*Chlamydia trachomatis* Manipulation of Protein Kinase A

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*Chlamydia trachomatis* Manipulation of Protein Kinase A

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Abstract: The most commonly reported bacterial sexually transmitted infection in the United States is *Chlamydia trachomatis* which can lead to pelvic inflammatory disease, tubal infertility and even increased risk of cervical cancer. *C. trachomatis* can only survive inside of the cell and lives in a parasitophorous vacuole. During infection, manipulation of different protein kinases aid in its replication and survival processes. One such enzyme is Protein Kinase A, PKA, which is an essential kinase in the host cell that phosphorylates other proteins for activation. Misregulation of PKA signaling has been identified in the development of many cancers. Not much is known about the intracellular Chlamydial manipulation of host cellular kinases, such as PKA during the infection process. The goal of this study was to determine the extent to which *C. trachomatis* manipulates PKA during the infection process. We hypothesize that *C. trachomatis* actively manipulates PKA signaling to regulate intracellular development and survival inside the host. We utilized western blot analysis of whole cell lysates (HeLa cells infected with *C. trachomatis*) collected at various time points to monitor phosphorylation changes of PKA kinases and substrates. Protein samples collected at various times of the infection process were separated by SDS-PAGE and transferred to nitrocellulose membranes. These membranes were probed by various phosphospecific antibodies to specific host PKA kinases, specific kinase substrates and total PKA substrates. The use of horse-radish peroxidase conjugated secondary antibodies allowed for visualization via the use of chemiluminescence. The results obtained confirmed that PKA and PKA substrates were indeed manipulated by *C. trachomatis* during infection and specifically that PKA activity was upregulated in the latter times of infection. These findings conclude that PKA enzymes serve an important role for the intracellular growth and development of *C. trachomatis.* Additional studies will help to determine if expression of specific PKA substrates is altered during *C. trachomatis* infection and if misregulation of these specific substrates is linked with cancer development. This can have a significant impact on human health and may identify certain factors that increase the risk of cervical cancer after *C. trachomatis* infection.

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# *Chlamydia trachomatis* Manipulation of Protein Kinase A

## Chapter 1: Introduction

### **1.1 Background on *Chlamydia trachomatis* infections**

*Chlamydia trachomatis* is the most commonly reported sexually transmitted disease in the United States with 1.75 million cases reported in 2018 alone (Centers for Disease Control and Prevention, 2018). An estimated 2.86 million infections occur annually in the United States of America with a large number of those cases going unreported due to a majority of people with *C. trachomatis* infections being asymptomatic. This is why it is commonly known as 'silent' infection with only about 10% of men and 5-30% of women with laboratory-confirmed chlamydial infections developing symptoms. Symptoms of chlamydial infections include penile and vaginal discharge and bleeding, as well as painful urination and intercourse. It infects both men and women and can cause serious damage to women's reproductive systems if left untreated. This can increase the difficulty for a pregnancy to occur and can even lead to permanent infertility. *C. trachomatis* is known to cause ectopic pregnancies (pregnancy that occurs outside the womb), pelvic inflammatory disease, and even increases the risk of cervical cancer (Josefson, 2001; Planned Parenthood, 2020). *C. trachomatis* infections are caused by vaginal, anal, and oral sex with someone who is already infected with *C. trachomatis.* Infections can also spread to babies during childbirth. Infections in both men and women can lead to urethritis and proctitis and may increase a person's chances of acquiring or transmitting HIV (Nusbaum, Wallace, Slatt, & Kondrad, 2004). Individuals who have has *C. trachomatis* infections in the past and been treated can get infected again by having sexual contact with another person infected with *C. trachomatis.* Chlamydial infections can be easily treated with

antibiotics, however with many patients being asymptomatic, many cases go untreated and can lead to serious medical complications later. Prevention of spreading sexually transmitted diseases like *Chlamydia* can be reduced by the use of latex condoms, abstaining from sexual intercourse, or to be in a long-term, monogamous relationship with a partner who has been tested and known to be uninfected.

## 1.2 Intracellular growth

*C. trachomatis* is a Gram-negative, obligate intracellular pathogen. It infects a host cell with elementary bodies (EBs), which are the infectious forms of *Chlamydia* that are metabolically inactive but highly infectious. This inactive state strictly means that it cannot grow or further develop its lifecycle to replicate, but rather can only infect other cells. After EBs are endocytosed, *C. trachomatis* prevents lysosomal fusion and forms a parasitophorous vacuole called an inclusion body which is the membrane-bound compartment that houses *C. trachomatis*. This inclusion body is usually found near the nucleus of the host cell. EBs are then converted to reticulate bodies (RBs), which are the metabolically active and replicative form of *Chlamydia* where the inclusion grows and develops further to multiply and replicate. RBs actively make proteins which are required for Chlamydial growth with additional proteins also secreted via a Type III Secretion system. *C. trachomatis* redirects exocytic vesicles that are in transit from the Golgi apparatus to the plasma membrane for nutritional sustainability for its own growth and survival in the cell. These processes are aided by the help of Chlamydial inclusion membrane proteins (Incs) which induce the formation of different membranous vesicular compartments and are localized at the inclusion membrane (Mital, Miller, Dorward, Dooley, & Hackstadt, 2013). The RBs begin to replicate exponentially in the inclusion and secrete additional effectors to

control other processes in the host cell. Some examples include Translocated Actin Recruiting Protein (Tarp)(Clifton et al., 2004), which localizes at the plasma membrane just below Chlamydial attachment, and CT847, which interacts with human Grap2 cyclin D-interacting protein (Kleba & Stephens, 2008). Upon stress, RBs can go into a dormant persistent stage and transition to enlarged aberrant bodies (Wyrick, 2010). Protein synthesis is halted in this stage and the removal of the stress reactivates the RBs. This stress stage is not always apparent in the Chlamydial life cycle. Eventually the cell will begin to die as *C. trachomatis* takes up all the nutrients for itself as RBs are naturally converted back to EBs in an asynchronous fashion. These EBs are secreted from the host cell to infect other cells through lysis of the host cell or by extrusion (Hybiske & Stephens, 2007). The life cycle of *Chlamydia* is depicted in Figure 1.

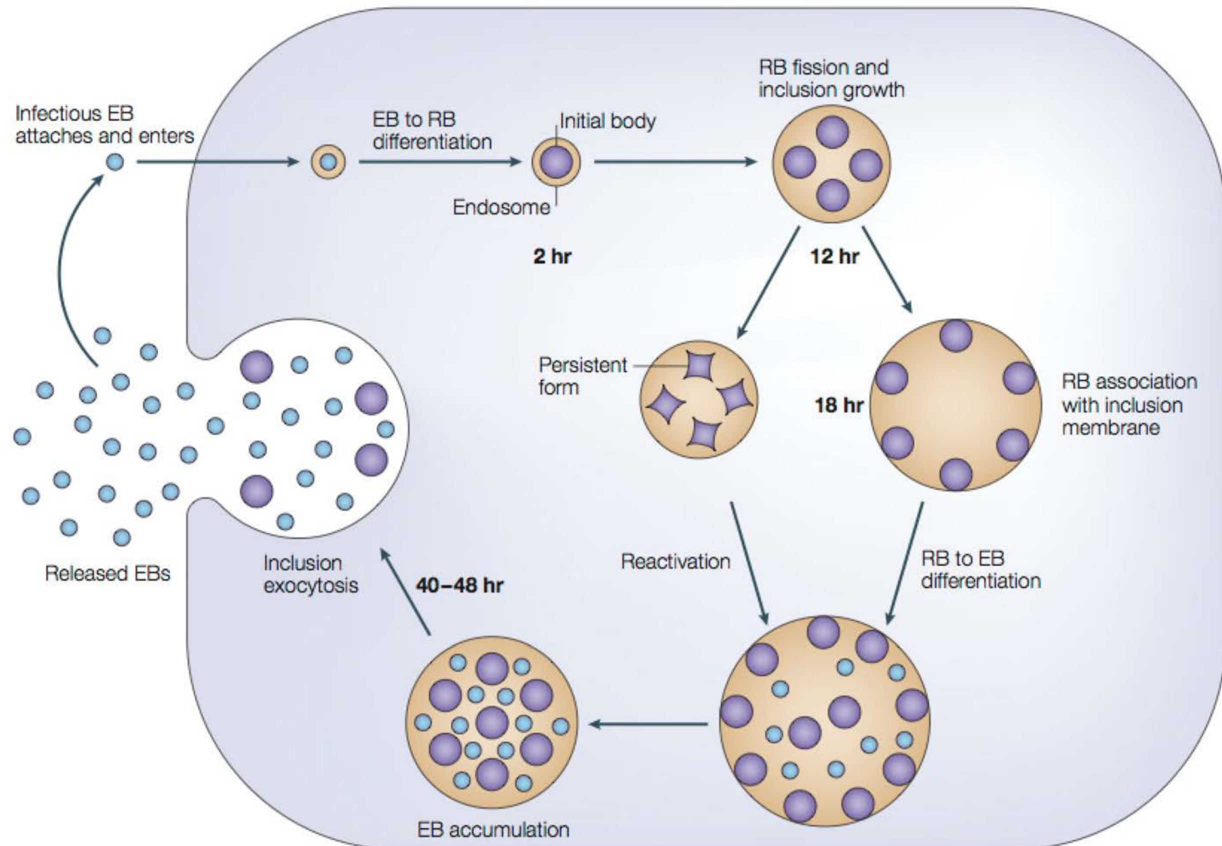


**Figure 1. The life cycle of *C. trachomatis.*** Shown are mechanisms of entry and exit as well as the EBs, RBs, and the persistent form (Potroz & Cho, 2015).

## 1.3 Host kinase manipulation by *C. trachomatis*

*C. trachomatis* manipulates multiple different signaling pathways and recruits many different host proteins to the inclusion membrane including host kinases. These kinases are responsible for activating other proteins through phosphorylation. Phosphorylation is a process where one protein activates another protein by transferring a phosphate group to it. Protein phosphorylation can control processes ranging from development, virulence, adaptive responses through enzyme activity, protein localization, signal transduction, and protein oligomerization (Grangeasse, Nessler, & Mijakovic, 2012; Pereira, Goss, & Dworkin, 2011). Upon *C. trachomatis* infection, several host-tyrosine kinases (platelet-derived growth factor receptor (PDGFR), and Abl kinase are phosphorylated and recruited to the site of bacterial attachment and may function redundantly

in entry (Elwell, Ceesay, Kim, Kalman, & Engel, 2008; Kim, Jiang, Elwell, & Engel, 2011). Src family kinases are host kinases that are abundant in these kinase rich microdomains and help to identify where these microdomains are located on the surfaces of the of inclusions (Mital et al., 2013). Protein Kinase C, a host cellular protein responsible for controlling phosphorylation of other proteins is found to be located in these Src domains (Sah, Nelson, Shaw, & Lutter, 2019). Interestingly, PKC phosphorylated substrates are also recruited to the Chlamydial inclusion but show a circumferential recruitment; not just at the Src kinase microdomains. Other host kinases include myosin light chain kinase and myosin phosphatase which are believed to play a part in Chlamydial host-cell exit mechanisms. These proteins phosphorylate and dephosphorylate myosin light chain respectively and are also found to be colocalized in Src domains (Lutter, Barger, Nair, & Hackstadt, 2013). Some host kinases thought to be responsible for the binding of *C. trachomatis* are Pkn1 and PknD (Claywell, Matschke, & Fisher, 2016). Pkn1 is predicted to reside in the cytoplasm and interacts with an inclusion membrane protein and PknD is an integral protein thought to bind to an unidentified ligand. After infection, the Chlamydial inclusion manipulates multiple host-cell trafficking pathways to redirect essential host-derived nutrients like amino acids, lipids and iron, while limiting detection by the innate immune system. These studies suggest that host kinase manipulation is integral to *C. trachomatis* infection and opens the door to the role of other host kinases during the infection cycle.

## 1.4 Implication of Protein Kinase A in *C. trachomatis* Infection

Kinase rich microdomains on the surface of these inclusions are where the inclusion recruits most of its host cell proteins/kinases. Given that multiple host kinases are known to be recruited to the inclusion microdomains, we predicted that *C. trachomatis* kinase utilization would also

include Protein Kinase A (PKA) similar to that of another obligate intracellular pathogen, *Coxiella burnetii* (Macdonald, Graham, Kurten, & Voth, 2014). *C. burnetii* has also been shown to utilize myosin light chain kinases similar to *C. trachomatis* (Hussain, Broederdorf, Sharma, & Voth, 2010). Which gives reason to believe that *C. trachomatis* might also utilize PKA similar to *C. burnetii.* PKA is a cellular protein that is involved in multiple different signaling cascade events ranging from cellular growth and proliferation to apoptosis, and it has even been shown to play a significant role in the development of different variants of cancers (Caretta & Mucignat-Caretta, 2011; Hedrick et al., 2013). Currently, very little is known about how *Chlamydia* manipulates oncogenic signaling.

### 1.5 Hypothesis

**We hypothesized that PKA is recruited during infection and that *C. trachomatis* actively manipulates the PKA signaling network.**

## Chapter 2: Materials and Methods

### 2.1 Cell Line and Bacterial Strain

HeLa cells are regularly used as a model for *C. trachomatis* infection and were utilized for infection and protein collection. HeLa cells were derived from a patient with a cervical cancer on February 8, 1951 (Scherer, Syverton, & Gey, 1953) and grown at 37$^{o}$C with 5% $CO_2$. *Chlamydia* infections: *Chlamydia trachomatis* L2/434/Bu was used in all experiments.

## 2.2 Aseptic Technique in Cell Culture

Cell culture preparation was performed in a tissue culture room following all protocols of BSL2+ safety precautions. Tyvex gowns with gloves were sprayed with 70% ethanol and worn at all times in the tissue culture room. A microscope was initially used to verify cell confluence prior to passaging cells and prior to infection. For cell passaging and infections the media Roswell Park Memorial Institute (RPMI) was used. Fetal Bovine Serum (FBS) was added to new RPMI to a final concentration of 5% v/v (25mL of FBS was added to 500mL of RPMI). An electronic serological pipettor and sterilize serological pipettes were used to transfer liquids. Warmed 1X Phosphate-Buffered Saline (PBS; 37ºC) was used to rinse cells and remove loosely adhered or dead cells prior to passaging and use. T25 tissue culture flasks were used to grow HeLa cells. Warmed trypsin (37 ºC) was used to disassociate adherent cells from the surface of the flask while in an incubator. Roccal-D was used as a disinfectant for all solid and liquid waste prior to autoclaving. Sharpies were used for labeling. A biohazard waste container was used to dispose of other solid materials. Ethanol (70%) was used as a disinfectant on all surfaces.

## 2.3 Cell Maintenance in Tissue Culture

RPMI, trypsin and 1X PBS were taken from a 4ºC fridge, -20°C freezer, and a room temperature shelf respectively, and placed in a 37°C water bath for 10-15 minutes to warm up and then dried off and sprayed with 70% ethanol before being placed in a Class II Type A2 Biosafety Cabinet. One unused, clean T-25 tissue culture flask was placed in the Class II Type A2 Biosafety Cabinet after being sprayed with 70% ethanol. The T12.5 flask in which HeLa cells were grown was taken from the incubator and checked for confluency. If cells were at 85-95% confluency, they would be split into new flasks or used for experiments.

Splitting of cells: The liquid in the T12.5 flask was poured off into the Roccal-D removing the dead floating cells, while leaving the live adherent cells stuck on the bottom of the flask. The flask was rinsed with 2-3 mLs of 1X PBS which was also poured off into the Roccal-D. Using a pipette gun and a 2mL pipette, .7-.8mL of trypsin was added to the T12.5 flask and then placed in the 37 $^{o}$C incubator for five minutes. During the five minutes, the new T-25 tissue culture flask was labeled with “HeLa”, the passage number (in this case “P2”), the initials of the researcher, and the date. Twenty milliliters of RPMI was added to the new flask via a 10mL pipette. After five minutes, the T12.5 flask was taken out of the incubator and smacked two times, one on each side of the flask to thoroughly knock off all of the cells from the bottom of the flask. The exterior of the flask was sprayed with 70% ethanol before being put back into the Class II Type A2 Biosafety Cabinet. Three milliliters of RPMI was added to the T12.5 flask and thoroughly mixed. The entirety of the T12.5 flask was poured into the new T-25 flask and the T-25 flask was placed in the incubator. The T12.5 flask was discarded into a biohazard waste bin, and all used pipettes were placed in the Roccal-D container. The surface of the hood was then thoroughly wiped down with 70% ethanol.

For all succeeding passages, 6-8mL of 1X PBS wash was used, and 2mL of trypsin was used instead. After the five-minute trypsin incubation period, 8mL of RPMI was added to the trypsin flask and thoroughly mixed. A new T-25 flask can then receive 1-3mLs of the mixed solution. Less volume was passaged for longer growth times, and more volume was passaged for faster growth times. All remaining media in the old flask was then poured into the Roccal-D container. Everything else was held constant from the first passage.

Splitting cells for seeding into 24 well plates: Cells were seeded in 24 well-plates in a Class II Type A2 Biosafety Cabinet in the tissue culture room. Instead of pouring out the rest of the culture left in the old flask after passaging, it was saved for seeding well-plates. First, 24 well-plates and a 50mL conical tube were sprayed with 70% ethanol before being placed in the Class II Type A2 Biosafety Cabinet. RPMI was added first to the conical tube at a ratio of 10:1 when mixed with the culture in the old flask respectively and inverted 4-5 times to mix. One milliliter was then transferred to each well in the 24 well-plate. Next, the 24 well-plate was let alone for ten minutes before being placed in the 37 ºC incubator. The Class II Type A2 Biosafety Cabinet surface, was then wiped down with 70% ethanol and the remaining solution was poured into the Roccal-D container along with the pipette tips while disposing the empty flask into a biohazard waste bin.

## 2.4 Protein Sample Preparation

HeLa cells grown in 24 well plates to a confluency of 90%, were infected with *C. trachomatis* L2/434/Bu EBs at a multiplicity of infection (MOI) of 1. Infected cells were grown in RPMI containing chloramphenicol (200µg/mL) or vehicle (Ethanol 1% v/v), add at 1-hour post infection (hpi). Infected cells were lysed at different time points during infection (4, 12, 24, 36, 48 hpi). Mock infected HeLa cells were used as controls. Cells were washed with 1X phosphate buffered saline (PBS) before lysis. One hundred µL of 8M Urea supplemented with 325 units/mL Benzoase nuclease (EMD Millipore) and 1X protease inhibitor cocktail (Thermo Scientific) was added per well of 24 well plates, and incubated on ice for 10 minutes. Lysate was collected, and 100µL of 2X Laemmli buffer was added and stored at -20°C

## 2.5 Reagents Used in Sodium Dodecyl (Lauryl) Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gel Preparation

Tris-glycine SDS-Polyacrylamide gels were made in the laboratory. The materials and recipes for the resolving gel solutions of 6%, 8%, 10%, 12%, and 15% are described in Table 1 and the 5% stacking gels are described in Table 2.

**Table 1. Solutions for preparing resolving gels for tris-glycine SDS-polyacrylamide gel electrophoresis.**

| **Solution Components** | **Per Gel** (5 mL) | | **Solution Components** | **Per Gel** |
|---|---|---|---|---|
| **6%** | | | **12%** | |
| - $H_2O$ | 2.6mL | | - $H_2O$ | 1.6mL |
| -30% acrylamide mix | 1.0mL | | -30% acrylamide mix | 2.0mL |
| -1.5 M Tris (pH 8.8) | 1.3mL | | -1.5 M Tris (pH 8.8) | 1.3mL |
| -10% SDS | 0.05mL | | -10% SDS | 0.05mL |
| -10% ammonium persulfate | 0.05mL | | -10% ammonium persulfate | 0.05mL |
| -TEMED | 0.004mL | | -TEMED | 0.002mL |
| | | | | |
| **8%** | | | **15%** | |
| - $H_2O$ | 2.3mL | | - $H_2O$ | 1.1mL |

| -30% acrylamide mix | 1.3mL | | -30% acrylamide mix | 2.5mL |
|---|---|---|---|---|
| -1.5 M Tris (pH 8.8) | 1.3mL | | -1.5 M Tris (pH 8.8) | 1.3mL |
| -10% SDS | 0.05mL | | -10% SDS | 0.05mL |
| -10% ammonium persulfate | 0.05mL | | -10% ammonium persulfate | 0.05mL |
| -TEMED | 0.003mL | | -TEMED | 0.002mL |
| | | | | |
| **10%** | | | | |
| - $H_2O$ | 1.9mL | | | |
| -30% acrylamide mix | 1.7mL | | | |
| -1.5 M Tris (pH 8.8) | 1.3mL | | | |
| -10% SDS | 0.05mL | | | |
| -10% ammonium persulfate | 0.05mL | | | |
| -TEMED | 0.002mL | | | |

**Table 2. Solutions for preparing 5% stacking gels for tris-glycine SDS-polyacrylamide gel electrophoresis.**

| **Solution Components** | **Per 5mL (1mL per Gel)** |
|---|---|
| $H_2O$ | 2.77mL |
| 30% acrylamide mix | 0.83mL |
| 0.5 M Tris-HCl (pH 6.8) | 1.26mL |
| 10% SDS | 0.05mL |
| 10% ammonium persulfate | 0.05mL |
| TEMED | 0.005mL |

Ninety-five percent ethanol and Kimwipes were used to get the glass spacers and plates squeaky clean. They were then washed with distilled $H_2O$, wiped clean and air dried. The stacking and resolving gels solutions were prepared in 50mL conical tubes based on appropriate recipes in Tables 1 and 2. For brevity, the making of the 10% SDS is described. Per gel, 1.9 mL of $H_2O$ and 1.7 mL of 30% acrylamide solution were mixed. Next, the gel spacers and holders were assembled in a gel casting apparatus. Then 10% APS and TEMED were added to the resolving gel solution and mixed by slowly rotating the conical tube. Using a P1000 pipette, 1mL solutions were added to the top of the spacer/plate totaling 5mL per spacer/plate. One milliliter of $H_2O$ was immediately placed on top of the apparatus-gel mix. After the extra solution left in the 50mL conical tube solidified, the $H_2O$ on top of each gel was poured off. The corners of sterile paper towels were used to remove excess $H_2O$ from the gels. 10% APS and TEMED were added to the stacking gel solution and slowly mixed 4-5 times. One milliliter was added to each gel, being careful to avoid bubbles. Gel combs were added immediately following the stacking gel solution to make 10 wells per gel. After checking for polymerization by looking at the extra solution left

in the conical tube, the gels were placed in a clean, damp glass container. The glass container had damp, sterile paper towels to keep the gels from drying out. The gels were then stored in a 4°C fridge.

## 2.6 SDS-PAGE

SDS-PAGE was performed to separate the proteins of interest. Frozen protein samples were heated in a heat block set to 100°C. The protein samples were placed in the heat block for five minutes, and immediately placed on ice to cool for five minutes. After cooling, samples were centrifuged briefly for 10-15 seconds. Two SDS-PAGE gels were acquired from a 4°C fridge, assembled into the running modules and put inside an SDS-PAGE container apparatus. When running samples, the samples treated with chloramphenicol were run on a separate gel from those that were not treated. The SDS-PAGE container was filled with 1X SDS running buffer made from diluting 10X SDS running buffer (30g of Tris base, 144g of glycine, 10g of SDS and 1000mL of $H_2O$). When dealing with SDS, a mask was worn until it was dissolved in solution. Combs were then taken out of the gels. Five microliters of Super Signal Molecular Weight Ladder (ThermoFisher Scientific) and 5$\mu$L of Kaleidoscope Protein Ladder (Biorad) were mixed and placed into the first well on each gel. Next to the ladder, 20$\mu$L of each protein sample was added. SDS-PAGE was run at 125V for 1.5 hours. After 1.5 hours, a blue color at the bottom of the gels was verified to ensure that the proteins had been separated. The SDS-PAGE apparatus was then turned off and unplugged.

**2.7 Western Blot**

Electroblotting was the first step in western blotting. The electroblotting container was filled with 1X transfer buffer which was diluted from 10X transfer buffer (30g of Tris base, and 144g glycine with 1000mL of $H_2O$). Specifically, 1X transfer buffer was then made by adding 100mL of 10X transfer buffer to 200mL of methanol and 700mL of $H_2O$ and mixed thoroughly with a magnetic stir bar. Next, four filter papers, four sponges, forceps, a gel extractor, a roller, and two nitrocellulose membranes were soaked in 1X transfer buffer for approximately ten minutes. The apparatus used for electroblotting was made by placing the sponge first on the back black part of the apparatus, followed by filter paper. Then the gel extractor was used to cut the well tops off of the gels before placing it next. Forceps were then used to carefully place the nitrocellulose membrane on top of the gel. Filter paper went next, followed by another sponge. The roller was used to make sure there were no air bubbles in the sandwich. The sandwich was then fully made by closing and locking the apparatus. The process was repeated for the other gel as well. The bottom side of the sandwich faced the back of the container. An ice pack was then acquired from a -20°C freezer and placed in the front part of the container to ensure that the container did not heat up too much. The top was closed and hooked into a power supply unit and electroblotting was run at 100V for one hour. The apparatus was then turned off and unplugged. The ice pack was cleaned and placed back in the -20°C freezer.

Blocking was the next step in western blotting. For blocking, two plastic containers were cleaned with 1X TBST (1mL of Tween 20 and 50mL of 20X TBST to 950mL of $H_2O$). Twenty X TBST was made by adding 160.13g NaCl, 4.025g KCl, and 46.03g of Tris base to 1000mL $H_2O$. The 20X TBST mixture was then autoclaved and stored at room temperature.  The nitrocellulose

membranes were then placed in clean containers respectively with forceps while checking for protein transfer by displaying the colored protein ladder facing up. Blots were then blocked with 10mL 5% BSA or 5% milk depending on the primary antibody used. For phosphorylated proteins we used 5% BSA while for non-phosphorylated proteins we used 5% milk. Five percent BSA and 5% milk were made by adding 2.5g BSA or 2.5g of milk respectively to 50mL of 1X TBST. Five percent BSA and 5% milk were stored in a 4°C fridge. Blots were then placed in a 4°C fridge overnight.

Washes and adding primary antibodies were the next steps in western blotting. After attaining the nitrocellulose membranes from the 4°C fridge, milk was poured out and 15mL 1X TBST was added. The blots were then placed on a rocker for five minutes to complete the first wash. Two more washes followed. Primary antibodies were then acquired based on the protein of interest. Primary antibody proteins used in this experiment include PKA, phosphor-PKA (p-PKA), p-PKA substrates, GAPDH, HSP60, GSK-3$\beta$, phospho-GSK-3$\beta$ (p-GSK-3$\beta$), CREB, and phospho-CREB (p-CREB) were used. PKA is the protein of interest, and upon phosphorylation, becomes active and thus creating p-PKA. P-PKA substrates were looked at to determine the activity of possible phosphorylation of subsequent proteins by PKA. GAPDH was used as a standard control as all HeLa cells have GAPDH. HPS60 is used as a control for cells infected with *C. trachomatis.* GSK-3$\beta$ is a protein directly phosphorylated by PKA at the Serine 9 position. CREB is another protein that is phosphorylated by PKA. It was important to note whether the antibodies used were rabbit or mouse antibodies. Primary antibodies were mixed into blocking buffer in 10mL volumes in 15mL conical tubes. Solutions were thoroughly mixed by slowly inverting the conical tubes 9-10 times. After the last wash was poured out, 5mL of the

antibody solution was placed on each blot, and blots were either placed on a rocker to evenly distribute the antibodies on the blot for one hour or put in a 4°C fridge overnight.

Secondary antibody addition was the next step. After acquiring the blots from the fridge, the primary antibody solutions were poured out and followed by three washes with 1X TBST. Secondary anti-antibodies made from Cell Signaling Technologies (Anti-rabbit IgG, HRP-linked Antibody or Anti-Mouse IgG, HRP linked Antibody) were attained from a -20°C freezer to match the primary antibodies. Secondary anti-antibody solutions were all made in a 15mL conical tube with 10mL of 5% milk and 10$\mu$L of the appropriate secondary anti-antibody. After mixing by inversion, and pouring out the last wash of 1X TBST, 5mL of the secondary anti-antibody solution was added to the blots. Blots were placed on a rocker at the same speed as before for one hour or put in a 4°C fridge overnight.

The final step in western blotting is developing the blot. Three more washes with 1X TBST were applied to each blot followed by adding fresh 1X TBST after the last wash. Blots were developed with Thermo Scientific (SuperSignal West Pico PLUS Chemiluminescent Substrate and SuperSignal West Femto). Femto was used with phosphorylated proteins as they were generally harder to visualize with chemiluminescence while Pico was used with non-phosphorylated proteins. Gloves were worn while adding 2mL of substrate via a P1000 pipette and pipette tips to each blot after pouring out the 1X TBST into a waste container. Substrate sat on blots for one minute (Pico) or five minutes (Femto) before being placed in a clean, laminated cover paper to keep the blot clean when placed in the imager. The imager used in this experiment was the

Fluorchem E FE0622 system (ProteinSimple). Exposure time varied for imaging each protein of interest form ten seconds to five minutes.

Chapter 3: Results

## 3.1 PKA substrates are altered during *C. trachomatis* infection

As PKA is a kinase known to phosphorylate many host proteins (Brandt, Kenny, Rohde, Martinez-Quiles, & Backert, 2009; Erazo, Yee, Banfield, & Kinchington, 2011), the first round of experiments were aimed to see if PKA substrate phosphorylation was altered during infection by *C. trachomatis.* Phopspho-PKA substrates were looked at to determine the activity of possible phosphorylation of subsequent proteins by PKA. Two conditions were assessed: one where chloramphenicol was added to inhibit bacterial protein synthesis and another where no chloramphenicol was added. Chloramphenicol is a bacteriostatic antibiotic which does not kill the pathogen, but only inhibits growth. Each experiment contained two controls: GAPDH and HSP60. GAPDH was used as a loading control to ensure that the same amount of protein sample was loaded into each well. GAPDH is a housekeeping gene that is found in HeLa cells and the presence of GAPDH verifies that each well had sample and the same amount of total protein. HSP60 is a heat shock protein found in *C. trachomatis* and is therefore used to detect the presence and abundance of *C. trachomatis* during infection. As the post infection time increased, so did the amount of HSP60 corresponding to increased growth of *C. trachomatis*. HSP60 was not found in any samples with chloramphenicol, verifying that *C. trachomatis* protein production and growth were impaired.

Assessment of PKA substrate phosphorylation along with the controls of GAPDH and HSP60 are shown in Figure 2. The first observation to note is that in the conditions lacking chloramphenicol, a trend of increased phosphorylation of PKA substrates is seen by the gradual increase of intensity of the dark bands on the blots. The second observation to note is that this increase is phosphorylation was only seen in the samples where chloramphenicol was not present, allowing for *C. trachomatis* infection to occur. This suggests that the altered PKA substrate phosphorylation was due to active *Chlamydia* infection. This is in contrast to when chloramphenicol was present, *C. trachomatis* protein synthesis is inhibited and the *Chlamydia* are unable to grow, and there is no increase in PKA substrate phosphorylation over 48 hours. Thus, these results show that *C. trachomatis* infection results in upregulation in the phosphorylation of PKA substrates in a time dependent manner. The results were consistent when two different PKA substrate antibodies were tested with both having the same outcome (Figure 2). Each of the two PKA substrate antibodies used detected different substrates that PKA phosphorylates with some overlap. P-PKA Substrate 9621 detects proteins containing a phospho-serine/threonine residue with arginine at the -3 position which detects substrates of the ACG family kinases which include PKA and PKC. P-PKA Substrate 9624 is less specific and detects proteins containing a phospho-serine/threonine residue with arginine at both the -2 and -3 position. Interestingly, even though most of the changes during the course of infection resulted in increased PKA substrate phosphorylation, there were a few proteins that appeared to decrease in phosphorylation as the infection progressed (Figure 2). Once again, this only occurred in conditions were *C. trachomatis* was actively growing (not treated with chloramphenicol).

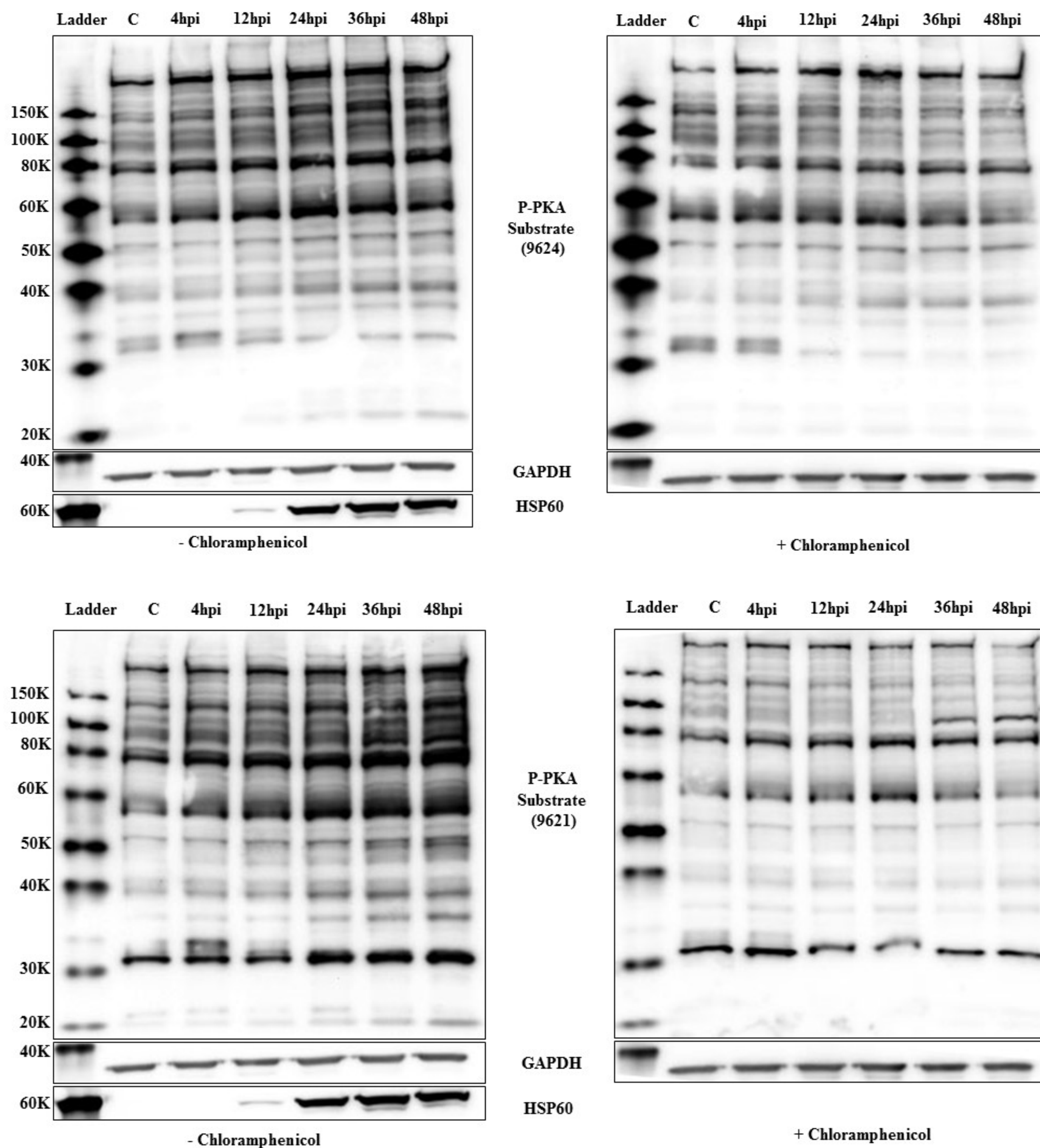


**Figure 2. Western blot analysis of phosphorylated PKA substrates during *C. trachomatis* infection.** Top) Phospho-PKA substrate (9624), Bottom) Phospho-PKA Substrate (9621). GAPDH loading controls and HSP60 to detect *C. trachomatis* are shown. No chloramphenicol

addition shown in the left panels and chloramphenicol addition shown in the right panels. HSP60 is not shown with the presence of chloramphenicol as there were no bands present.

### 3.2 PKA and P-PKA levels are altered during *C. trachomatis* infection

Given the substantial changes in PKA substrate phosphorylation we wanted to determine if this was due to overall changes in PKA protein or activated PKA (P-PKA) during infection. To do this, the same protein samples were analyzed by western blot using primary antibodies to PKA and P-PKA (an antibody detecting PKA phosphorylated at T197 which corresponds to activity (Cauthron, Carter, Liauw, & Steinberg, 1998)) and are shown in Figure 3. The total amount of PKA seems to be uniform in both conditions (with and without the addition of chloramphenicol) and for the duration of experiment suggesting that *C. trachomatis* did not alter the total amount of PKA in the host cells. However, some differences can be observed with P-PKA in that the amount of phosphorylated PKA seems to increase in a time dependent manner when cells are infected with *C. trachomatis.* No such activity is observed when infected cells are treated with chloramphenicol. This shows that *C. trachomatis* is upregulating the phosphorylating PKA, albeit only moderately.

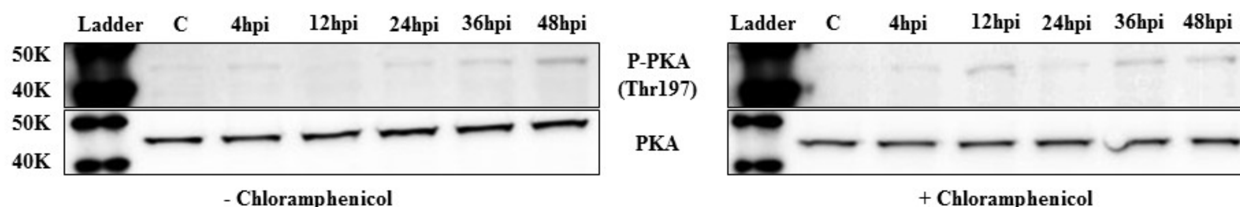


**Figure 3. Western blot analysis of Phospho-PKA (Thr197) & total PKA during *C. trachomatis* infection.** Top) Phospho-PKA (Thr197), Bottom) Total PKA, Left) Lacking chloramphenicol, Right) Treated with chloramphenicol.

**3.3 Phosphorylation of GSK-3$\beta$ is altered during *C. trachomatis* infection**

There are many potential PKA substrates that can be detected using the phosphospecifc antibodies used in Figure 2. To verify these results, the amount of p-GSK-3$\beta$ and GSK-3$\beta$ were looked at. GSK-3$\beta$ is a substrate of PKA that is phosphorylated by PKA at the site of serine 9 (Li et al., 2000). As the post infection time increased, the amount of p-GSK-3$\beta$ increased for cells infected with *C. trachomatis*. There is no change in the infected cells treated with chloramphenicol. The total amount of GSK-3$\beta$ is shown to be held constant in cells infected with *C. trachomatis* and mock infected cells. This shows that GSK-3$\beta$ gets phosphorylated by PKA as the post infection time increases.

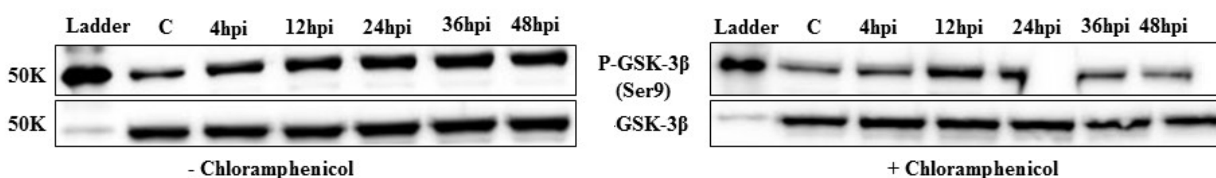


**Figure 4. Western blot analysis of Phospho-GSK-3$\beta$ (Ser9) & total GSK-3$\beta$ during *C. trachomatis* infection.** Top) phospho-GSK-3$\beta$ (Ser9), Bottom) Total GSK-3$\beta$, Left) Lacking chloramphenicol, Right) Treated with chloramphenicol.

**3.4 Phosphorylation of CREB is not altered during *C. trachomatis* infection**

CREB is another protein that can be phosphorylated by PKA at the serine 133 position (Naqvi, Martin, & Arthur, 2014). The amount CREB that gets phosphorylated into P-CREB increases with post infection time. However, the same holds true for samples with chloramphenicol where *C. trachomatis* is not present. Therefore, one cannot deduce that the increase in P-CREB is caused by *C. trachomatis* and may simply be due to a gradual increase in cell confluency and

number. The amount of CREB is shown to slightly increase as post infection time increases for cells infected with *C. trachomatis.* However, no such distinction is evident in mock infected cells (condition with chloramphenicol).

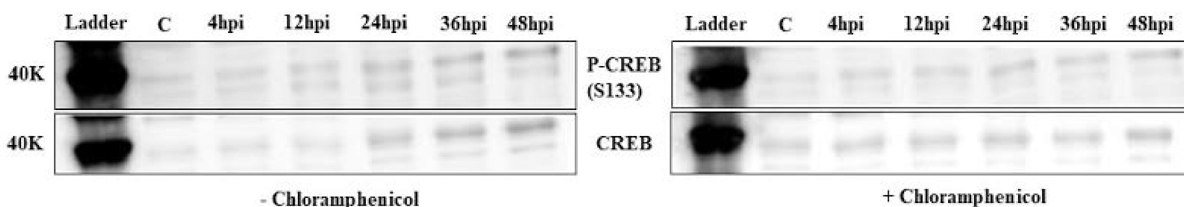


**Figure 5. Western blot analysis Phospho-CREB (S133) & total CREB during *C. trachomatis* infection.** Top) p-CREB (S133), Bottom) Total CREB, Left) Lacking chloramphenicol, Right) Treated with chloramphenicol.

**4.0 Discussion**

The results show that *C. trachomatis* is indeed manipulating host cellular PKA signaling. This is evidenced in the assessment of total PKA substrates and also a specific PKA substrate, GSK-3$\beta$**.** This suggests that PKA may be used or needed in the latter parts of the *C. trachomatis* life cycle, especially since phosphorylation was time dependent. The life cycle of *C. trachomatis* starts with EBs, then goes to RBs, then back to EBs to infect other cells. Once the host cell can no longer provide enough nutrients for *Chlamydia*, the RBs get converted back to EBs to infect other cells via lysis of the host cell or extrusion of the EBs. Lysis of cells releases EBs all at once while the extrusion process consists of a budding of the host cellular membrane filled with EBs that go on to infect other host cells. Multiple extrusions can come from a single infected host cell. PKA may play a role in the activation of EBs in the extrusion process. Additional evidence gathered by a graduate student in the lab shows that PKA is recruited to the Src rich domains of the

parasitophorous vacuole and that the recruitment is time dependent with the greatest amount of recruitment occurring late in infection. This shows more relevance as to why it may be involved in the extrusion process since it is on the membrane of the Chlamydial inclusion. However, the extrusion process for *C. trachomatis* occurs at 48 hours post infection while the upregulation of phosphorylated PKA starts before this. Other late stage events include turning RBs back to EBs or transitioning to enlarged aberrant bodies for a dormant persistent stage upon stress. PKA may also be used for regulating transcription factors to turn genes on and off in the later stages of the infection process. Overall, PKA phosphorylates many proteins, so its uses may not be limited to one purpose.

Interestingly, PKA substrates were shown to become both dephosphorylated and phosphorylated during the course of infection with *C. trachomatis* with most substrates showing a general trend for the latter. This raises the potential that some PKA substrates may be only needed at certain times during the Chlamydia life cycle and not at others. Future experiments exploring the temporal need of PKA substrates may provide some valuable insights perhaps into the requirements for the growth and differentiation of EBS to RBs and then RBs back to EBs.

In a different study on another strain, *C. pneumonia*, GSK-3$\beta$ was found to be interacting with an Inc (Flores & Zhong, 2015). Another study shows that GSK-3$\beta$ was identified as a potential interacting partner for TepP in *C. trachomatis* (Bugalhao & Mota, 2019). TepP is a type III secreted effector protein for *C. trachomatis*. This led to the belief that *C. trachomatis* might manipulate this protein as a potential target as well. This study showed GSK-3$\beta$ to have increased phosphorylation during the late stages of the infection cycle as well. GSK-3$\beta$, like

PKA, is also involved to phosphorylate many other proteins involved in nearly every process of the cell (Sutherland, 2011). This makes it hard to identify any specific reason over another as to what its manipulation might entail. One protein that is commonly manipulated by GSK-3$\beta$ is glycogen synthase. After phosphorylating glycogen synthase, a reduction in glycogen synthesis occurs (Sutherland, 2011). This may be relevant to *C. trachomatis* so that it does not waste more energy on synthesis in order to spend it on cell exit mechanisms. Therefore, it is not surprising that we see an increase in GSK-3$\beta$ as there are a few reasons as to why one could hypothesize *C. trachomatis* would want to manipulate GSK-3$\beta$ as mentioned earlier, but we are not sure as to why.

CREB is a protein that is also involved in many other processes in the cell as it a transcription factor (Wen, Sakamoto, & Miller, 2010). It is known to play key roles in cell proliferation, differentiation and survival. This protein was tested for upregulation in phosphorylation as post time infection increased because it has been shown to do so in other intracellular pathogens like *Salmonella, Shigella,* and *Yersinia* (Wen et al., 2010). By manipulating CREB, these pathogens have been able to evade the apoptotic mechanisms that macrophages use to kill abnormal cells. *C. trachomatis* is an intracellular pathogen that must evade apoptosis for its own survival, so CREB was measured to possibly show a reason as to how *C. trachomatis* evades host cellular apoptosis. CREB showed only a slight increase in phosphorylation in cells without chloramphenicol. This small increase is not substantial enough to conclude that *C. trachomatis* alters the phosphorylation of CREB. This observation falls in line with other studies. Specifically, *Coxiella burnetii*, another intracellular pathogen, has also been shown to upregulate

phosphorylation of PKA without altering phosphorylation of CREB (MacDonald, Kurten, & Voth, 2012).

As mentioned earlier, altered PKA phosphorylation has been implicated in multiple variants of cancer, which is an uncontrolled growth of cells. Ultimately, we wanted to explore if Chlamydial manipulation of PKA could be a potential avenue that might link *C. trachomatis* to the increased risk of cervical cancer. This study suggests that PKA is somehow manipulated during infection, albeit to what end is still to be determined. More research is required to identify exact reasons for PKA manipulation in *C. trachomatis* infected cells.

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