receptor (EGFR) and human transferrin receptor (hTfR) were labelled with specific monoclonal antibodies. Scale bar: 2 μ m. The frequency of colocalization of adhered EBs (at least 75), CTxB and the human EGFR/hTfR is given in percent ($n = 2$).

C. Recombinant CPn0473 adheres to CTxB-positive domains. HEP-2 cells were incubated for 15 min at 4°C with rCPn0473 (20 μ g ml⁻¹) and FITC-labelled cholera toxin B-subunit (CTxB, 10 μ g ml⁻¹) to stain lipid rafts. Cells were then fixed using PFA, permeabilized with 2% saponin and rCPn0473 was detected with a monoclonal anti-His antibody. The boxed area in the lower row is shown at higher magnification on the right (enlarged). Scale bar: 5 μ m.

and CTxB-negative areas (Fig. 5C, lower row), most likely induced by CTxB as we also observed this in cells treated with CTxB and BSA (data not shown). Analysis of other adhesion-competent CPn0473 variants revealed that they, like the full-length protein, strictly followed the CTxB staining pattern (data not shown), suggesting that the CPn0473 binding domain is the main determinant of binding to CTxB-positive PM microdomains.

As described before, the establishment and maintenance of lipid raft microdomains in the human membrane is dependent on the cortical actin cytoskeleton (Goswami *et al.*, 2008), [reviewed in Chichili and Rodgers (2009)]. To investigate the role of the actin and microtubule cytoskeleton on the lipid rafts/CPn0473-distribution at the host membrane, cells were treated with cytoskeleton destabilizing drugs prior to adhesion of CPn0473. In untreated cells actin filaments at the host plasma membrane accumulated underneath the distinct CTxB-positive domains, colocalizing with bound rCPn0473 (Fig. S5A, upper row). The inhibition of actin polymerization in the host cell by cytochalasin D led to a decrease in CTxB staining as well as to a reduction in the distinct staining pattern towards a more uniform CTxB distribution (Fig. S5A, lower row). Interestingly, the rCPn0473 adhesion pattern very closely followed the CTxB signal pattern. In contrast disruption of the host microtubule cytoskeleton by nocodazole did not significantly influence the distribution of CTxB or rCPn0473 (Fig. S5B). This finding again clearly indicates that CPn0473 preferentially associates with lipid raft microdomains.

Discussion

We have characterized CPn0473 as a novel EB surface protein that binds to cholesterol-rich regions in the bilayer of the host cell PM and contributes to internalization of *C. pneumoniae* via lipid rafts. CPn0473 is a *C. pneumoniae* specific protein with no homologues in other organisms that is expressed late during the infection cycle. The protein can first be detected within chlamydial particles at 48 hpi, when chlamydial RB to EB re-differentiation has started, and is located on the surface of infectious EBs at 72 hpi when *C. pneumoniae* EBs start to leave their host cells for new infections (Fig. 2). Antibodies raised against rCPn0473 reduce infection comparable to antibodies directed against the OmcB adhesin. However, CPn0473 is not part of the cOMC as it appeared to be Sarkosyl soluble and does not contain any cysteine residues (Fig. 3). Interestingly, C-terminal fragments of CPn0473 can be extracted from EBs with 1% Triton, but not with PBS. Thus, CPn0473 may be processed and released from the EB during infection, perhaps after the highly cross-linked surface has been disrupted by disulfide reduction. Host protein disulfide isomerase (PDI) activity is necessary for internalization of *C. trachomatis* L2, and disulfide reduction of outer

membrane proteins is necessary for surface display of chlamydial Hsp70 (Raulston *et al.*, 2002; Abromaitis and Stephens, 2009). Thus, human PDI may make CPn0473 accessible for cleavage by a chlamydial or a host cell protease upon attachment of the EB.

Surprisingly, recombinant CPn0473 dose-dependently enhances the internalization of *C. pneumoniae* EBs resulting in an increased infectivity. To our knowledge this is the first example of a pathogen-derived protein which is able to promote the uptake process when added to the infection. Typically, blocking experiments with a soluble recombinant adhesin attenuate the host cell entry process because the specific receptor is blocked by the protein. Thus, the CPn0473-induced boosting phenotype suggests an unusual mode of action for this adhesin. The molecular basis for this phenomenon is currently not understood but is the subject of a subsequent study. Interestingly, increasing the amount of chlamydial adhesins on the EB surface, by incubating the EBs with recombinant Pmp21 and CPn0473 prior to an infection, enhances subsequent infection significantly. Presumably, the more adhesion molecules available on the EB surface, the more interaction partners can be recruited to the EB binding site on the host cell membrane, which then might allow the bacteria to infect its host more efficiently.

The CPn0473 domain analysis revealed the existence of at least two functionally distinct domains within the protein. The adhesion to human cells requires a 50 amino acids long stretch in the C-terminus (aa 307 to aa 356) (Fig. S1). The internalization enhancing effect is depending on the adhesion of CPn0473 to the human cell, but in addition requires the N-terminal 150 amino acids (Figs 3 and S3). Thus, most likely, CPn0473 action can be divided in two steps. First CPn0473 may bind via the C-terminal binding domain to the human host cell, followed by the action of the N-terminal part that supports the uptake process. The identity of the human interaction partner/s to whom CPn0473 is binding to remains to be identified. In agreement with others, we found that uptake of *C. pneumoniae* EBs is dependent on lipid rafts and requires the cortical actin cytoskeleton (Clifton *et al.* 2005) (Stuart *et al.*, 2003; Korhonen *et al.*, 2012). However, we show for the first time that lipid-raft domains are enriched at *C. pneumoniae* entry points as well as in the early inclusion membrane (Fig. 5). Moreover, the CPn0473-induced boost on EB internalization is directly dependent on the integrity of lipid rafts (Fig. 4). Finally, recombinant CPn0473 preferentially associates with CTxB-positive domains of HEp-2 cells, suggesting that CPn0473 has a higher affinity for lipid rafts than for other domains of the PM. Both CTxB and rCPn0473 signal intensities are decreased in the absence of cortical actin (Fig. S5). Inhibition of dynamin reduces susceptibility to infection, and rCPn0473 also boosted residual infectivity following inhibition of dynamin, indicating that it acts

upstream of the pinching-off of the endocytotic vesicle. Depletion of dynamin-II by siRNA also reduces uptake of *C. trachomatis* L2, while a dominant-negative K44A mutant of dynamin I had no effect (Boleti *et al.*, 1999; Hybiske and Stephens, 2007). Thus, dynamin-II may be a key player in chlamydial endocytosis generally.

In light of these findings, we propose that the initial contact with the target cell is mediated by OmcB, followed by specific adhesion of Pmp21 and possibly other Pmps to the EGFR associated with lipid rafts (Molleken *et al.*, 2013). CPn0473, either located on the EB surface or released from it, may interact with its yet unknown interaction partner associated with the cholesterol-rich lipid-raft domains, which then serve as sites of entry for *C. pneumoniae*. Indeed, we found that EGFR accumulates in the early inclusion membrane and this coincides with the lipid rafts. Interestingly, in the presence of high concentrations of ligand, EGFR has been reported to be endocytosed in a lipid raft-dependent manner (Sigismund *et al.*, 2005). The LOX-1 receptor previously reported to be essential for infection by *C. pneumoniae* (Campbell *et al.*, 2012) also localizes to, and requires lipid rafts for its activity (Matarazzo *et al.*, 2012). Furthermore, the fact that the *C. pneumoniae*-specific CPn0473 boosts infection by *C. trachomatis* E – but not L2 (Fig. S2) – implies that the two biovars use different mechanisms to invade host cells. Indeed, infection by serovar E is dependent on lipid rafts, while L2 shows no such dependency (Stuart *et al.*, 2003).

Lipid rafts are targeted by several invasive pathogens, which often recruit them to the point of entry, so as to build up a large signalling platform that promotes invasion of the host cell [reviewed in (Vieira *et al.*, 2010)]. Here we have characterized a novel chlamydial protein that is involved in the lipid raft-dependent uptake of the EB and may participate in the accumulation of lipid rafts at the entry site of *C. pneumoniae*. The identification of the human interaction partner of CPn0473 will be the aim of future studies, as well as analyzing the precise role of CPn0473 during chlamydial infection. Furthermore, targeting the ability of CPn0473 to bind to the host cell and to enhance the chlamydial infection may offer a useful novel strategy to diminish the virulence of *C. pneumoniae*.

Experimental procedures

Chemicals and antibodies

For sources of all chemicals and antibodies used in this study refer to Supplement Experimental Procedures section in the Supporting Information.

DNA manipulations and protein expression

Escherichia coli XL₁ blue (Stratagene) was used for plasmid amplification and the Origami strain (Novagen) for protein expression. Plasmids were constructed by *in vivo* homologous recombination in *Saccharomyces cerevisiae* strain

YKM2, a GFP-expressing version of EBY100 (Invitrogen) as described previously (Moelleken and Hegemann, 2008). *Yersinia pseudotuberculosis* invasin was expressed as the C-terminally His-tagged Inv₄₇₉ variant (aa 490–969) for purification of recombinant protein or as the Inv₁₉₇ variant (aa 772–969) for the yeast adhesion assay as described earlier (Moelleken and Hegemann, 2008; Moelleken *et al.*, 2010). The binding domain of OmcB (aa 40–100) from *C. pneumoniae* was expressed with a C-terminal His-tag and fused N-terminally to GST. GST and CPn0473 were expressed as, C-terminally His-tagged proteins. GST- and His-tagged proteins were purified with the protocols supplied by Sigma Aldrich and Qiagen respectively, and analysed on Western blots.

Immunofluorescence microscopy

Microscopy was performed with a Zeiss Cell Observer® SD confocal spinning-disc microscope or Nikon Eclipse Ti-E C2 confocal microscope. Confocal images were assembled using ZEN2011 (Zeiss) or NIS Element (Nikon) software. All other images were assembled using Image Pro Plus software. For further details refer to Supplement Experimental Procedures section in the Supporting Information.

Propagation of chlamydial strains

The HEp-2 cell line (epithelial larynx carcinoma, ATCC No.: CCL-23) was used for propagation of chlamydial strains and for adhesion and infection experiments. The cells were cultured in DMEM GlutaMax supplemented with FCS, vitamins, non-essential amino acids, amphotericin B (2.5 µg ml⁻¹) and gentamicin (50 µg ml⁻¹) (Invitrogen). *C. pneumoniae* GiD and the *C. trachomatis* serovars L2 (L2/434/Bu) and E (DK-20) were propagated in HEp-2 cells in the presence of 1.2 µg ml⁻¹ cycloheximide, and EBs were purified using 30% gastrografin (Schering).

Adhesion and infection assays

Unless otherwise stated, all adhesion assays were carried out with fully confluent layers of 10⁶ HEp-2 cells grown on glass coverslips at 37°C in an atmosphere containing 6% CO₂.

CPn0473 was identified by an artificial adhesion assay performed using a yeast display system as previously described (Moelleken and Hegemann, 2008; Moelleken *et al.*, 2010).

The mode of surface localization of CPn0473 was assessed by differential detergent extraction of EBs. For further details refer to Supplement Experimental Procedures section in the Supporting Information.

Adhesion of soluble recombinant proteins as well as of protein-coated beads was assessed by overlaying HEp-2 cells with culture medium containing the protein of interest (100 µg ml⁻¹), and bound protein was quantified by immunoblotting. For further details refer to Supplement Experi-

mental Procedures section in the Supporting Information. The effect of prior exposure to rCPn0473 on EB adhesion was measured by flow cytometric analysis of purified chlamydial EBs labelled with CFSE (Schnitger *et al.*, 2007).

To assess the effects of selected recombinant proteins on infectivity, HEp-2 cells were overlaid with culture medium containing the relevant protein prior to infection. Conversely, in another set of experiments, infectious EBs were pre-coated with selected recombinant proteins prior to infection. Infectivity was then quantified 50 hpi by the standard microscopy assay. The impact of selected antibodies on infectivity was measured in a similar way, after infection with EBs (MOI=20) that had been preincubated in preimmune serum or antiserum.

To quantify effects of recombinant proteins on EB internalization, cells were overlaid with cell culture media containing recombinant protein and infected with purified EBs (MOI=20). Internalization was quantified by qPCR using a previously described protocol (Kim *et al.*, 2011). For further details refer to Supplement Experimental Procedures section in the Supporting Information.

Surface biotinylation of *C. pneumoniae* EBs

The 1×10^7 purified infectious *C. pneumoniae* EBs were biotinylated with 5 mM Sulfo-NHS-Biotin for 2 hours on ice. EBs were lysed and incubated with Streptavidin-Agarose at 4°C overnight. The agarose was washed 5 times with PBS to remove unbiotinylated proteins, then resuspended in 150 µl PBS and analysed for bound (biotinylated) proteins by immunoblotting using anti-CPn0473, anti-Pmp21, anti-EF-Tu and anti-DnaK antibodies. As total protein lysate of 5×10^5 untreated EBs was used as an input control.

Disruption of endocytotic processes with chemical inhibitors

HEp-2 cells were incubated with different inhibitors prior to infection with purified EBs (MOI=20), or exposure to recombinant protein ($100 \mu\text{g ml}^{-1}$) and the same inhibitor. Infectivity was quantified 50 hpi as described in the preceding texts. For further details refer to Supplement Experimental Procedures section in the Supporting Information.

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Author's contribution

JHH conceived the study; TF and JNG performed, analysed, and interpreted the experiments. TF, JNG and JHH discussed the experiments and wrote the manuscript. The authors declare no competing financial interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. CPn0473 binds to human cells via a 50 aa region in its C-terminus.

Fig. S2. Detection of CPn0473 with the polyclonal anti-CPn0473 antibody.

Fig. S3. The rCPn0473- dependent boost of infection is mediated by its first 150 amino acids.

Fig. S4. Binding of *C. pneumoniae* is affected by cholesterol sequestering agents and recombinant CPn0473 boosts the infection by *C. pneumoniae* and *C. trachomatis* serovar E but not serovar L2.

Fig. S5. CTxB-positive domains are actin-dependent and targeted by rCPn0473.