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The *Chlamydia trachomatis* CT149 protein exhibits esterase activity *in vitro* and catalyzes cholesteryl ester hydrolysis when expressed in HeLa cells

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Abstract

Chlamydia, like other intracellular bacteria, are auxotrophic for a variety of essential metabolites and obtain cholesterol and fatty acids from their eukaryotic host cell, however not many *Chlamydia*-specific enzymes have been identified that are involved in lipid metabolism. *In silico* analysis of one candidate *C. trachomatis* enzyme, annotated as a conserved putative hydrolase (CT149), identified two lipase/esterase GX SXG motifs, and a potential cholesterol recognition/interaction amino acid consensus (CRAC) sequence. His-tag purified recombinant CT149 exhibited ester hydrolysis activity in a nitrophenyl acetate-based cell-free assay system. When cholesteryl linoleate was used as substrate, ester hydrolysis occurred and production of cholesterol was detected by high performance liquid chromatography. Exogenous expression of transfected CT149 in HeLa cells resulted in a significant decrease of cytoplasmic cholesteryl esters within 48 hrs. These results demonstrate that CT149 has cholesterol esterase activity and is likely to contribute to the hydrolysis of eukaryotic cholesteryl esters during intracellular chlamydial growth.

Keywords

Chlamydia; lipids; cholesterol esterase; cholesteryl esters

1. Introduction

Chlamydia trachomatis is an important human pathogen responsible for ocular and genital infections. *C. trachomatis* serovars D-K are the most common bacterial sexual transmitted diseases world-wide with more than a million new reported cases in 2010 in the USA alone (STD surveillance report 2010, CDC website: <http://www.cdc.gov/std/stats10/>).

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C. trachomatis is an obligate intracellular bacterial pathogen with a unique biphasic developmental cycle characterized by an extracellular infectious, but metabolically inert elementary body (EB), and an intracellular metabolically active, but non-infectious reticulate body (RB). In eukaryotic host cells chlamydiae are found in a membrane-surrounded vesicle called the inclusion, which is non-fusogenic with lysosomes [1]. After numerous rounds of RB replication differentiation of RBs back to EBs occurs, releasing infectious forms to invade additional host cells.

Like other strict obligate intracellular organisms, *Chlamydia* lack genes that code for a number of important metabolic enzymes, making them dependent on their host cell for key essential metabolites [2–8]. For example, *Chlamydia* acquire a subset of essential lipids directly from their host cell, while they are able to synthesize others independently. *Chlamydia* can synthesize phospholipids *de novo* that are typically found in prokaryotes (e.g. phosphatidylethanolamine [PE], phosphatidylglycerol [PG], and phosphatidylserine [PS]). They also utilize lipids normally associated with eukaryotes, including phosphatidylcholine [PC], phosphatidylinositol [PI], sphingomyelin [SM], and cholesterol [C], which they may salvage directly from host cell stores [9]. In eukaryotic cells lipids are acquired either by receptor-mediated uptake mechanisms (e.g. LDL-receptor or the scavenger receptor B (CD36)), or are synthesized *de novo* in mitochondria (PG, cardiolipin), the Golgi apparatus (SM), or endoplasmic reticulum (PC, PE, PS, PI, C). Previous studies have shown that *C. trachomatis* obtain lipids from various intracellular sources. For example, lipid-containing vesicles may be directly transported to inclusions where lipids are deposited in the vesicle lumen [10, 11]. The exocytic pathway is utilized by *Chlamydia* to obtain Golgi-derived sphingomyelin in this way [12]. In addition, late endosomes or multivesicular bodies from the host cell cytoplasm have been shown to be re-routed and directed to the inclusion [13]. Also, a Brefeldin A-independent Golgi stack transport to the inclusion has been reported [9, 14].

We hypothesize that *Chlamydia* possesses a panel of proteins that can interact and metabolize eukaryotic lipids within the chlamydiae, or even in the host cell during the developmental cycle. Some potential proteins have been annotated by comparison to other bacterial species. However the majority of proteins have not been tested in a biochemical assay to determine their activities. A tractable genetic system was not available to directly manipulate *Chlamydia* gene function until recently [15, 16] and gene transfer approaches have not been applied to *Chlamydia* functional studies. Therefore using bioinformatics software to search for specific motifs is one effective way to predict potential functions of chlamydial proteins. We conducted *in silico* analysis on the putative chlamydial hydrolase (CT149) and used *in vitro* biochemical assays with a His-tag purified recombinant protein and expression of CT149 in HeLa cells to confirm cholesterol esterase activity. Not only will these results contribute to the identification of chlamydial protein functions and will further help to understand the interaction of chlamydiae with their host cell during the developmental cycle. Our results may also help to develop strategies to treat or even prevent chlamydial infections using the for *Chlamydia* important lipid metabolism in chlamydiae as target for drugs.

2. Material and Methods

2.1. Chemicals

Unless stated otherwise all chemicals were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Cell culture and bacterial strains

The human epithelial cell line Hela 229 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle medium (DMEM, Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum, 0.01 mg/ml Gentamicin (Gibco, Grand Island, NY), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM Hepes and 0.055 mM β -mercaptoethanol at 37°C with 5% CO₂ in a humidified atmosphere. *C. trachomatis* serovar D was routinely grown in Hela cells at 37°C, 7% CO₂ and stocks were purified as previously described [17]. *E. coli* Rosetta blue (Novagen, Madison, WI) was cultured at 37°C with agitation in Luria Bertani (LB)-medium and 50 μ g/ml kanamycin (Kan) when appropriate.

2.3. Plasmid construction

For N-terminal His-tag fusion protein expression, full length *ct149* (a putative hydrolase, Genbank ID AAC67740.1) was cloned into the bacterial expression vector pET30a (+) (Novagen, Madison, WI) using EcoRI restriction sites. The resulting recombinant plasmid was electroporated into *E. coli* Rosetta blue. For eukaryotic protein expression a truncated version of *ct149* lacking the first 18 N-terminal amino acids was cloned into pIRES2-DsRed2 (Clontech, Mountain View, CA) using the restriction site EcoRI as site for recombination with the In-Fusion Advance kit (Clontech, Mountain View, CA) as described in the manufacturer's protocol.

2.4. Protein expression and His-tag purification with NiNTA-beads

E. coli Rosetta blue bacteria containing pET30a (+)-*ct149* were grown at 37°C overnight and diluted 1:50 in LB-medium containing Kan. Cultures were grown at 30°C shaking to OD₆₀₀ 0.4–0.6. Protein expression was induced using 0.1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) at RT overnight. Bacteria were centrifuged at 6,000 $\times$ g for 10 min at 4°C, resuspended in Tris-NaCl (TN)-buffer (20 mM Tris- pH 7.4, 150 mM NaCl) and sonicated 3 times at 20 W output. The insoluble fraction with protein aggregates containing the recombinant protein was centrifuged at 13,000 rpm for 10 min and 4°C and washed under stringent conditions with TN-buffer containing 2% Triton-X100. The protein aggregates were solubilized overnight with 6 M guanidinium hydrochloride (GndCl, Fisher Science, Pittsburgh, PA) in TN buffer containing 2 mM dithiothreitol. His-tag protein purification was performed with NiNTA-beads (Sigma Aldrich, St. Louis, MO) under denaturing conditions as described in the manufacturer's protocol. Briefly, the beads were washed in a chromatography column with water and equilibrated for 15 min in 100 mM potassium phosphate pH 8 and 6 M GndCl. The protein containing lysate was incubated with rotating at 4°C for 2 hrs and the lysate removed from the column by gravity. The beads were washed under stringent conditions with wash buffer (100 mM potassium phosphate pH 6.3 and 6 M GndCl) until no protein was detected in the flow through at 280 nm absorbance. The beads were incubated for 30 min rotating in elution buffer (100 mM potassium phosphate pH 5 and 6 M GndCl) and eluted by gravity flow. Since proteins often require native folding for activity, CT149 was refolded by gradual re-naturation as described [18]. Briefly, the eluate was slowly added in aliquots to 100 mM potassium phosphate pH 7 and 0.2% Triton-X100 to a final dilution of 1:10. However, it is likely that substantially less than 100% of the protein refolded back to its native state and lower activity compared to an authentic enzyme was to be expected. The protein concentration was measured with the BCA protein kit (Pierce Biotechnology, Rockford, IL) as described in the manufacturer's manual. Protein was separated in a 4–20% SDS-PAGE gel (BioRad, Hercules, CA) and either stained with Coomassie Brilliant blue stain or transferred to a PVDF membrane.

2.5. Esterase assays

Carboxylic esterase activity was measured as described [19] with modification. Briefly, the assay was done in a reaction buffer composed of 100 mM potassium phosphate pH 7, 0.2% Triton-X100, 300 mM GndCl, and 3 mM *ortho*-nitrophenyl acetate (*o*-NPA, Fisher Science, Pittsburgh, PA) as substrate. The protein concentration of CT149 was adjusted to 0.1 mg/ml in reaction buffer and 50 – 100 μ l used in the assays. The reaction was started by adding CT149 protein to the reaction mix and the production of *ortho*-nitrophenol (*o*-NP) measured in a CE2041 spectrophotometer (Cecil Instruments, Cambridge, England) at 405 nm at RT. To calculate the amount of product the Lambert-Beer law was used with a molar absorbance coefficient of 3100 M⁻¹cm⁻¹ for *o*-NP at 405 nm. For inhibition studies phenylmethanesulfonylfluoride (PMSF) was used in the *o*-NPA assays up to a concentration of 1 mM. This concentration results in specific inhibition of the serine in the GX SXG motif found in both serine-proteases and carboxylic esterases, as is commonly used to inhibit esterases by others [20, 21].

To test for cholesterol esterase activity, cholesteryl linoleate in isopropanol was used as substrate and diluted in reaction buffer to a final concentration of 0.5 mg/ml. *Pseudomonas* cholesterol esterase (CEH) from the Amplex Red Cholesterol assay (Invitrogen, Carlsbad, CA) was used as positive control in the assays.

2.6. Reverse phase high performance liquid chromatography (HPLC)

Heat inactivated samples from the cholesterol esterase assay were extracted with a 1:1 (v:v) mix of dichloromethane/methanol. The samples were mixed 1:1 with dichloromethane/methanol, vortexed and phases separated at 13,000 rpm for 10 min at 4°C. The lower organic phase was transferred to a new tube and dried at 37°C. After drying, the sample was resolved in 1 ml acetonitrile. Extracts were subjected to reverse phase HPLC using a Waters Breeze 2 HPLC apparatus. 100 μ l extracts were injected in duplicate and separated using a Bondapak C18 column with 3.9 $\times$ 300 mm dimensions (Waters, Milford, MA) in a 1:1 mix of methanol/acetonitrile as running buffer with a flow rate of 1 ml/min. Absorbance was monitored with a Waters 2998 photodiode array at 210 nm. Signals were collected and analyzed with the Breeze 2 software (Waters, Milford, MA). Peaks were identified using HPLC-grade standards run under the same conditions as the samples. The unknown cholesterol peak areas at a retention time of 9.5 min were compared to a cholesterol standard curve (0–10 μ g) measured by HPLC for triplicate injections.

2.7. Transfection of plasmid DNA

Cells were seeded at a density of 1 $\times$ 10⁵ per well in 24 well-plates and transfected 24 hrs later with HighPerfect (Qiagen, Valencia, CA) as described in the manufacturer's protocol. Briefly, 100 ng plasmid DNA was diluted in Hepes buffered saline (HBS, 20 mM Hepes and 150 mM NaCl) and incubated with transfection reagent for 10 min at RT. The transfection mix was added dropwise to the cell culture medium with slow shaking of the plate. Medium was replaced 24 hrs post transfection with fresh cell culture medium. For transfection efficiency DsRed2 expressing cells were compared to total cells. The transfection efficiency in all samples ranged between 70 and 80%.

2.8. Lipid extraction and measurement of free cholesterol and cholesteryl esters

Lipids were extracted as previously described [22]. Briefly, transfected cells were washed with PBS and fixed with 0.5% paraformaldehyde for 10 min at RT. Cells were washed with PBS and lipids extracted with 100% ethanol for 30 min. To measure the concentration of cholesterol and cholesteryl esters the Amplex Red Cholesterol assay (Invitrogen, Carlsbad, CA) was used as described in the manufacturer's protocol. To measure total acylated

cholesterol and total free cholesterol, diluted samples were mixed with 2× reaction mix containing 300 μM Amplex red reagent, 2 U/ml cholesterol oxidase, 0.2 U/ml cholesterol esterase, and 2 U/ml horseradish peroxidase. The free cholesterol pool was measured with a reaction mix in the absence of cholesterol esterase. The difference between free and total cholesterol was significant in the empty vector control using a pairwise t-test with a p-value

0.05. The amount of cholesteryl esters were calculated from the difference of total and free cholesterol. The reaction was incubated protected from light for 30 min at 37°C and measured with a HTS 7000 Bio assay reader (Perkin Elmer, Waltham, MA) at 535 nm excitation and 595 nm emission. All experiments were done in triplicate.

2.9. Antibodies

Murine polyclonal monospecific serum against *C. trachomatis* D CT149 was obtained upon immunization of Balb/c mice with affinity-purified recombinant His-tagged CT149. The serum was used at a 1:200 dilution. A goat anti-mouse antibody labeled with Alexa 555 fluorophore (Invitrogen, Carlsbad, CA) at a 1:20,000 dilution was used as a secondary antibody. Control serum was from an unimmunized mouse diluted and stained with secondary antibody as above.

2.10. *C. trachomatis* infection and Immunofluorescence microscopy

Hela cells were seeded at a density of 1×10^5 cells per well in 24 well plates on 10 mm cover slips 24 h prior to infection. The cells were washed in HBSS (Gibco, Grand Island, NY) and incubated with 1× DEAE-Dextran in HBSS for 10 min at 37°C. The cells were washed in HBSS, and infected with *C. trachomatis* diluted in sucrose-phosphate-glutamic acid- buffer (SPG, 0.22 M sucrose, 3.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 5 mM L-glutamic acid; pH7.4). Infected cells were centrifuged at 1200 rpm for 1 hr at 30°C and incubated for 30 min at 37°C with rocking. The inoculum was removed (start of infection), fresh cell culture medium was added, and the infected cells were further incubated at 37°C with 7% CO₂. Cells were washed in PBS at 24 hrs post infection (p.i.), and fixed in methanol for 10 min at RT. CT149 was stained using monospecific mouse serum with a 1:200 dilution as primary antibody and a goat anti-mouse Alexa 555 with a 1:20,000 dilution as secondary antibody. *C. trachomatis* was identified in immunofluorescence microscopy using a *Chlamydia* genus specific staining kit (Argene, North Massapequa, NY) as described in manufacturer's protocol. The stained cells were washed in PBS and mounted on glass slides with 20% glycerol in PBS before examination under a Nikon Eclipse TE200-U fluorescence microscope using a 200× magnification. Images were obtained with a Retiga 1300 cooled 12-bit camera (QImaging, Surrey, Canada) using the IPLab software version 3.9.2 (Becton, Dickinson and Company, Franklin Lakes, NJ).

2.11. Statistical analysis

Statistical analysis was performed with either a pairwise two-tailed student t-test or an ANOVA test in Excel 2010 (Microsoft). Only results with a p-value 0.05 were considered as significantly different.

3. Results

3.1. CT149 contains lipase/esterase motifs and a potential cholesterol recognition/interaction amino acid consensus (CRAC) sequence in the alpha/beta-hydrolase domain

Previously CT149 was annotated as a hydrolase. It contains an α/β-hydrolase domain, which belongs to a super family of enzymes including lipases and esterases. We identified two GX SXG lipase/esterase motifs at positions 118–122 and 158–162. This motif is found in mammalian lipases and esterases (e.g. carboxylic esterase 1, CES1) and bacterial

carboxylic ester hydrolases (CEH) [23, 24]. Three other annotated members of the esterase/lipase family (predicted outer membrane protein CT073, Lysophospholipase esterase CT136, and predicted acyltransferase CT206) had either zero or only one GX SXG motif in their protein sequence. In addition we did not find other annotated hydrolases in other bacterial species, including *Mycobacterium* and *E. coli* that contained more than one GX SXG motif (data not shown). We also found a cholesterol recognition/interaction amino acid consensus (CRAC) sequence L/A X₁₋₅-Y X₁₋₅-R/K in CT149. The CRAC motif is localized to position 169 – 176 and is in close proximity to the second lipase/esterase motif. Additionally, SignalP software version 3 [25] identified an N-terminal signal peptide between amino acids 18 and 19 with a cleavage site probability of 1.0 and 0.949 in gram-negative bacteria and eukaryotes respectively.

Fig. 1A shows the identified motifs in a schematic drawing. We searched for homologs in other chlamydial species using ClustalX2 (version 2.0.12, [26]) analysis for alignment. Fig. 1B shows the alignment of homologs to CT149 in *C. trachomatis* serovar L2 strain 434/Bu (CTL0404), *C. trachomatis* A/HAR-13 (CTA_158), *C. muridarum* Nigg (TC_426), *C. pneumoniae* AR39 (CP_0620), and *C. caviae* GPIC (CCA_00614). The identical motifs are not only conserved in chlamydiae, but also the localization of the genes coding for CT149 homologs is syntenic with respect to the monooxygenase CT148 and the hypothetical protein CT147 and their homologs in other chlamydial species (Fig. 1C). Also, despite the same genetic orientation of CT148 and CT149 these genes are based on the Operon database (operondb.cbcb.umd.edu) not part of an operon. To search for homologs in eukaryotic cells we performed a BLASTP [27] search against the non-redundant protein sequence database of eukaryotes (taxid no. 2759) and homo sapiens (taxid no. 9606). CT149 showed homology only within the α/β -hydrolase domain in eukaryotes and had an overall low homology to homo sapiens esterases (data not shown).

3.2. Recombinant CT149 has carboxylic esterase activity and is inhibited by PMSF

To analyze the function of CT149 we cloned the full length *ct149* into the bacterial expression vector pET30a (+). The chlamydial protein was expressed and lysates prepared with 6 M GndCl to solubilize the expressed protein. The N-terminal His-tagged protein was purified with NiNTA-beads under denaturing conditions and refolded as described in materials and methods. To test for enzymatic activity we used *o*-NPA as substrate, which is commonly used to test for esterase and lipase activity. The substrate is hydrolyzed by carboxylic esterases to *o*-NP and can be detected spectrophotometrically.

Fig. 2A shows the kinetics of *o*-NP production by CT149 during a 30 min incubation period demonstrating carboxylic esterase activity. An identical sample treated at 65°C for 15 min to inactivate enzymatic activity without proteolysis (Coomassie stain and anti-His Western are shown in Fig. 2B) is included as negative control.

To further investigate if the observed carboxylic esterase activity requires the predicted GX SXG motif, we used the serine-protease inhibitor, PMSF [28]. Inhibition of cholesterol ester hydrolases by PMSF has been demonstrated in eukaryotes [29, 30] and prokaryotes [28, 31]. Samples were incubated with increasing amounts of PMSF in the esterase assay and *o*-NP production measured after 10 min incubation with 5 μ g CT149 at RT. Fig. 2C shows the percentage of produced *o*-NP compared to control with no inhibitor. Esterase activity of CT149 was significantly reduced in a dose dependent manner and was completely abolished at 1 mM PMSF, an effective concentration of inhibitor as described for other esterases [20, 21]. This demonstrates that the esterase motif GX SXG is part of the predicted catalytic triad, which is required to hydrolyze *o*-NPA, and that CT149 is a carboxylic esterase.

3.3. Recombinant purified CT149 hydrolyzes cholesteryl linoleate

To compare the activities of CT149 and CEH in the cholesterol esterase assay we calculated the activity per mg of protein for CT149 and the authentic enzyme based on the *o*-NPA assay. The enzyme activity in units (U) is defined as the amount of converted substrate in μmol per minute. The activities for both enzymes were calculated within the initial 10 min using the linear slope between 2 and 7 min (Fig. 3A). The activity per mg protein was 0.06 U mg^{-1} and 18 U mg^{-1} for CT149 and authentic CEH respectively. The low activity of purified CT149 versus CEH is probably a result of inefficient renaturation during processing of the chlamydial protein, but it may also be that CT149 requires another preferred substrate for improved activity. Cholesterol esterases often show preference to cholesterol esters with long fatty acid chains, such as cholesteryl oleate (C18:1) and cholesteryl linoleate (C18:2). To determine if CT149 hydrolyses cholesteryl esters we used cholesteryl linoleate as the substrate in the esterase assay. We incubated $100 \mu\text{g}$ CT149 (6 mU activity in *o*-NPA assay) with cholesteryl linoleate at 37°C and collected samples to indicated time points. We used buffer only and CEH (1 mU activity in the *o*-NPA assay) as controls.

Fig. 3B shows the cholesterol production after reverse-phase HPLC separation at 0, 1, and 2 hrs. Incubation with either CT149 or CEH resulted in an increase of cholesterol while the amount of cholesterol in the negative control did not increase. The specific activity of CEH and CT149 with cholesteryl linoleate could not be determined with this method. Changes over time of the peak area for free cholesterol in the HPLC assay were not significant for short incubations, which are required to assess initial rate kinetics and calculate the specific activity. However, when compared to CEH the activity of CT149 was 50 fold greater with cholesteryl linoleate compared to *o*-NPA. These results demonstrate that CT149 has cholesteryl esterase activity and that cholesteryl linoleate is a preferred to *o*-NPA as a substrate.

3.4. Expression of CT149 in HeLa cells results in a decrease of intracellular cholesteryl esters and an increase of intracellular free cholesterol

Next we tested if CT149 has activity when expressed in host cells and affects intracellular cholesterol and cholesteryl ester levels. HeLa cells were transfected with either empty pIRIS2-DsRed2 vector or a construct containing a truncated version of CT149 lacking the putative secretion signal sequence (first 18 N-terminal amino acids, see Fig. 1A). The proteins were expressed in HeLa cells for 48 hrs and total lipids were extracted. Total cholesterol, free cholesterol, and cholesteryl ester amounts were determined in the lipid extracts and normalized to total protein amounts.

Fig. 4 shows a decrease of cholesteryl esters in samples expressing cleaved CT149 as compared to control cells. In addition, the amount of free cholesterol increased in the CT149 sample, while the total amount of cholesterol (cholesteryl esters and free cholesterol) stayed constant. The decreasing cholesteryl ester levels and increasing free cholesterol resulted in a change of free cholesterol/cholesteryl ester-ratio from 3.8 in control cells to 18.3 in cells expressing CT149, demonstrating that CT149 has esterase activity in HeLa cells.

3.5 CT149 is localized within chlamydiae and partially co-localizes with LPS

To test for the localization of CT149 during the developmental cycle we infected HeLa cells with a multiplicity of infection of 0.5 and fixed the infected cells 24 hrs post infection (p.i.). Sera from a mouse immunized with purified His-tagged CT149 or from a non-immunized mouse were used at a 1:200 dilution as primary antibody, and a goat anti-mouse Alexa 555 antibody in a 1:20,000 dilution was used as secondary antibody. For the detection of *C. trachomatis* D a commercial available anti-LPS staining kit was used as described in material and methods.

Figure 5 A shows immunofluorescence images taken at 600× magnification from two independent fields with polyclonal, CT149-specific serum compared to normal serum from a non-immunized mouse. In figure 5 B the two single inclusions 1 and 2 were electronically magnified by factor 25× from figure 5A. The images show that CT149 is not detected in the eukaryotic cytoplasm and is only found within chlamydiae with the majority of the protein detected within the larger developmental RB form. Figure 5 A and B also show that the CT149-specific staining partially co-localizes (yellow) with the *Chlamydia* LPS-specific staining.

4. Discussion

C. trachomatis must acquire essential lipid precursors to grow in eukaryotic host cells. The requirement for *Chlamydia* to scavenge lipids is likely a result of the gene-loss during chlamydial evolution to the strictly obligate intracellular existence. To identify proteins that are important for lipid metabolism of *Chlamydia*, a bioinformatic approach was used to select CT149 for further analysis. CT149 is annotated as putative α/β -hydrolase. Members of this family include esterases and lipases. From four annotated esterase/lipase proteins in *C. trachomatis* (CT073, CT136, CT149, and CT206) only the gene product from *ct149* contains two GX SXG motifs found in lipases/esterases [23, 24]. Also, compared to other bacterial species, we did not find another esterase/lipase with two GX SXG motif suggesting that CT149 has rare or even unique features. We also identified a potential cholesterol recognition/interaction amino acid consensus (CRAC) sequence found in the human peripheral type benzodiazepine receptor [32] and the HIV-1 viral gp41 protein [33], suggesting that the *Chlamydia* enzyme has a motif that may interact with eukaryotic lipids. More detailed functional studies will require the use of recombinant mutant versions of CT149 expressed in a surrogate system. These studies are currently under development. Using polyclonal, CT149-specific serum from a mouse immunized with purified His-tagged CT149 we found CT149 only localized in the chlamydial inclusion within the chlamydiae after 24 hrs p.i., but not in the eukaryotic cytoplasm. In addition, the staining for CT149 partially co-localized with the *Chlamydia* anti-LPS staining. It is possible that the predicted N-terminal signal sequence (Fig. 1A) would allow CT149 either the transport to the periplasmic compartment or secretion to the outer membrane where it can use substrates delivered to the inclusion lumen as described by others [9, 11–14].

A His-tagged purified recombinant CT149 demonstrated that the enzyme exhibits carboxylic esterase activity using α -NPA as substrate. The observed activity with α -NPA was low in comparison to the authentic CEH. This is likely a result of the partial refolding or missfolding, which resulted in lower than optimal activity. CT149 contains nine cysteine that may build a high degree S-bonds, which is problematic during expressing in *E. coli*. It can also be possible that the cholesteryl linoleate used in this assay is not the preferred substrate of CT149. A similar low activity was found for a cholesterol esterase in *Streptomyces*, where the activity with *para*-NPA was lower than 0.01 U/mg protein, but had nearly 8000 times higher activity with cholesteryl linoleate [34]. Generally, esterases prefer substrates with short fatty acid chains while lipases are more active on substrates with long chain fatty acids. However, cholesterol esterases in a number of prokaryotes have more activity for cholesteryl esters with long chain fatty acids such as cholesteryl linoleate or cholesteryl palmitate [31, 34–36]. We demonstrated in *in vitro* assays that CT149 hydrolyzes cholesteryl linoleate, which is the main cholesteryl ester in LDL [37], to cholesterol and linoleate with an apparent substantially improved enzymatic activity by 300 fold in comparison to CEH. In addition, expression of CT149 in HeLa cells also showed that the chlamydial enzyme functioned in the eukaryotic host cell by catalyzing a decrease in host cell cholesteryl esters while the total amount of cholesterol stayed constant, confirming cholesterol esterase activity of CT149.

Cholesteryl esters are usually found in lipid storage droplets or in secretory vesicles destined for transport to the exterior of the cell, while free cholesterol is mainly found in the cell membrane. It is known that redirecting of lipid droplets and multivesicular bodies from the secretory pathway to the inclusion lumen occurs during chlamydial development [10, 11, 13]. Since our data suggest that CT149 functions as a cholesterol esterase *in vivo*, the hydrolysis of cholesteryl esters by CT149 would benefit *Chlamydia* by providing cholesterol and fatty acids in the inclusion lumen. Each of these lipid classes are known to be specifically required for *Chlamydia* growth [9, 38] and would be made available from cholesteryl esters via the activity of CT149.

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Fig. 1.

Bioinformatic analysis shows the putative CT149 hydrolase is a potential secreted protein with a lipase/esterase motif. A) The protein sequence of CT149 was searched with the SignalP for bacterial or eukaryotic signal sequences with default settings. A potential cleavage site (black arrowhead) was identified between amino acids 18 and 19. The lipase/esterase specific protein motif GXSXG [23, 24] and the CRAC sequence [32, 33] were identified within the hydrolase domain. B) Protein alignment of CT149 and the homologs in *C. trachomatis* serovar L2 strain 434/Bu (CTL0404, acc. no. YP_001654488.1), *C. trachomatis* A/HAR-13 (CTA_158, acc. no. YP_327950.1), *C. muridarum* Nigg (TC_0426, acc. no. AAF39282.1), *C. pneumonia* AR39 (CP_0620, acc. no. AAF38435.1), and *C. caviae* GPIC (CCA_00614, acc. no. AAP05356.1) in ClustalX2 with default settings. The black arrow marks the predicted cleavage site in all sequences. The GXSXG lipase/esterase and the CRAC sequence are marked with solid and dashed line box respectively. C) Locus comparison of CT149 in *C. trachomatis* and its homologs in other chlamydial species. Same colors in the drawing represent genes of homologues proteins. All schematic drawings are not in scale.

Fig. 2.

Expressed recombinant CT149 shows carboxylic esterase activity *in vitro* and is inhibited by PMSF in a dose dependent manner. Carboxylic esterase activity was measured using *o*-NPA as substrate and the production of *o*-NP after hydrolysis monitored at 405 nm at RT. A) Carboxylic esterase activity of His-tag purified and refolded recombinant CT149 was monitored for 30 min. B) Active and heat-inactivated CT149 was separated in a 10% SDS-PAGE gel. Protein was either detected with Coomassie blue stain (1 μg total protein) or with an anti-His antibody in a Western blot analysis (0.5 μg total protein). C) Carboxylic esterase activity was measured using *o*-NPA as substrate and the production of *o*-NP after hydrolysis monitored at 405 nm at RT after 10 min. Activity was measured in the absence or presence of 0.01 mM, 0.1 mM, and 1 mM PMSF. The hydrolysis of *o*-NPA by CT149 was measured in triplicate. Error bars represent standard deviation of triplicate samples (n=3). Error bars

represent standard deviation. To calculate statistical significance a group-wise ANOVA test with $p < 0.01$ and a pairwise t-test ($*p < 0.01$, $**p < 0.05$) were performed.

Fig. 3.

Recombinant CT149 hydrolyses cholesteryl linoleate *in vitro*. A) Activity of refolded recombinant CT149 was monitored for 10 min. and activity calculated from the initial slope between 2 and 7 min. The hydrolysis of *o*-NPA was measured in triplicate and activity was calculated from the molar amount of produced *o*-NPA per min per mg protein. B) Cholesteryl linoleate was incubated with 100 μg CT149, buffer or carboxylic ester hydrolase (CEH) at 37°C. Samples were taken at t 0, 1, and 2 hrs and the proteins heat-inactivated for 15 min at 65°C. Lipids were extracted with dichloromethane/methanol and dried at 37°C. The extracts were dissolved and separated in reverse-phase HPLC. A representative HPLC run for CT149 is shown with 0 hr (small dashed line), 1 hr (medium dashed line), and 2 hrs (solid line) of incubation. The peaks at 9.5 min and 58 min retention time were identified as cholesterol (C) and cholesteryl linoleate (CL) respectively using standards. C) The amount of produced cholesterol in the sample was calculated from the area of the peak at 9.5 min retention time using a cholesterol standard curve. Each sample was measured in duplicate injections ($n=2$). Error bars represent standard deviation. To calculate statistical significance a group-wise ANOVA test with $p < 0.01$ and a pairwise t-test (* $p < 0.01$, ** $p < 0.05$) were performed.

Fig. 4.

Expression of CT149 in HeLa cells resulted in a decrease of cholesteryl esters and an increase of free cholesterol. HeLa cells were transfected with empty pIRIS2-DsRed2, pIRIS2-DsRed2-CT149 as full length protein or missing the cleavable N-terminal 15 amino acids and incubated at 37°C for 48 hrs. Lipids were extracted and the amounts of cholesteryl esters, free cholesterol and total cholesterol normalized to total protein determined. The experiment was done in triplicate and free and total cholesterol measured in triplicate ($n=3$). The amount of cholesteryl esters was calculated from the difference of total and free cholesterol. Error bars represent standard deviation and a pairwise t-test to calculate statistical significance ($p < 0.05$).

Fig. 5.

Immunofluorescence staining with monospecific serum against CT149 demonstrates that CT149 is only localized in the chlamydial inclusion within the chlamydiae 24 hrs p.i., but is not found in the eukaryotic cytoplasm. HeLa cells were infected with a multiplicity of infection of 0.5 and incubated at 37°C. After 24 hrs cells were fixed and CT149 detected with mouse serum from purified His-tagged CT149 immunized mouse compared to normal serum from a non-immunized mouse as control. A goat anti-mouse Alexa 555 antibody was used as secondary antibody. *C. trachomatis* D was detected using a commercial available anti-LPS kit labeled with FITC. The image shows A) a 600× magnification of two independent fields. B) Two single inclusions numbered 1 and 2 were electronically magnified by factor 25× from image A. The experiment was done in triplicate.