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Chlamydia trachomatis-derived deubiquitinating enzymes in mammalian cells during infection

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Summary

Chlamydia trachomatis **is an obligate intracellular bacterium that causes a variety of diseases in humans.** ***C. trachomatis*** **has a complex developmental cycle that depends on host cells for replication, during which gene expression is tightly regulated. Here we identify two** ***C. trachomatis*** **proteases that possess deubiquitinating and deneddylating activities. We have designated these proteins** ***Chla*****Dub1 and** ***Chla*****Dub2. The genes encoding** ***Chla*****Dub1 and** ***Chla*****Dub2 are present in all** ***Chlamydia*** **species except for** ***Chlamydia pneumoniae*****, and their catalytic domains bear similarity to the catalytic domains of other eukaryotic ubiquitin-like proteases (Ulp). The** ***C. trachomatis*** **DUBs react with activity-based probes and hydrolyse ubiquitinated and neddylated substrates.** ***Chla*****Dub1 and** ***Chla*****Dub2 represent the first known bacterial DUBs that possess both deubiquitinating and deneddylating activities.**

Introduction

Chlamydia trachomatis is an obligate intracellular bacterium responsible for a number of human diseases. Despite its sensitivity to antibiotics, *C. trachomatis* remains a major cause of morbidity. *C. trachomatis* has a well-characterized biphasic developmental cycle. The infectious form of *C. trachomatis* is the elementary body (EB). After internalization into host cells the bacterium converts to the metabolically active reticulate body (RB). RBs replicate within a specialized vacuolar compartment known as the inclusion and they convert back to EBs around 24–36 h post infection (hpi). By 48 hpi the integrity of the infected cell is compromised and EBs are released into the extracellular space to begin a new round of infection and replication (Stephens, 1999).

Because *C. trachomatis* has an absolute dependence on the host cell for survival, it has developed many strategies to exploit host cells. *C. trachomatis* sequesters itself within the inclusion, yet it acquires nutrients such as ATP, ribonucleotides, amino acids and phospholipids from the cytosol and Golgi apparatus of the host cell (Stephens, 1999). Additionally, *C. trachomatis* can block phagolysosomal fusion with the inclusion, inhibit apoptosis, downregulate the levels of major histocompatibility complex (MHC) molecules, and remodel the host cell cytoskeleton (Fields and Hackstadt, 2002). To manipulate these host cell functions, *C. trachomatis* deploys into the host cell cytoplasm a number of virulence factors that can affect host cell processes, either directly or indirectly. These bacterial proteins must survive the degradative machinery of the host cell long enough so that they can act. One way by which *C. trachomatis* may manipulate the turnover of bacterial or host cell proteins is through alteration of key components of the ubiquitin-proteasome pathway.

Many cellular processes are under the control of the ubiquitin-proteasome pathway. These include cell cycle progression, transcriptional regulation, antigenic peptide production, receptor internalization and signal transduction. While addition of ubiquitin is coordinated through the activities of the E1, E2 and E3 enzymes (Glickman and Ciechanover, 2002), removal of ubiquitin is mediated by a large family of deubiquitinating enzymes (DUBs), also referred to as ubiquitin-specific proteases (USPs). DUBs for the most part are cysteine proteases and their functions may include the rescue of proteins from proteasome-mediated degradation or the reversal of ubiquitin-mediated signalling or trafficking (Amerik and Hochstrasser, 2004).

Expression of DUBs is not limited to eukaryotic cells and some pathogens produce DUBs, presumably to manipulate cellular functions to their own advantage. For example, the N-terminal portion of the Herpes Simplex Virus-1 (HSV-1) tegument protein, UL36, possesses deubiquitinating activity (Kattenhorn *et al.*, 2005), and this activity is conserved in the UL36 homologues found in murine cytomegalovirus and Epstein Barr virus (Schlieker *et al.*, 2005). YopJ, a *Yersinia* virulence factor, also has

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deubiquitinating activity that results in deubiquitination of IκB and hence inhibition of NF-κB signalling (Orth *et al*., 2000; Zhou *et al*., 2005). Because *C. trachomatis* completely depends on the host for survival and replication, we postulated that *C. trachomatis* might use cellular DUBs or express its own DUBs in order to regulate the function of host and/or bacterial proteins.

Ubiquitin-based probes react specifically with the active-site cysteine of DUBs (Borodovsky *et al*., 2001; 2002; Misaghi *et al*., 2005) and can be used to identify new DUBs or measure their activities (Hemelaar *et al*., 2004a; Galardy *et al*., 2005; Kattenhorn *et al*., 2005; Schlieker *et al*., 2005). Using such probes, we here report the identification of two DUBs produced by *C. trachomatis*, *Chla*Dub1 and *Chla*Dub2, which are encoded by open reading frames (ORFs) CT868 and CT867 respectively (Stephens *et al*., 1998). When expressed in mammalian cells or produced by *in vitro* translation, *Chla*Dub1 and *Chla*Dub2 reacted with probes designed to bind deubiquitinating and deneddylating enzymes, suggesting that they function as deubiquitinating and deneddylating enzymes. In addition, *Chla*Dub1 and *Chla*Dub2 were capable of hydrolysing ubiquitinated and neddylated substrates.

Results

Chlamydia trachomatis *infection induces a protein that binds a probe specific for deubiquitinating enzymes*

To address whether *C. trachomatis* alters the expression of DUBs within infected cells, we used HA-UbVME (ubiquitin-vinylmethylester), an electrophilic ubiquitin derivative. We have used these activity-based probes to identify cellular and viral DUBs (Borodovsky *et al*., 2002; Schlieker *et al*., 2005). HeLa cells were either mock infected or infected with *C. trachomatis* for 0 or 24 h. Lysates of cells were reacted with HA-UbVME and, after resolution by SDS-PAGE, were analysed by immunoblotting with an anti-HA antibody (Fig. 1A). In the absence of the HA-UbVME probe, a 75 kDa polypeptide was detected by the anti-HA antibody in uninfected cells. After 24 h of *C. trachomatis* infection, this species appeared as a 40 kDa polypeptide (Fig. 1A, left panel and data not

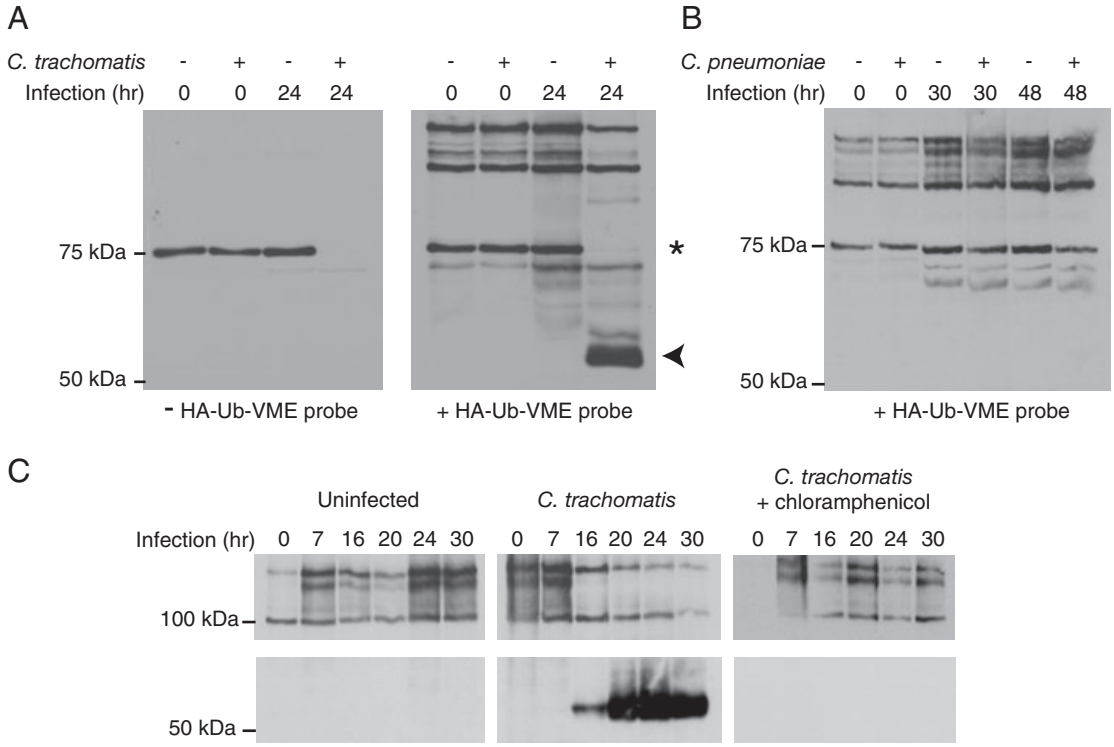

Fig. 1. Detection and expression profile of a *C. trachomatis*-induced DUB.
A. HeLa cells were either infected with *C. trachomatis* or left untreated and the levels and activity of DUBs were assessed using HA-UbVME. Uninfected and infected cells were collected at 0 or 24 hpi, lysed, and either left untreated (left panel) or treated with HA-UbVME (right panel), followed by SDS-PAGE (8%) and immunoblotting (using the anti-HA antibody).
B. HeLa cells were infected with *C. pneumoniae* or left untreated and cell lysates were collected at 0, 30 and 48 hpi and treated with HA-UbVME as in (A).
C. Uninfected cells and *C. trachomatis* infected cells, which were left either untreated or treated with chloramphenicol (100 μg ml^{-1} final) 3 hpi, were collected at the indicated time points. Cells were lysed and treated with HA-UbVME followed by SDS-PAGE and immunoblotting as in (A). The *C. trachomatis*-induced DUB is indicated by an arrowhead. The asterisks depict the background bands.

shown). The detection of these polypeptides was obviously independent of HA-UbVME and the labelling of these polypeptides is therefore considered non-specific. Labelling of cell lysates with HA-UbVME resulted in detection of several active cellular DUBs and a prominent *C. trachomatis*-induced probe-adduct (~55 kDa) at 24 hpi (Fig. 1A, right panel). When HA-UbVME labelling was performed in the presence of the alkylating agent N-ethylmaleimide (NEM), reactivity of the probe with all cellular DUBs and the *C. trachomatis*-induced protein was blocked (data not shown). These results suggested that the prominent HA-UbVME-reactive protein detected in *C. trachomatis*-infected cells was a DUB. We also detected a number of other HA-UbVME-reactive proteins in *C. trachomatis*-infected cells, albeit at lower intensity.

To determine whether infection of cells with other *Chlamydia* species induced expression of the 55 kDa DUB, HeLa cells were either mock infected or infected with *Chlamydia pneumoniae* for 30 or 48 h. These late time points were chosen to compensate for the longer developmental cycle of *C. pneumoniae* (48–72 h) as compared with *C. trachomatis* (36–48 h) (Stephens, 1999). Infected or mock-infected cells were then analysed as above. Unlike *C. trachomatis*, infection of HeLa cells with *C. pneumoniae* did not alter the pattern of DUB labelling when compared with mock-infected cells (Fig. 1B). Even after 72 h of infection with *C. pneumoniae*, we failed to observe the 55 kDa HA-UbVME-reactive protein induced upon *C. trachomatis* infection (data not shown). The significance of this finding will be revisited below.

The complex developmental cycle of *C. trachomatis* requires tight temporal control of gene expression over time. We therefore examined when in the course of infection we could first detect this *C. trachomatis*-induced DUB. The 55 kDa DUB was first detected 16 hpi (Fig. 1C, middle panel) and the levels and/or activity of this DUB increased for the remainder of the *C. trachomatis* developmental cycle. When a similar time course experiment was performed in the presence of chloramphenicol, an antibiotic that inhibits bacterial but not host protein synthesis, the *C. trachomatis*-induced DUB could no longer be detected (Fig. 1C, right panel). We found no significant changes in labelling of the cellular DUBs with HA-UbVME, even in the presence of chloramphenicol. These results establish that the *C. trachomatis*-induced DUB is a bacterial protein and that its expression requires active bacterial protein synthesis.

Affinity purification and mass spectrometry identifies the deubiquitinating enzyme as a C. trachomatis *gene product*

To determine the identity of the *C. trachomatis*-induced DUB, we labelled lysates from 150×10^6 infected cells with HA-UbVME and isolated this DUB from the *C. trachomatis*-infected cell by affinity purification (Kattenhorn *et al*., 2005). The immuno-purified *C. trachomatis*-induced DUB was resolved by SDS-PAGE and visualized by Coomassie staining (data not shown). The polypeptide corresponding to the *C. trachomatis*-induced DUB was excised from the gel, digested *in situ* with trypsin and the released peptides were analysed by mass spectrometry. Four peptides derived from a single *C. trachomatis* ORF, CT868, were identified by mass spectrometry (data not shown). The CT868 ORF was predicted to be a membrane thiol protease of approximately 47 kDa (Stephens *et al*., 1998). Based on sequence alignment with known thiol proteases, its catalytic residues likely include H288, D305 and C358. These results establish that CT868 is the *C. trachomatis*-induced DUB we observed in our experiments. The difference in size between the predicted molecular weight (MW) of the CT868 gene product (~47 kDa) and that estimated for the *C. trachomatis*-induced DUB (~55 kDa) detected by labelling with HA-UbVME, closely corresponds to covalent modification of the CT868 protein (~47 kDa) with a single HA-UbVME entity (~8 kDa).

Sequence alignment showed that the CT868 ORF had similarity to ORFs across several different species, strains and serovars within the *Chlamydia* genus, including *C. trachomatis*, *C. muridarum* and *C. psittaci* (Fig. 2 and data not shown). The only pathogenic species that lacked a homologue of CT868 was *C. pneumoniae*, for which no HA-UbVME-labelled specific fragments were detected in infected cells (Fig. 1B).

An ORF adjacent to CT868 in the *C. trachomatis* genome sequence, CT867, is homologous to CT868 and had been predicted to be a membrane thiol protease as well (Stephens *et al*., 1998). The CT867 ORF was also conserved across all different strains and serovars of *Chlamydia*, again with the exception of *C. pneumoniae* (Fig. 2 and data not shown). Furthermore, the predicted catalytic domains of CT868 and CT867 proteins share similarity with the Ulp domain of SUMO-specific proteases (SENPs) (Fig. 2), a family of proteases that possess either deneddylating or desumoylating activities (Hay, 2005). The predicted catalytic His, Asp, Cys and Gln (contributing to the formation of the oxyanion hole) residues of CT868 and CT867 are conserved among all SENPs (Fig. 2). As a result of these findings, we designated the CT868 and CT867 ORFs as the *C. trachomatis* genes *cdu1* and *cdu2*, which encode proteins that we refer to as *Chla*Dub1 and *Chla*Dub2 respectively.

Chla*Dub1 is a deubiquitinating and deneddylating enzyme*

To verify that the protein encoded by *cdu1* indeed

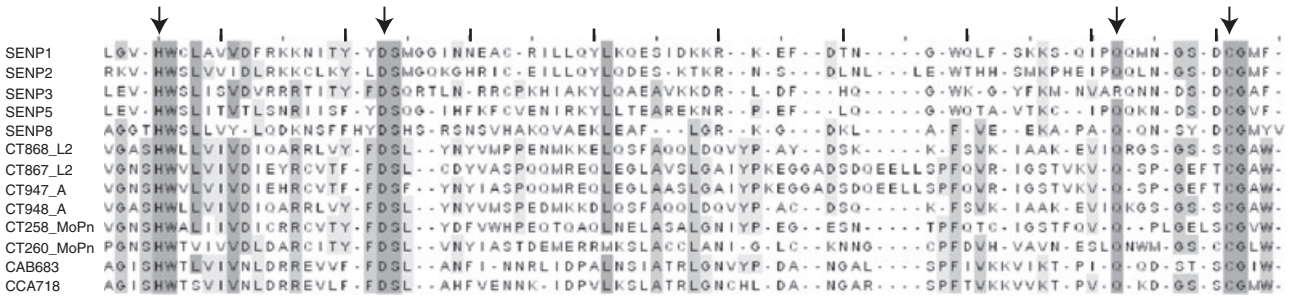

Fig. 2. Sequence alignment of the *C. trachomatis*-induced DUB. Sequence alignment analysis revealed that the predicted product of ORF CT868 shares similarity with both the Ulp domain of SENPs and the catalytic domain of the predicted product of CT867. In addition, the genes encoded by CT868 and CT867 are conserved among all strains and species of the *Chlamydia* genus, except for *C. pneumoniae*. Regions of similarity are shown in grey where the degree of similarity directly correlates with increasing shades of grey. The H, D, C and the oxyanion hole (Q), depicted by arrows, are the predicted catalytic residues. L2 and A belong to the species *trachomatis*, MoPn belongs to the species *muridarum*, CAB and CCA belong to the species *psittaci*.

possesses DUB activity, we transiently transfected HeLa cells with a HA-tagged *cdu1* construct. After 24 h the cells were lysed and reacted with the UbVME probe in the presence or absence of NEM. Immunoblotting analysis of these samples using an anti-HA antibody showed that the recombinant *Chla*Dub1-HA protein reacted with UbVME, as revealed by an increase of ~8 kDa in its MW (Fig. 3A, left panel). Consistent with its activity as a cysteine protease, reactivity of *Chla*Dub1-HA with UbVME was sensitive to inclusion of NEM. To confirm that the C358 residue is the active-site cysteine, we changed cysteine 358 to an alanine by site-directed mutagenesis. Unlike the wild-type protein, the C358A mutant protein failed to react with UbVME (Fig. 3A, middle panel). We detected two HA-reactive polypeptides in HeLa cells expressing the C358A mutant of *Chla*Dub1-HA, with the smaller species having the same MW as the wild-type CT868 protein (as seen by comparing the left and middle panels of Fig. 3A). Because the MW of *Chla*Dub1-HA corresponds to the polypeptide with lower MW, the larger polypeptide may have arisen through as yet unidentified covalent modification(s) of the smaller polypeptide, in a manner that is independent of HA-UbVME.

Reactivity of *Chla*Dub1-HA with HA-UbVME was confirmed using a cell-free *in vitro* transcription/translation system. ^{35}S-labelled *Chla*Dub1-HA was reacted with the HA-UbVME probe in the presence or absence of NEM and immunoprecipitated using a HA-specific antibody. We observed reactivity of the *in vitro*-translated wild-type CT868 protein with the HA-UbVME probe (Fig. 3A, right panel). The mutant *Chla*Dub1(C358A)-HA protein failed to react with HA-UbVME (data not shown). Consistent with our observations using transient transfection, two polypeptides corresponding to the *Chla*Dub1(C358A)-HA protein were detected.

Several other ubiquitin-like proteins such as Nedd8, SUMO and ISG15 may be covalently linked to target proteins and alter their stability and/or function (Jentsch and Pyrowolakis, 2000). A number of proteases, previously believed to be ubiquitin-specific, can in fact recognize some of these ubiquitin-like modifiers. Without exception, reaction of proteases with electrophilic probes derived from ubiquitin or ubiquitin-like modifiers has perfectly correlated with their enzymatic activity. To test whether *Chla*Dub1 was capable of recognizing other ubiquitin-like proteins, we examined its ability to react with three other ubiquitin-like activity-based probes, SUMO-VME, Nedd8-VS and ISG15-VME (Hemelaar *et al*., 2004b). The *in vitro* expressed *Chla*Dub1-HA protein reacted not only with UbVME but also with Nedd8-VS and did so in a NEM-sensitive manner (Fig. 3B). We did not observe any interactions of *Chla*Dub1 with SUMO-VME or ISG15-VME (Fig. 3B), while these probes did form adducts with known proteases (Hemelaar *et al*., 2004b).

To establish enzymatic activity of *Chla*Dub1 by an independent method, we tested the ability of purified *Chla*Dub1-HA to hydrolyse the fluorogenic substrates ubiquitin-7 amino-4-methylcoumarin (Ub-AMC) and Nedd8-AMC. We expressed *Chla*Dub1-HA in 293T cells by transient transfection and purified the protein using anti-HA-conjugated beads. Purified *Chla*Dub1-HA was then incubated with Ub-AMC or Nedd8-AMC and substrate hydrolysis was measured by increase in fluorescence release (Fig. 3C). Like UCH-L3, the only presently known DUB with dual specificity for ubiquitin and Nedd8 (Wada *et al*., 1998; Hemelaar *et al*., 2004b), *Chla*Dub1-HA hydrolysed both Ub-AMC and Nedd8-AMC. As a control, proteins purified from 293T cells transfected with the pcDNA3.1+ vector alone did not show any deubiquitinating or deneddylating activities (denoted in the figure as 'Mock'). Combined, these experiments establish that *Chla*Dub1, encoded by the *C. trachomatis cdu1* gene, possesses deubiquitinating and deneddylating activities.

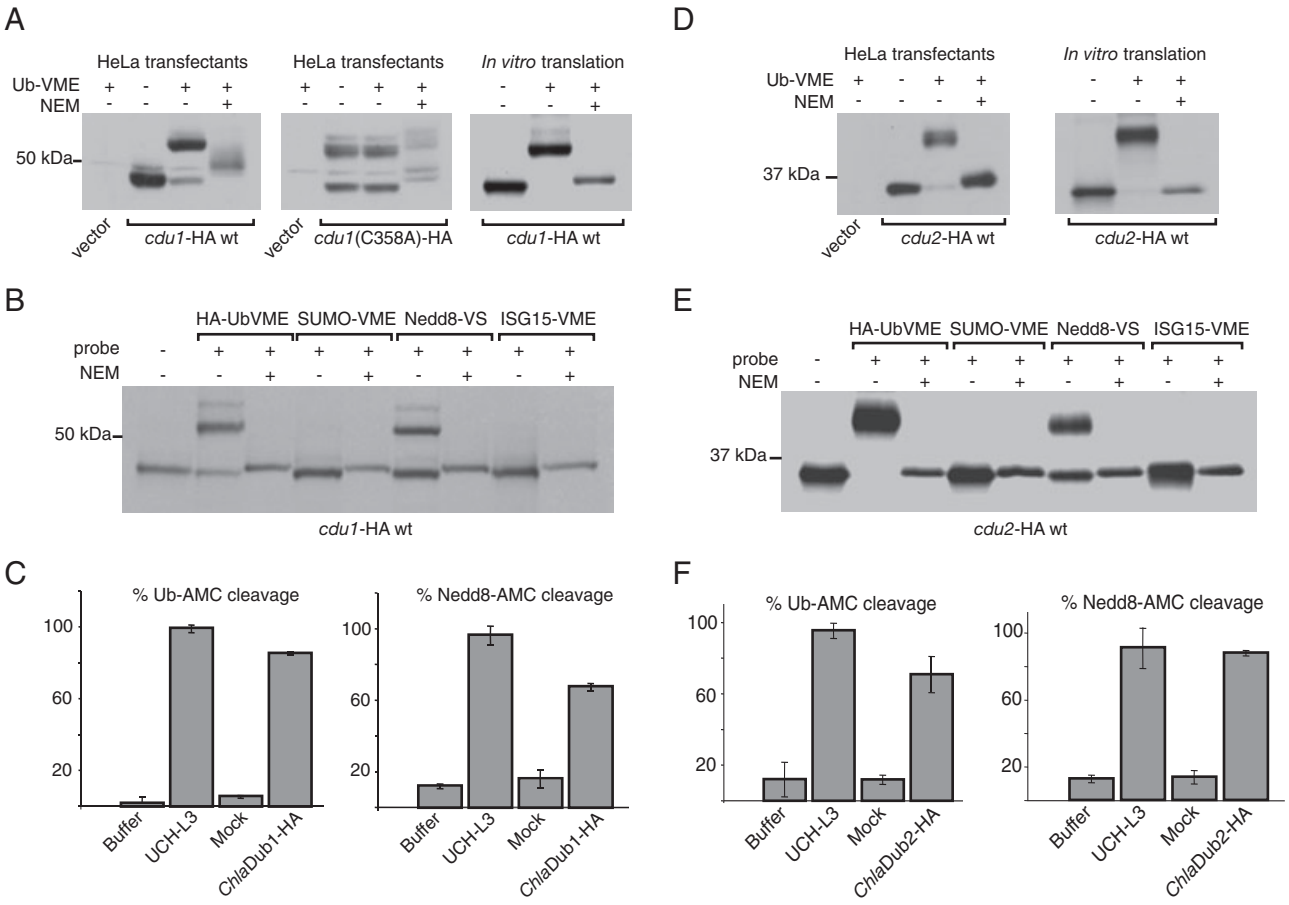

Fig. 3. *Chla*Dub1 and *Chla*Dub2 are deubiquitinating and deneddylating enzymes.
A. Wild type (left panel) or C358A (middle panel) *Chla*Dub1-HA was expressed in HeLa cells and cell lysates were labelled with UbVME in the presence or absence of NEM, followed by SDS-PAGE (9%) and immunoblotting with the anti-HA antibody. *Chla*Dub1(C358A) is expressed as a doublet and is discussed in the text. Vector alone (pcDNA3.1+) was used as a control. Right panel, wild-type ^{35}S-labelled *Chla*Dub1-HA was expressed *in vitro* and reacted with HA-UbVME. The labelled proteins were immunoprecipitated with the HA-specific 12CA5 antibody and subjected to SDS-PAGE followed by autoradiography.
B. Reactivity of *Chla*Dub1 with other ubiquitin-like probes was tested by *in vitro* transcription and translation as explained above.
C. Immuno-affinity purified *Chla*Dub1-HA was tested for its ability to cleave Ub-AMC (over 3 h) and Nedd8-AMC (over 4 h). UCH-L3 was used as a positive control, while the eluate from 293T cells transfected with pcDNA3.1+ was used as the negative control (mock). Experiments were performed in triplicate and all values have been normalized against the highest value in the positive control.
D. Wild-type *Chla*Dub2-HA was expressed in HeLa cells (left panel) and ^{35}S-labelled *Chla*Dub2-HA was expressed *in vitro* (right panel). Samples were processed further as in (A).
E. Reactivity of *Chla*Dub2 with other ubiquitin-like probes was tested by *in vitro* transcription and translation.
F. Immuno-affinity purified *Chla*Dub2-HA was tested for its ability to cleave Ub-AMC and Nedd8-AMC (over 5 h) as in (C).

The catalytically inactive but not the active version of *Chla*Dub1 manifests itself as a prominent doublet on SDS-PAGE (Fig. 3A, middle panel). Autocatalytic removal of either ubiquitin or Nedd8 by the active enzyme could, in principle, account for this difference. We were unable to detect either ubiquitin or Nedd8 modifications when we immunoprecipitated these polypeptides and probed them with anti-ubiquitin or anti-Nedd8 antibodies (data not shown), nor did addition of purified *Chla*Dub1-HA to the *in vitro* expressed *Chla*Dub1(C358A)-HA result in disappearance of the higher MW species (data not shown). Currently, we have no explanation for the origin(s) of these higher MW species.

Chla*Dub2 also has deubiquitinating and deneddylating activities*

Because the gene adjacent to *cdu1* in the genome, *cdu2*, encodes a protein (~37 kDa) with sequence similarity to *Chla*Dub1, we examined whether the *Chla*Dub2 protein also possesses deubiquitinating or deneddylating activity. We transiently transfected HeLa cells with a construct that encodes *Chla*Dub2-HA. Cell lysates were reacted with the UbVME probe, followed by immunoblot analysis using an anti-HA antibody. Like *Chla*Dub1, *Chla*Dub2-HA also reacted with the UbVME probe in a NEM-sensitive manner, resulting in ~8 kDa increase in its MW (Fig. 3D, left

panel). These results suggested that the *C. trachomatis* *Chla*Dub2 protein is also a DUB. ^{35}S-labelled *Chla*Dub2-HA, expressed by *in vitro* transcription and translation, also reacted with the HA-UbVME probe in a NEM-sensitive manner (Fig. 3D, right panel).

*Chla*Dub2-HA protein also reacted with both Nedd8-VS and HA-UbVME, while it did not show any reactivity toward SUMO-VME or ISG15-VME (Fig. 3E). To further confirm deubiquitinating activity of *Chla*Dub2, we immuno-affinity purified this protein from 293T cells and examined its ability to cleave Ub-AMC and Nedd8-AMC. Both purified *Chla*Dub2-HA protein and UCH-L3 (positive control) successfully cleaved Ub-AMC and Nedd8-AMC, while the mock control (proteins purified from 293T cells transfected with the pcDNA3.1+ vector alone) failed to cleave these substrates (Fig. 3F). Our results thus show that the *C. trachomatis cdu2* gene encodes a second protein, *Chla*Dub2, that can function, in principle, as a deubiquitinating and deneddylating enzyme.

Our inability to detect *Chla*Dub2 in the initial studies in which infected cell lysates were labelled with HA-UbVME (Fig. 1) may suggest that *Chla*Dub2 is expressed at lower levels during *C. trachomatis* infection. Alternatively, *Chla*Dub1 may have higher affinity than *Chla*Dub2 for the HA-UbVME probe. *Chla*Dub1 may thereby sequester much of the available HA-UbVME, limiting detection of other DUBs, including *Chla*Dub2, in the infected cell lysate. Nevertheless, our studies using either cells transfected with *Chla*Dub2 or *in vitro* translated *Chla*Dub2 (Fig. 3D–F) suggest that this enzyme does, indeed, possess deubiquitinating and deneddylating activities.

Discussion

We have identified two *Chlamydia*-specific DUBs, encoded by ORFs CT868 (*cdu1*) and CT867 (*cdu2*), that we refer to as *Chla*Dub1 and *Chla*Dub2 respectively. *Chla*Dub1 was isolated from *C. trachomatis*-infected cells using HA-UbVME and was then identified by mass spectrometry as the product of ORF CT868. When expressed in cells or by *in vitro* translation, *Chla*Dub1 reacted with both Ub-VME and Nedd8-VS, which specifically trap active deubiquitinating and deneddylating enzymes respectively. Partially purified *Chla*Dub1 cleaved Ub-AMC and Nedd8-AMC, establishing that *Chla*Dub1 possesses intrinsic deubiquitinating and deneddylating activities. *Chla*Dub2 also reacted with both Ub-VME and Nedd8-VS probes and cleaved Ub-AMC and Nedd8-AMC in a cell-free assay. These findings suggest that *C. trachomatis* possesses at least two enzymes that can function to deubiquitinate or deneddylate target proteins, at least as judged by the above criteria. Physiological substrates for *Chla*Dub1 and *Chla*Dub2 remain to be identified.

Ubiquitin modification of proteins is a process restricted to eukaryotes. Consequently, all of the enzymes that act on ubiquitin were believed to be confined to eukaryotes as well. The only currently known exception among the prokaryotes is the YopJ protein encoded by *Yersinia pseudotuberculosis*, originally suggested to be a SUMO-specific protease (Orth *et al*., 2000), and more recently characterized as a USP (Zhou *et al*., 2005). YopJ activity inhibits a NF-κB-dependent inflammatory response by deubiquitinating proteins such as TRAF2, TRAF6 and IκB. Viruses also possess DUB-like activities, as reported first for Adenoviruses (Balakirev *et al*., 2002) and more recently for Herpes (Kattenhorn *et al*., 2005; Schlieker *et al*., 2005) and Corona (Barretto *et al*., 2005; Lindner *et al*., 2005) viruses. The occurrence of viral examples is easily understood in the context of the strict reliance of viruses on host cell machinery for their successful propagation. It therefore is not surprising that *Chlamydia*, with an intracellular replication cycle, has managed to modulate the host ubiquitin-proteasome pathway through expression of its own USPs. The fact that all known ubiquitin- or Nedd8-modified proteins carry this modification in the topological equivalent of the cytoplasm suggests that the active forms of *Chla*Dub1 and *Chla*Dub2 should somehow be able to access this compartment.

We have tried to demonstrate directly cytosolic localization of *Chla*Dub1 and *Chla*Dub2 during *C. trachomatis* infection. However, attempts to express and purify recombinant protein in *Escherichia coli* and other expression systems have proven challenging. We also generated antibodies against peptides from *Chla*Dub1 and attempted to use them in immunofluorescence studies to demonstrate localization of endogenous *Chla*Dub1 in *C. trachomatis*-infected cells. However, these antibodies were not able to recognize native *Chla*Dub1. Finally, although cytosolic localization of bacterial proteins can often be addressed through cellular fractionation approaches, these studies are confounded when *C. trachomatis*-infected cells are used. Because the *C. trachomatis* vacuole is fragile and easily disrupted, bacterial organisms contaminate fractions of the host cytosol even after lysis in mild detergents.

Nedd8 is an ubiquitin-like protein that regulates the function of cullins, whose activity is enhanced through neddylation. Cullins participate in formation of SCF-E3 ubiquitin ligases, implicated in ubiquitination of a number of cellular proteins involved in diverse processes, including cell cycle control (Willems *et al*., 2004). COP9/signalosome (CSN), a multiprotein complex, mediates removal of Nedd8 from cullins (Lyapina *et al*., 2001). A number of intestinal bacteria, including *Salmonella* and enteropathogenic *E. coli*, interfere with neddylation of cullin-1 by an as yet unknown mechanism (Collier-Hyams *et al*., 2005). This results in inactivation of cullin-1 and

therefore stabilization of IκB, which leads to inhibition of NF-κB. Our results suggest the possibility that ubiquitin-specific or Nedd8-specific proteases of *Chlamydia* could likewise take on such a role. Unfortunately, uncovering the role of *Chla*Dub1 and *Chla*Dub2 during *C. trachomatis* infection is challenging in the absence of tools to genetically manipulate *Chlamydia*. Structural analysis of *C. trachomatis* DUBs may allow the design of inhibitors of *Chla*Dub1 and *Chla*Dub2 that could help define their role during *C. trachomatis* infection.

Infection of human endothelial cells with *C. pneumoniae* activates NF-κB, while infection with *C. trachomatis* prevents NF-κB activation (Molestina *et al*., 2000). If deubiquitinating or deneddylating activities of the *C. trachomatis* DUBs result in suppression of NF-κB signalling, then their absence from *C. pneumoniae* may account for its inability to block NF-κB activation. In addition, *C. trachomatis* inhibits host cell apoptosis, an effect correlated with *C. trachomatis*-induced degradation of proapoptotic proteins in a proteasome-dependent manner (Fan *et al*., 1998; Fischer *et al*., 2004). This process may also be regulated by *Chla*Dub1 or *Chla*Dub2 during *C. trachomatis* infection. Other functions that rely on modulation of the levels of ubiquitin-modified proteins, such as protein degradation, vesicular trafficking and multivesicular body formation, remain obvious possibilities as well. For example, recognition of *C. trachomatis*-infected cells by T lymphocytes depends on degradation and processing of bacterial proteins for presentation on host MHC molecules. One possible function of *Chla*Dub1 and *Chla*Dub2 might be to protect proteins secreted by *C. trachomatis* from the antigen degradation and processing pathways, thereby limiting T cell recognition.

While we have yet to determine the role of the *Chlamydia* DUBs during infection, some insight may be gained by examining their conservation across different strains and species of the *Chlamydia* genus. Homologues of *Chla*Dub1 and *Chla*Dub2 are present within all strains of *C. trachomatis* (ocular, genital and lymphogranuloma venereum) as well as within the genomes of *C. muridarum* and *C. psittaci* (Fig. 2). Furthermore, sequence alignment analysis shows that the catalytic domain of *C. trachomatis* DUBs shares similarity with the Ulp domain found in SENPs (Fig. 2). Remarkably, no homologues of these proteins exist within the *C. pneumoniae* genome, which is consistent with our observation that a *Chlamydia*-induced DUB was absent from *C. pneumoniae*-infected cells (Fig. 1B). However, the *C. pneumoniae* genome apparently encodes a cysteine protease from the Otubain (OTU)-like family of proteins that is absent from other *Chlamydia* species (Makarova *et al*., 2000). A few members of the OTU-family of proteases possess deubiquitinating activity (Balakirev *et al*., 2003; Nanao *et al*., 2004) and it is plausible that the OTU-protease, encoded by *C. pneumoniae*, serves a function analogous to that of DUBs in *C. trachomatis*.

Experimental procedures

Cell culture, Chlamydia *propagation and infection*

HeLa cells and 293T cells were cultured in DMEM and the murine fibroblast (McCoy) cell line was grown in Eagle's MEM (Steele *et al*., 2004). EBs of *C. trachomatis* serovar L2434/Bu were propagated in McCoy cell monolayers as previously described (Howard *et al*., 1974), aliquoted and stored at −80°C in SPG buffer (250 mM sucrose, 10 mM sodium phosphate and 5 mM L-glutamic acid, pH 7.2). Aliquots of *C. pneumoniae* serovar/strain K6 were generously provided by Benjamin Wizel, University of Texas at Tyler. HeLa cells were infected with *C. trachomatis* or *C. pneumoniae* by adding EBs diluted in SPG buffer to cells and centrifuging at 37°C for 1 h (2000 *g*). The multiplicity of infection (MOI) of 10:1 and 4:1 were used for *C. trachomatis* and *C. pneumoniae* respectively. At these MOIs, 85–90% of cells were infected with *C. trachomatis* and 20–30% were infected with *C. pneumoniae*, as assayed by immunfluorescence microscopy. Increasing the MOI above 4:1 for *C. pneumoniae* did not enhance infection efficiency. Chloramphenicol was added (100 μg ml^{-1} final) 3 h after infection, for the duration of the experiment. SPG buffer alone was used for mock infection.

Plasmid construction, transfection and antibodies

The *cdu1* and *cdu2* genes were amplified from *C. trachomatis* L2 genomic DNA using the following primers:

cdu1 forward:
5′CCGGAATTCGCCATGTTGTCTCCCACCAACTCAAC3′
cdu1 reverse:
5′CCGCTCGAGGAAAAGAGCTTTTGCTTCAGGCC3′
cdu2 forward:
5′CATGGGATCCGCCATGGAACCAATTCATAATCCTCC3′
cdu2 reverse:
5′CCGCTCGAGATCCGTAGTTGGCCAGCTC3′

Amplified inserts were cloned into the EcoRI and XhoI sites of pcDNA3.1+ bearing a C-terminal HA-tag and stop codon between the XhoI and XbaI sites (Misaghi *et al*., 2004) to generate *cdu1*-HA and *cdu2*-HA. *cdu1*(C358A)-HA was generated by site-directed mutagenesis (QuikChange, Stratagene).

cdu1(C358A) forward:
5′TCCGGATCCAGCGCCGGCGCTTGGTGC3′
cdu1(C358A) reverse:
5′GCACCAAGCGCCGGCGCTGGATCCGGA3′

HeLa and 293T cells were transiently transfected with Fugene 6 (Roche) and *Trans*IT-293T (Mirus Bio). 12CA5 (Roche) is a mouse anti-HA monoclonal antibody.

HA-UbVME labelling, large-scale purifications and mass spectrometry

Labelling of cell lysates (24 hpi) with HA-UbVME and large-

scale purification of the labelled DUBs were performed as described (Kattenhorn *et al.*, 2005). The band corresponding to the *C. trachomatis*-induced DUB was excised from the gel and digested with trypsin. An aliquot of the resulting extract was analysed by tandem mass spectrometry using a Waters CapLC XL HPLC coupled to a Micromass micro Q-TOF mass spectrometer. The resulting data were analysed using MASCOT to determine the protein identities. TNT-Quick (Promega) kit was used as instructed for *in vitro* expression of ^{35}S-labelled *Chla*Dub1 and *Chla*Dub2. Twenty microlitres of reticulocyte lysate/sample were reacted with HA-UbVME or other ubiquitin-like probes (NEM, 10 mM final, added 15 min prior to addition of the probe). Samples were diluted with 1 ml of NP40 buffer (0.5%), immunoprecipitated using anti-HA antibody, and subjected to SDS-PAGE and autoradiography.

Immunopurification of Chla*Dub1 and* Chla*Dub2 and Ub-AMC or Nedd8-AMC assay*

*Chla*Dub1 and *Chla*Dub2 were purified from transiently transfected 293T (30×10^6 cells), using pcDNA3.1+ as a negative control. Cells were lysed in 30 ml of NP40 buffer, and 140 µl of 12CA5-conjugated beads (50% slurry) were incubated with the lysate overnight. Beads were washed first with NP40 buffer and then with 50 mM Tris pH 8/100 mM NaCl. The proteins were eluted from the beads using 300 µl of anti-HA peptide (2 mg ml^{-1}) in 50 mM Tris pH 8/100 mM NaCl. Functionality of *Chlamydia*-expressed DUBs and absence of other cellular DUBs were confirmed by reacting the eluted *Chla*Dub1 and *Chla*Dub2 with HA-UbVME, followed by SDS-PAGE and immunoblot (data not shown). Ub-AMC or Nedd8-AMC (Boston Biochem) were used at a final concentration of 100 nM, diluted in 200 µl of buffer A (50 mM HEPES, 0.5 mM EDTA, pH 7.5 and 1 mM DTT) containing the partially purified DUBs. Purified UCH-L3 was used as a positive control (0.4 nM final), and partially purified proteins from cells transfected with pcDNA3.1+ were used as the negative control. Five to 30 µl (based on relative concentration) of partially purified *Chla*Dub1 or *Chla*Dub2 were used in each assay and data were collected as indicated. All experiments were performed in triplicate.

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