

Developmental Regulation of *Chlamydia trachomatis* Class I Accessible Protein-1, a CD8⁺ T Cell Antigen

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In this report, we show that the *Chlamydia trachomatis*-specific CD8⁺ T cell antigen class I accessible protein-1 (Cap1) is expressed during the early stages of the *C. trachomatis* developmental cycle. We provide additional evidence suggesting that Cap1 may be important in early immune recognition of the organism. Understanding the temporal and spatial expression of CD8⁺ T cell antigens such as Cap1 may be beneficial in designing multisubunit vaccines to stimulate a vigorous immune response against *C. trachomatis*.

Chlamydia trachomatis is an obligate intracellular bacterium that causes a wide range of diseases in humans and animals. *C. trachomatis* has a unique biphasic developmental cycle [1]. The elementary body, the infectious form of the bacterium, shows no metabolic activity. Once elementary bodies enter the host cell, they begin to convert to noninfectious reticulate bodies that are metabolically active and that replicate within a specialized vacuole known as an inclusion. Approximately 20 h after infection, reticulate bodies begin to convert back to elementary bodies. By 36 h after *C. trachomatis* infection, the host cell lyses, and elementary bodies are released into the extracellular environment to begin further rounds of infection and replication.

The resolution of *C. trachomatis* infection and resistance to reinfection depends on the coordinated activity of B cells, CD4⁺

T cells, and CD8⁺ T cells (reviewed in [2]). We and others have been particularly interested in understanding how CD8⁺ T cells control *C. trachomatis* infection. The recent discovery of a number of *C. trachomatis*-specific CD8⁺ T cell antigens [3–5] has begun to facilitate these studies.

One of the first *C. trachomatis*-specific CD8⁺ T cell antigens to be identified was class I accessible protein-1 (Cap1), a 31-kDa protein of unknown function [4]. Fling et al. [4] initially demonstrated that Cap1 is expressed in fibroblasts at the *C. trachomatis* inclusion membrane 24 h after infection. More recently, we have begun to use Cap1 as a tool to study the initiation of the *C. trachomatis*-specific CD8⁺ T cell response [6]. Once activated, CD8⁺ T cells differentiate into effector cells and acquire the important task of recognizing *C. trachomatis*-infected cells and targeting them for destruction. However, because *C. trachomatis* has a strictly coordinated developmental cycle, many antigens may be regulated in their expression and may not always be available for recognition by CD8⁺ T cells. Therefore, to determine which classes of *C. trachomatis* proteins may contribute most to CD8⁺ T cell-mediated clearance of this pathogen, it is essential to understand the temporal and spatial expression of a diversity of these antigens in their host cells.

Here, we characterize the subcellular compartmentalization and temporal expression of Cap1 in *C. trachomatis*-infected host cells in vitro. Using a combination of different techniques, we address the questions of when Cap1 is initially expressed at both the transcript and protein level, whether the protein remains localized to the inclusion membrane throughout the *C. trachomatis* developmental cycle, and when infected host cells are first able to be recognized by Cap1-specific CD8⁺ T cells.

Materials and methods. McCoy, HeLa, and BALB 3T3 cells (American Type Culture Collection) were infected with *C. trachomatis* serovar L2 434/Bu in a sucrose-phosphate-glutamate media by centrifugation of bacteria with cells at 1200 g for 1 h at 37°C. After centrifugation, the inoculum was removed and replaced with Dulbecco's modified Eagle medium (HeLa), MEM (McCoy), or RPMI 1640 medium (BALB/3T3) containing 10% fetal bovine serum.

For real-time polymerase chain reaction (PCR) analysis, RNA was isolated from cells using the Ultraspec RNA system (Biotex Laboratories). One microgram of total RNA from each sample was reverse-transcribed using 3'-specific primers to generate cDNA specific for *cap1*, *incA*, and 16s rRNA. Real-time PCR was then performed on each cDNA sample, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The primers and probes used in these studies were designed using

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Primer Express software (Applied Biosystems). The initial amount of *cap1* or 16s rRNA cDNA template in each sample was calculated by comparison with a standard curve in which known dilutions of *C. trachomatis* L2 genomic DNA were amplified using the same gene-specific primers and probe.

Cells analyzed by fluorescent in situ hybridization (FISH) were grown and infected on glass coverslips. Cells were fixed and processed for FISH, essentially as described elsewhere [7, 8]. Cells were hybridized at 35°C with a Cy5-labeled probe complementary to 18 nt of the *cap1* mRNA sequence, counterstained with the nucleic acid stain 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and visualized with a Nikon E1000 automated upright epifluorescence microscope (Nikon Imaging Facility, Harvard Medical School, Boston).

For immunofluorescence studies, cells were grown and infected on glass coverslips. Cells were fixed in 100% methanol for 10 min at room temperature, washed in PBS, and blocked with 10% donkey serum and 2% bovine serum albumin. Cells were then costained with a mouse monoclonal antibody against the *C. trachomatis* major outer membrane protein, MOMP (Accurate Chemical and Scientific), and a rabbit polyclonal antibody against Cap1 [4]. The anti-MOMP antibody was visualized in combination with a fluorescein isothiocyanate-conjugated donkey anti-mouse IgG antibody, whereas the anti-Cap1 antibody was visualized in combination with a rhodamine red X-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories).

To evaluate processing and presentation of Cap1 antigen, BALB/3T3 cells were fixed at different times after infection with 1% paraformaldehyde for 15 min at room temperature before being cultured with the Cap1-specific CD8⁺ T cell line 69 [9]. Line 69 was maintained and expanded by weekly stimulation on *C. trachomatis*-infected cells, as described elsewhere [9]. Supernatants from the cocultured cells were analyzed for levels of interferon (IFN)- γ , using an IFN- γ Minikit (Pierce).

Results. To examine the transcriptional regulation of *cap1*, we performed real-time PCR analysis on *C. trachomatis*-infected cells. McCoy cells, a murine fibroblast cell line, were infected with *C. trachomatis* at an MOI of 1:1 in the presence of 1.5 μ g/mL cycloheximide. At various times after infection, total RNA was isolated and reverse-transcribed to generate cDNA specific for *cap1*, *incA*, and 16s rRNA. Real-time PCR was then performed on each cDNA sample. Because production of 16s rRNA transcripts by individual organisms remains relatively constant during infection [10], the amount of *cap1* or *incA* in each sample was divided by the amount of 16s rRNA in the sample, to normalize for the increase in the number of *C. trachomatis* organisms over time. For each time point, the fold change in *cap1* mRNA levels relative to that present in the initial inoculum (0 h) is plotted in figure 1A. As early as 4 h after infection, *cap1* transcript levels were up-regulated. *cap1*

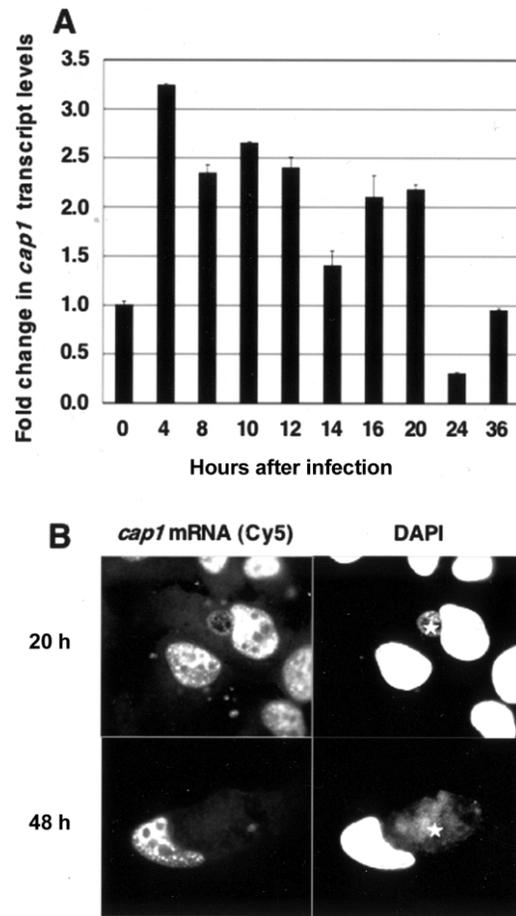


Figure 1. Transcription of *cap1* early in the *Chlamydia trachomatis* developmental cycle. *A*, Infection of McCoy cells with *C. trachomatis* for various lengths of time. Total RNA was isolated at each time point and was used to generate cDNA specific for *cap1* and 16s rRNA. Real-time polymerase chain reaction was then performed in duplicate on the cDNA to determine the amount of *cap1* and 16s rRNA present in each sample. *cap1* levels were normalized against the levels of 16s rRNA in each sample, to control for the increase in the total number of *C. trachomatis* organisms present over time. Bars represent the fold change in *cap1* transcript levels for each time point relative to the amount of *cap1* mRNA present in the initial inoculum (0 h after infection). The graph depicts data from 1 of 3 representative experiments. Error bars represent the range of data collected for each time point in this particular experiment. *B*, Infection of HeLa cells with *C. trachomatis* for either 20 h or 48 h. Cells were fixed and hybridized with a Cy5-labeled oligomer that recognizes *cap1* mRNA. Cells were also counterstained with the nucleic acid stain DAPI and then visualized by epifluorescence microscopy. White stars are included to depict the location of the *C. trachomatis* inclusion.

mRNA levels remained elevated until 20 h after infection, after which time transcript levels dropped below that present in the initial elementary body inoculum. *cap1* transcript levels modestly rose again between 24 and 36 h after infection. This profile of *cap1* transcription was in contrast to that observed for *incA*, which encodes an inclusion membrane protein previously shown to be expressed at the middle of the *C. trachomatis* developmental

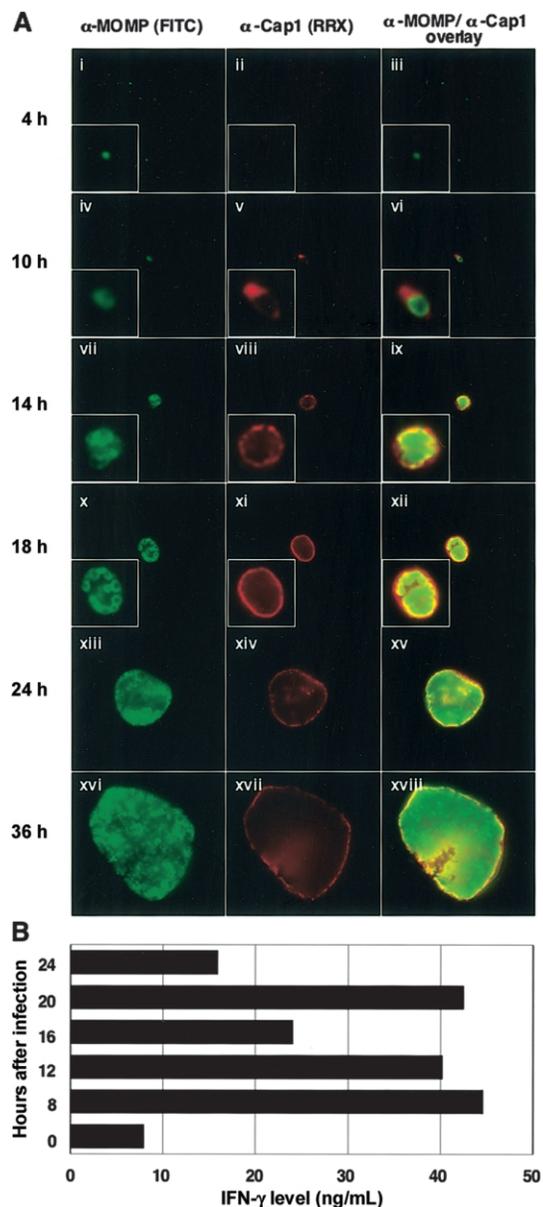


Figure 2. Expression of Cap1 protein at significant levels within an infected cell as early as 8–10 h after infection. *A*, Infection of HeLa cells with *Chlamydia trachomatis* for 4 h (*i–iii*), 10 h (*iv–vi*), 14 h (*vii–ix*), 18 h (*x–xii*), 24 h (*xiii–xv*), or 36 h (*xvi–xviii*). Cells were fixed and costained with a mouse monoclonal antibody against the *C. trachomatis* major outer membrane protein (MOMP) (fluorescein isothiocyanate [FITC]-conjugated secondary antibody; *i*, *iv*, *vii*, *x*, *xiii*, and *xvi*) and a rabbit polyclonal antibody against Cap1 (rhodamine red X [RRX]-conjugated secondary antibody; *ii*, *v*, *viii*, *xi*, *xiv*, and *xvii*) and then visualized by epifluorescence microscopy. FITC (green) and RRX (red) images were overlaid to determine the relative localization of MOMP and Cap1 (*iii*, *vi*, *ix*, *xii*, *xv*, and *xviii*). *B*, Infection of BALB/3T3 epithelial cells with *C. trachomatis* for the indicated times. Cells were fixed and coincubated with a Cap1-specific CD8⁺ T cell line. After 16 h of coincubation, the amount of interferon (IFN)- γ secreted into the supernatant was measured by ELISA. Detection of IFN- γ in the cultures indicates the recognition of infected BALB/3T3 cells by the T cell line. The data shown are representative of 3 separate experiments.

cycle [11]. Similar to what was described by Scidmore-Carlson et al. [11], we observed that *incA* mRNA was first expressed between 12 and 14 h after infection (data not shown). *incA* levels peaked by 20 h after infection (>100-fold up-regulation), declined significantly by 24 h after infection, and then rose again by 36 h after infection.

These real-time PCR studies suggest that there is early transcription of *cap1* after *C. trachomatis* infection. This finding is consistent with existing data generated by microarray analysis in which Belland et al. [12] identified the *cap1* homologue from *C. trachomatis* serovar D as an immediate-early gene. However, during later stages of infection, when *C. trachomatis* inclusions become more asynchronous in their development, it becomes more difficult to use real-time PCR or microarray analysis to accurately evaluate the transcriptional regulation of *cap1*. The overall changes in *cap1* mRNA levels detected by either of these methods could result from transcriptional regulation within individual reticulate bodies or could simply reflect the conversion of a number of reticulate bodies to metabolically inactive elementary bodies. Therefore, to begin to address this concern, we used FISH to examine *cap1* mRNA expression at the level of the individual inclusion.

HeLa cells, a human cervical epithelial cell line, were infected with *C. trachomatis* at an MOI of 3:1 in the presence of 1.5 μ g/mL cycloheximide. At various times after infection, cells were fixed and stained with a fluorescently labeled probe recognizing *cap1* mRNA. To confirm that our fluorescent probe was specifically binding *cap1* mRNA and not genomic DNA, FISH analysis was also performed on infected cells using a fluorescently labeled oligomer complementary to the *cap1* probe. Minimal signal was detected using this oligomer (data not shown). Unfortunately, FISH proved far less sensitive than real-time PCR in detecting *cap1* transcripts in infected cells. In fact, *cap1* mRNA was not detected by FISH until 18 h after *C. trachomatis* infection (data not shown). Furthermore, because we were unable to visually distinguish reticulate bodies from elementary bodies within an inclusion, the transcriptional regulation of *cap1* by individual organisms could not be compared. Nevertheless, we observed that although *cap1* mRNA was present within the inclusion at 20 h after infection, transcripts were no longer detectable within large inclusions at 42 h after infection (figure 1B). Our FISH analysis suggests that transcription of *cap1* most likely declines in individual inclusions late in the *C. trachomatis* developmental cycle and remains undetectable for the duration of the cycle. The subsequent increase in *cap1* transcription observed in our real-time PCR studies at 36 h after infection (figure 1A) most likely reflects the asynchrony of *C. trachomatis* infection. By 36 h, many inclusions have already lysed, and organisms have begun new rounds of infection and transcriptional up-regulation of early genes such as *cap1*. These studies suggest that a combination of real-time PCR and FISH analysis may

allow for enhanced sensitivity in measuring transcriptional regulation of *C. trachomatis* genes throughout the entire developmental cycle of the organism.

To determine whether this early transcription of *cap1* also correlates with early protein expression, we then used indirect immunofluorescence microscopy to study the temporal and spatial expression of Cap1 protein. HeLa cells were infected with *C. trachomatis* at an MOI of 10:1. At different times after infection, cells were fixed and costained with antibodies specific for the Cap1 protein [4] and MOMP. Antibodies against MOMP detect individual elementary bodies and reticulate bodies within a *Chlamydia* inclusion. As shown in figure 2A, Cap1 protein was initially detected at the *C. trachomatis* inclusion membrane at 8–10 h after infection, when single reticulate bodies are first detected within the inclusion. Because immunofluorescence analysis is somewhat limited in its sensitivity, it is also possible that Cap1 is expressed earlier within infected cells. Cap1 protein levels continued to increase by 14–18 h after infection and remained stably expressed as late as 36 h after infection. Cap1 appeared to localize predominantly at the inclusion membrane throughout the course of the *C. trachomatis* developmental cycle.

Our analysis of *cap1* expression indicates that this gene is transcribed and translated soon after *C. trachomatis* infects non-professional antigen-presenting cells. Cap1 protein may, therefore, be available for processing and presentation on major histocompatibility complex (MHC) class I relatively early in the *C. trachomatis* developmental cycle. To test this hypothesis, a murine epithelial cell line, BALB/3T3, was infected with *C. trachomatis* at an MOI of 100:1. At various times after infection, cells were fixed and cocultured with the Cap1-specific CD8⁺ T cell line 69 [4]. Recognition of infected BALB/3T3 cells by this T cell line was measured using an IFN- γ ELISA (figure 2B). These T cells recognized Cap1 on the surface of infected BALB/3T3 cells as early as 8 h after *C. trachomatis* infection and continued to recognize infected cells as late as 20 h after infection. In evaluating these findings, it is important to consider that recognition of infected cells by T cells is more reliable for evaluating initial protein expression than for measuring the decline in protein expression, because of the substantial half-life of MHC-peptide complexes at the cell surface [13, 14].

Discussion. In this study, we examined the temporal and spatial expression pattern of the *C. trachomatis*-specific CD8⁺ T cell antigen Cap1. Using a variety of techniques, we determined that Cap1 is expressed at the early stages of infection. The fact that, by 8 h after infection, sufficient Cap1 protein is expressed to stimulate CD8⁺ T cells suggests that Cap1 expression may be critical in early immune detection of *C. trachomatis*-infected cells.

Cap1 is one of a growing number of proteins that are being described as CD8⁺ T cell antigens. The interest in identifying

and characterizing *C. trachomatis*-specific CD8⁺ T cell antigens stems, in part, from a desire to develop an effective vaccine against this pathogen. Past studies have focused on boosting antibody titers and the CD4⁺ T cell response against *Chlamydia* (reviewed in [15]). However, the additional inclusion of a number of different CD8⁺ T cell antigens into a vaccine may prove to be a promising way of inducing long-lasting immunity against *C. trachomatis*. The temporal and spatial expression studies we performed with Cap1 can be expanded to other *C. trachomatis*-specific T cell antigens. Choosing a diversity of antigens that overlap in their temporal expression would ensure that there is no period during the *C. trachomatis* developmental cycle when the organism could escape recognition by the host immune system. This approach may increase the possibility of developing a vaccine that achieves sterilizing immunity against *C. trachomatis*.

In addition, because genetic manipulation of *C. trachomatis* remains elusive, it has been difficult to attribute functions to most *C. trachomatis*-specific proteins. However, knowing the developmental regulation and subcellular compartmentalization of *C. trachomatis* proteins allows one to hypothesize a range of putative functions for these proteins. This information, together with insights into the cellular biology of *C. trachomatis* infection and protein-protein interactions, may help to elucidate the role of specific proteins in the pathogenesis of *C. trachomatis*.

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