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RESEARCH ARTICLE

Chlamydia trachomatis recruits protein kinase C during infection

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One sentence summary: *Chlamydia trachomatis* is an obligate intracellular pathogen that uses host proteins such as protein kinase C for intracellular survival.

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ABSTRACT

Chlamydia trachomatis is a significant pathogen with global and economic impact. As an obligate intracellular pathogen, *C. trachomatis* resides inside the inclusion, a parasitophorous vacuole, and depends on the host cell for survival and transition through a biphasic development cycle. During infection, *C. trachomatis* is known to manipulate multiple signaling pathways and recruit an assortment of host proteins to the inclusion membrane, including host kinases. Here, we show recruitment of multiple isoforms of protein kinase C (PKC) including active phosphorylated PKC isoforms to the chlamydial inclusion colocalizing with active Src family kinases. Pharmacological inhibition of PKC led to a modest reduction of infectious progeny production. PKC phosphorylated substrates were seen recruited to the entire periphery of the inclusion membrane. Infected whole cell lysates showed altered PKC phosphorylation of substrates during the course of infection. Assessment of different chlamydial species showed recruitment of PKC and PKC phosphorylated substrates were limited to *C. trachomatis*. Taken together, PKC and PKC substrate recruitment may provide significant insights into how *C. trachomatis* manipulates multiple host signaling cascades during infection.

Keywords: *Chlamydia*; protein kinase C; microdomains; phosphorylation

INTRODUCTION

Chlamydia trachomatis is a Gram-negative, obligate intracellular pathogen causing a variety of infections in humans. The genus consists of distinct serovars categorized into three biovars based on tissue tropisms. Serovars A–C cause trachoma, which is the leading infectious cause of preventable blindness worldwide (Burton and Mabey 2009). Serovars D–K represent the most common sexually transmitted infection in the USA and are associated with sequelae such as infertility, ectopic pregnancy and pelvic inflammatory diseases (Gerbase, Rowley and Mertens 1998; Da Ros and Schmitt Cda 2008). Serovars L1, L2 and L3 cause invasive urogenital infections termed lymphogranuloma venereum (Schachter 1999). Infections with *C. trachomatis* have also been associated with increased risk for cervical cancer (Zhu *et al.* 2016).

The chlamydial developmental cycle involves alternation between two morphologically and physiologically distinct forms, elementary bodies (EBs) and reticulate bodies (RBs). EBs are the highly infectious form that historically were defined as metabolically inactive; however, this paradigm is gradually shifting toward EBs having some metabolic activity (Omsland *et al.* 2012; Cosse, Hayward and Subtil 2018). RBs on the other hand are non-infectious, replicating and metabolically active form. EBs, upon endocytosis into the host cell, convert into RBs that

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replicate by polarized cell division (Abdelrahman *et al.* 2016) and the phagosome is rapidly modified by *Chlamydia*-encoded proteins to form a parasitophorous vacuole referred to as an inclusion (Moulder 1991; Wyrick 2000; Coombes and Mahony 2002; Abdelrahman and Belland 2005). Chlamydial development occurs exclusively inside the inclusion and as the infection proceeds, RBs convert back to EBs. At the end of the life cycle, EBs are released via cell lysis and/or extrusion to infect neighboring cells (Hybiske and Stephens 2007).

As an intracellular pathogen, *C. trachomatis* manipulates several host signaling networks including kinase pathways and governs these activities at the inclusion membrane, the key host cell−pathogen interface. Active Src family kinases are recruited to inclusion membrane microdomains throughout the infection process and are important for inclusion development and infectious progeny formation (Mital *et al.* 2010; Mital and Hackstadt 2011). Src kinases are known to regulate the phosphatidylinositol 3-kinase (PI3K) signaling cascade, which is associated with increased host survival via AKT/protein kinase B activation during *C. trachomatis* infection (Verbeke *et al.* 2006; Olive *et al.* 2014; Carpenter *et al.* 2017). Likewise, *C. trachomatis* recruits host myosin light chain kinase (MLCK) to inclusion microdomains for the phosphorylation of myosin light chain 2 (MLC2) promoting the extrusion mechanism of host cell exit (Lutter *et al.* 2013). The negative regulator of MLC2 phosphorylation, myosin phosphatase (MYPT1), is recruited by the chlamydial inclusion membrane protein, CT228 (Lutter *et al.* 2013); thus, *Chlamydia* host cell exit is modulated by a pathogen-directed balance of host kinase (MLCK) and phosphatase (MYPT1) activities targeting MLC2 (Lutter *et al.* 2013; Shaw *et al.* 2018). Upstream regulation of these host signaling pathways that affect infectious progeny formation as well as host cell exit is largely unknown, although protein kinase C (PKC) has been implicated as a regulator of MYPT1 activity (Toth *et al.* 2000).

PKC enzymes are classified according to the nature of their regulatory domains, calcium dependency and activators as either conventional (PKCα, PKCβ and PKCλ), novel (PKCδ, PKCθ, PKCε and PKCη) or atypical (PKCμ, PKC1/λ and PKCζ) (Johannes *et al.* 1994; Wu-Zhang and Newton 2013). Phosphorylation of specific residues of PKC activates the enzyme to subsequently phosphorylate a variety of host proteins and signaling pathways (Keranen, Dutil and Newton 1995; Nishizuka 2001). Activated PKC enzymes transduce various extracellular signals that lead to the generation of the lipid second messenger diacylglycerol (DAG) in order to regulate various cellular functions, such as growth and proliferation, migration, survival and apoptosis (Keranen, Dutil and Newton 1995; Nishizuka 1995; Nishizuka 2001; Wu-Zhang and Newton 2013). Usurping PKC pathways may benefit chlamydial during its parasitic growth within the host cell as well as provide alternative exit modes for bacterial dissemination. Previous studies demonstrated the enrichment of both DAG, an activator of PKC, and Green Fluorescent Protein (GFP)-tagged PKC variants proximal to the chlamydial inclusion (Tse *et al.* 2005), suggesting a role for PKC in *C. trachomatis* infection.

Taken together, we hypothesized that activated/ phosphorylated PKC would localize to microdomains of the chlamydial inclusion and affect bacterial growth. In this study, we demonstrate recruitment of different isoforms of active PKCs to microdomains while PKC substrates localize throughout the periphery of the *C. trachomatis* inclusion. Moreover, we show that PKC substrates are differentially phosphorylated during infection. Inhibition of PKC led to a modest reduction of infectious progeny production suggesting the requirement of PKCs during *C. trachomatis* intracellular development.

MATERIALS AND METHODS

Chlamydia strains and cell culture

HeLa cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum (FBS) at 37°C with 5% CO_2. *Chlamydia trachomatis* serovars D, B/Jali20/OT and L2/434/Bu, *Chlamydia muridarum* mouse pneumonitis MoPN, *Chlamydophila caviae* GPIC and *Chlamydia pneumoniae* AR-39 were propagated in HeLa 229 cells and purified by Renografin density gradient centrifugation as previously described (Caldwell, Kromhout and Schachter 1981).

Antibodies

Anti-Src-pY419 (Clone 9A6, Cat# 05-677; Millipore Sigma, Burlington, MA) was used to detect active Src-family kinases. Phospho-PKC antibody sampler kit (Cat# 9921; Cell Signaling Technology, Danvers, MA) was used to detect phosphorylated PKC isoforms. Anti-PKC substrate (Cat# 2261; Cell Signaling Technology) and Anti-Akt substrate (Cat# 9614; Cell Signaling Technology, Danvers, MA) were used to detect phosphorylated PKC and Akt substrates, respectively. Anti-phospho PKC pan (Cat# PA5-38428; ThermoFisher Scientific, Waltham, MA) was used to detect phosphorylated PKC isoforms. Anti-Hsp60 (Clone A57-B9 Cat# MA3023; ThermoFisher Scientific, Waltham, MA) was used to detect *Chlamydia* by western blotting and anti-GAPDH (Cat #25778) was used to detect GAPDH. Anti-rabbit or anti-mouse DyLight 594 and DyLight 488 (Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies for immunofluorescence. Anti-rabbit-HRP or anti-mouse HRP (Cell Signaling Technology, Danvers, MA) was used for western blot analysis. Anti-EB rabbit polyclonal antibody (Cat# PA1-73069; ThermoFisher Scientific, Waltham, MA) was used to stain EBs and anti-rabbit DyLight 594 was used as a secondary for progeny count.

Immunofluorescence microscopy

HeLa cells were cultured in 24-well plates (CellTreat Scientific, Pepperell, MA) containing round cover slips and infected with *C. trachomatis* serovars L2 D, B/Jali20/OT, *C. muridarum*, *C. pneumoniae* and *C. caviae* GPIC at MOI of ~0.5. HeLa cells infected with L2, *C. muridarum* and *C. caviae* were fixed at 18 hours post-infection while cells infected with serovar D, B-Jali/20 and *C. pneumoniae* were fixed at 42 hours post-infection. For the time course of infection experiments, HeLa cells were infected with an MOI of ~0.5 and fixed at 12, 24, 36 and 48 hours post-infection. All cells were fixed with methanol or 4% paraformaldehyde followed by permeabilization with Triton X-100, washed in phosphate buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA). Cells were treated with primary antibodies against PKC isoenzyme, phospho-Akt substrate, phospho (Ser) PKC substrate, phospho-PKC-pan, anti-*Chlamydia* LPS or Src family kinases followed by wash in PBS and blocking with BSA. After the PBS washes, anti-mouse/rabbit secondary antibodies and DAPI were added. Coverslips were mounted onto slides using Dako Mounting Medium (Agilent Technologies, Santa Clara, CA) and observed using a Leica DMI6000B (Leica, Buffalo Grove, IL).

PKC inhibition and infectious progeny enumeration

HeLa cells in 24-well plates (CellTreat Scientific, Pepperell, MA) were infected with *C. trachomatis* serovar L2 EBs at MOI of 1 and treated with inhibitor/control at 4 hours post-infection. Staurosporine (Cat# S4400-.1MG; Sigma-Aldrich, St Louis, MO) and Go6983 (Cat# G1918-1MG; Sigma-Aldrich, St Louis, MO) were used at 0.5 μM concentration to inhibit PKCs and DMSO was used as a vehicle control. The 0.5 μM concentration of PKC inhibitors did not affect cell viability. Cells were incubated for 48 hours at 37°C in the presence of 5% CO_2. After incubation, cells were lyzed with sterile water and serially diluted (10^{-1} to 10^{-6}) in Hank's balanced salt solution (HBSS). Fresh HeLa cell monolayers in 24-well plates were infected with 200 μL of each dilution and incubated for 24 hours followed by methanol fixation and staining with anti-EB antibody and anti-rabbit DyLight 594. Inclusions were counted on 30 microscopic fields for each time point using a Leica DMI6000B microscope and total inclusion forming units (IFUs)/mL were calculated.

Western blotting

HeLa cells, in 24-well plates, were infected with *C. trachomatis* L2 elementary bodies (EBs) at MOI of 1. Infected cells were grown in RPMI containing chloramphenicol (200 μg/mL) or vehicle (ethanol) 1-hour post-infection (hpi). Infected cells were lyzed at different time points during infection (4, 12, 24, 36, 48 hpi). Mock infected HeLa cells were used as control. For time points 24, 36 and 48 hpi, cells were treated with RPMI containing 150 μM CPAF inhibitor (Clasto-lactacystin β-lactone, Cat# 426102; Millipore Sigma, Burlington, MA) for 1 hour before lysis. Cells were washed with 1× PBS before lysis. One hundred microliters of 8 M urea supplemented with 325 units/mL Benzoase nuclease (Millipore Sigma, Burlington MA) and 1× protease inhibitor cocktail (ThermoFisher Scientific, Waltham MA) was added per well of 24-well plates and incubated on ice for 10 minutes. Lysate was collected and 100 μL of 2× Laemmli buffer was added. Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk in 1× Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. After blocking, membranes were incubated with rabbit polyclonal antibodies against PKC substrates diluted in 5% BSA in 1× TBST at 4°C overnight. Reacting proteins were detected using horseradish peroxidase conjugated anti-rabbit antibodies and observed by enhanced chemiluminescence using SignalFire ECL reagents (Cell Signaling Technology, Danvers, MA). Immunoblot images were acquired using Fluorchem E FE0622 system (ProteinSimple, San Jose, CA).

Statistical analysis

Statistical analysis was carried out using Prism 5.0 (GraphPad Software, San Diego, CA). One-way ANOVA was performed with *post hoc* Tukey test for the comparison of infectious progeny in the presence and absence of PKC inhibitors.

RESULTS

PKC is recruited to the *C. trachomatis* inclusion

Many different isoforms of PKC are produced in eukaryotic cells (Wu-Zhang and Newton 2013); hence, there are several candidates for recruitment to the *C. trachomatis* inclusion. To assess whether PKC is recruited to the inclusion during *C. trachomatis* L2 infection, a general phospho-PKC (pan) antibody that detects levels of multiple phosphorylated isoforms of PKC (PKCα, PKCβ, PKCδ and PKCε) was utilized initially. This antibody detects the PKC isoforms phosphorylated at a C-terminal residue homologous to the threonine residue at position 497. Figure 1A shows that phospho-PKC was recruited to the inclusion at small discrete punctate regions that resemble active Src kinase microdomain staining (Mital *et al.* 2010; Mital and Hackstadt 2011). Uninfected neighboring cells contain only diffuse staining of phospho-PKC and clearly lack any discrete or punctate staining. The timing of phospho-PKC recruitment was monitored via a time course of infection at 12, 24, 36 and 48 hours post-infection (Fig. 1B). At 12 hours post-infection, phospho-PKC is seen to be diffuse throughout the infected cells, similar to uninfected cells. However, by 24 hours post-infection phospho-PKC is seen recruited to the chlamydial inclusion in discrete microdomains, which become more pronounced as infection progresses.

Pharmacological inhibition of PKC results in decreased recoverable IFUs

The presence of PKC in inclusion membrane microdomains suggests specific and active recruitment by *C. trachomatis* during infection. To determine whether PKC is important for chlamydial intracellular growth and survival, we pharmacologically inhibited PKC enzymatic activity and assessed the possibility of growth defects. Two different PKC pharmacological inhibitors, staurosporine and Go 6893, were added at 4 hours post-infection and maintained in the cell culture supernatant for the duration of the infection. At 48 hours the cells were lyzed, serially plated on fresh HeLa cells and infectious progeny enumerated. Treatment by both inhibitors, staurosporine and Go 6893, modestly reduced the recoverable infectious progeny by ~20% compared to the control (no inhibitor treatment) (Fig. 1B; $P < 0.001$) indicating the importance of host PKC for optimal chlamydial development.

Multiple phosphorylated isoforms of PKC are recruited to inclusion microdomains

Recruitment of different isoforms and phosphorylation states of PKC to the chlamydial inclusion were assessed by immunofluorescent microscopy. The endogenous forms of PKCα, PKCδ and PKD/PKCμ were tested for colocalization with active Src Kinases (Fig. 2A and B). The antibodies employed detected total levels of PKC isoforms and are not specific to any phosphorylation. PKC enzymes are regulated by phosphorylation at specific sites, which determines their consequent enzymatic activity. To determine whether the PKC isoforms recruited to the chlamydial microdomains were in their activated state, immunofluorescent microscopy was performed utilizing phosphospecific antibodies to different isoforms of PKC (Fig. 2C). As can be clearly seen in Fig. 2C, each phosphorylated PKC isoform also colocalized with active Src kinases in the microdomains.

Phosphorylated PKC substrates are recruited throughout the periphery of the inclusion membrane

The phosphorylated PKC isoforms recruited to the inclusion microdomains correlated with the active state of the PKC

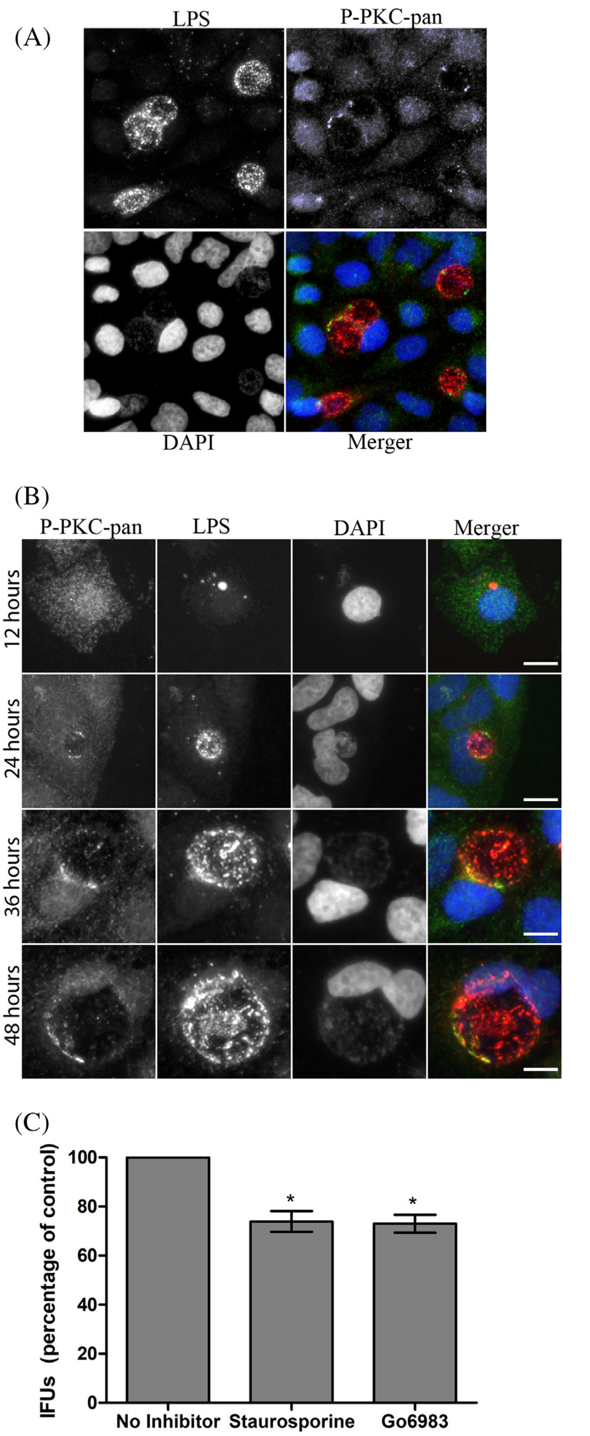

Figure 1. PKC is recruited to the chlamydial inclusion and PKC inhibitors reduce *C. trachomatis* IFUs. **(A)** HeLa cells were infected with *C. trachomatis* for 36 hours and prepared for immunofluorescence microscopy. Phospho-PKC-pan antibody was used to detect endogenous levels of phosphorylated PKCs recruited to the chlamydial inclusion. **(B)** Phospho-PKC-pan recruitment was monitored over a time course of infection. Shown are 12, 24, 36 and 48 hours post-infection. **(C)** HeLa cells were infected with *C. trachomatis* L2 and treated with staurosporine (0.5 μM) or Go6893 (0.5 μM) with the inhibitors being present throughout infection. At 48 hours post-infection, the cells were lyzed to release *Chlamydia* and cell lysates were serially diluted and used to infect HeLa cell monolayers. Infection was allowed to proceed for 18 hours, cells were fixed with cold methanol, processed for microscopy and 30 fields of view were counted for each condition in triplicate. Error bars indicated standard deviation. Scale bar, 10 μm. $^{*}P < 0.001$.

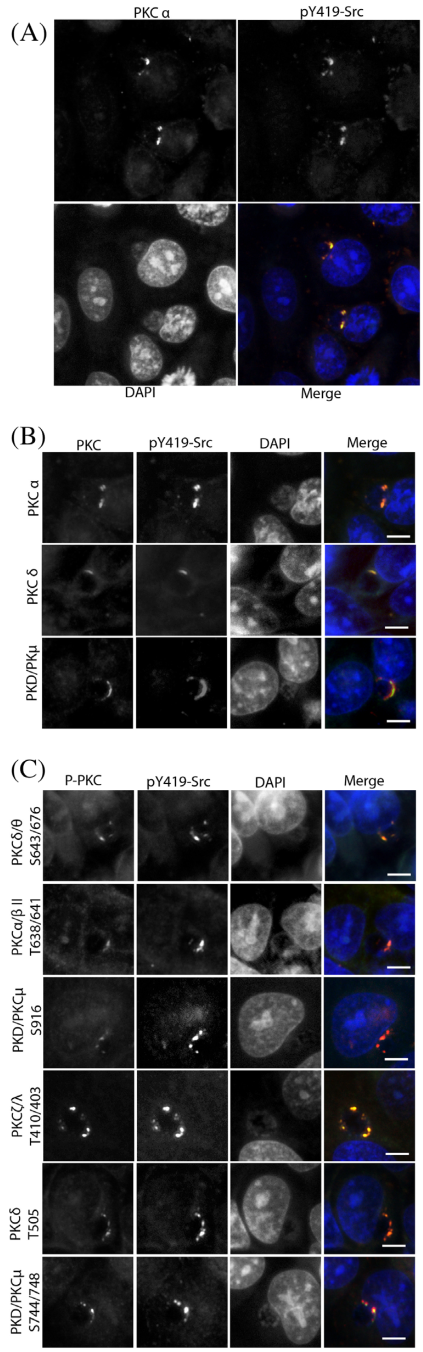

Figure 2. Multiple isoforms of PKC are recruited to the inclusion microdomains by *C. trachomatis*. HeLa cells were infected with *C. trachomatis* L2 for 18 hours, fixed and prepared for immunofluorescence microscopy. **(A)** Endogenous levels of PKCα showing colocalization with active Src kinases (pY419-Src). Multiple infected and uninfected cells are shown. **(B)** Endogenous levels (irrespective of phosphorylation state) of PKCα, PKDδ and PKD/PKCμ were assessed for colocalization with active Src kinases (pY419-Src). **(C)** Phosphospecific antibodies were used to detect the different phosphorylated isoforms of PKC also colocalizing with active Src Kinases (pY419-Src). Scale bar, 10 μm.

enzymes since the antibodies used were specific to the phosphorylated sites and do not cross react with non-phosphorylated sites. PKC enzymes are Serine/Threonine kinases known to phosphorylate their substrates at specific residues. A commercial phosphoserine PKC antibody detects various proteins phosphorylated at serine residues that are flanked by an Arginine or

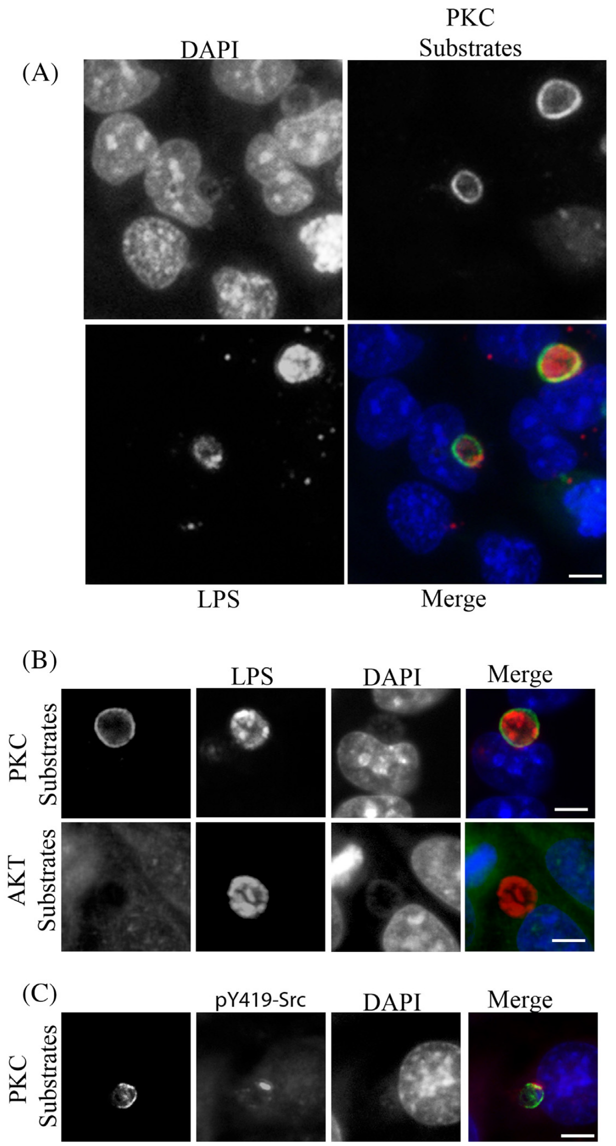

Figure 3. PKC phosphorylated substrates are recruited to the entire periphery of the *C. trachomatis* inclusion. HeLa cells were infected with *C. trachomatis* L2 at an MOI of 0.5 for 18 hours, fixed in cold fixative and processed for immunofluorescence microscopy. **(A)** Phospho (Ser)-PKC substrates are shown surrounding the chlamydial inclusion (*Chlamydia* detected with anti-*Chlamydia* LPS). Multiple infected and uninfected cells can be seen for comparison. **(B)** Phospho (Ser)-PKC substrates and Akt substrates were detected with phosphospecific antibodies for recruitment to the chlamydial inclusion (*Chlamydia* detected with anti-*Chlamydia* LPS). **(C)** Active Src kinases (pY49-Src) are shown colocalizing in discrete microdomain overlapping the phospho-(Ser)-PKC. Scale bar, 10 μm.

Lysine at the −2 and +2 positions, respectively, with a hydrophobic residue at the +1 position. Positive staining with this phosphospecific PKC substrate antibody would suggest PKC phosphorylation of substrates. Immunofluorescent microscopy of *C. trachomatis* L2-infected HeLa cells revealed abundant staining of PKC phosphorylated substrates throughout the entire periphery of the inclusion (Fig. 3A, B and C). An antibody against Akt specific substrates was used as a comparative control to demonstrate the specificity of PKC substrates recruited to the chlamydial inclusion (Fig. 3B). Active Src kinases were seen to form microdomains that colocalized with the PKC substrates at the inclusion membrane (Fig. 3C)

PKC and PKC substrate recruitment are limited to *C. trachomatis* serovars

The species-specific recruitment of PKC and PKC substrates to the chlamydial inclusion during infection was examined using two different serovars of *C. trachomatis* (serovars B and D) as well as *C. muridarum*, *C. caviae* and *C. pneumoniae*. HeLa cells were infected with each species, fixed at designated times post-infection and probed with either the PKC-pan antibody (to detect endogenous forms of phosphorylated PKC isoforms) or the phosphoserine PKC substrate antibody. Interestingly, only the *C. trachomatis* serovars B and D displayed recruitment of phosphorylated PKC and PKC substrates to the inclusion during infection (Fig. 4). PKC was recruited to the inclusion in small discrete microdomain-like regions for serovar B and D (Fig. 4A) in the same manner as what was observed for serovar L2 (Fig. 1). Likewise, the PKC phosphorylated substrates were recruited to the entire periphery of chlamydial inclusions in *C. trachomatis* serovars B and D (Fig. 4B), consistent with the recruitment detected in serovar L2 (Fig. 3). *Chlamydia pneumoniae*, *C. muridarum* and *C. caviae* were negative for recruitment of both phosphorylated PKC isoforms and PKC phosphorylated substrates.

Phosphorylation of PKC substrates is altered during *C. trachomatis* infection

PKC is an essential host protein known to regulate numerous downstream targets and signaling pathways. The various isoforms of PKC are all regulated by phosphorylation (Reyland 2009) and exhibit overlapping roles within the cell. Due to the complexity of PKC isoforms recruited to the *C. trachomatis* inclusion, total PKC activity was monitored by visualizing the phosphorylation of downstream substrates (Fig. 5). HeLa cells were infected with *C. trachomatis* L2 with and without chloramphenicol treatment and whole cell lysates collected at 4, 12, 24, 36 and 48 hours post-infection (mock infected HeLa lysates served as a negative control) followed by immunoblot analysis using the phospho (Ser)-PKC substrate antibody to detect substrates phosphorylated at serine residues. Changes in the phosphorylation of PKC substrates were observed as early as 4 hours post-infection with multiple proteins increasing in phosphorylation status during the course of infection (Fig. 5). This is especially evident at 24, 36 and 48 hours post-infection when the bacterial burden was the greatest within the cell. No increased phospho (Ser)-PKC substrate phosphorylation was observed in the chloramphenicol-treated infected cells, suggesting that *Chlamydia* were actively manipulating PKC activity during infection. GAPDH was monitored as a control during infection to control for total protein content.

DISCUSSION

Recruitment of PKC to the inclusion microdomains is not surprising as other obligate intracellular pathogens, such as *Coxiella burnetti*, require PKC for its intracellular development (Hussain *et al.* 2010) and PKC activates NF-κB signaling during *Rickettsia rickettsii* infections (Sahni *et al.* 1999; Sahni and Rydkina 2009). In this study, all PKC isoforms, including PKC isoforms phosphorylated at specified residues, were recruited to the inclusion microdomains wherein multiple kinases reside: Src, Yes and Fyn (Mital *et al.* 2010; Mital and Hackstadt 2011) and MLCK (Lutter *et al.* 2013). The additional recruitment of multiple phosphospecific isoforms of PKC to the already kinase-rich microdomains

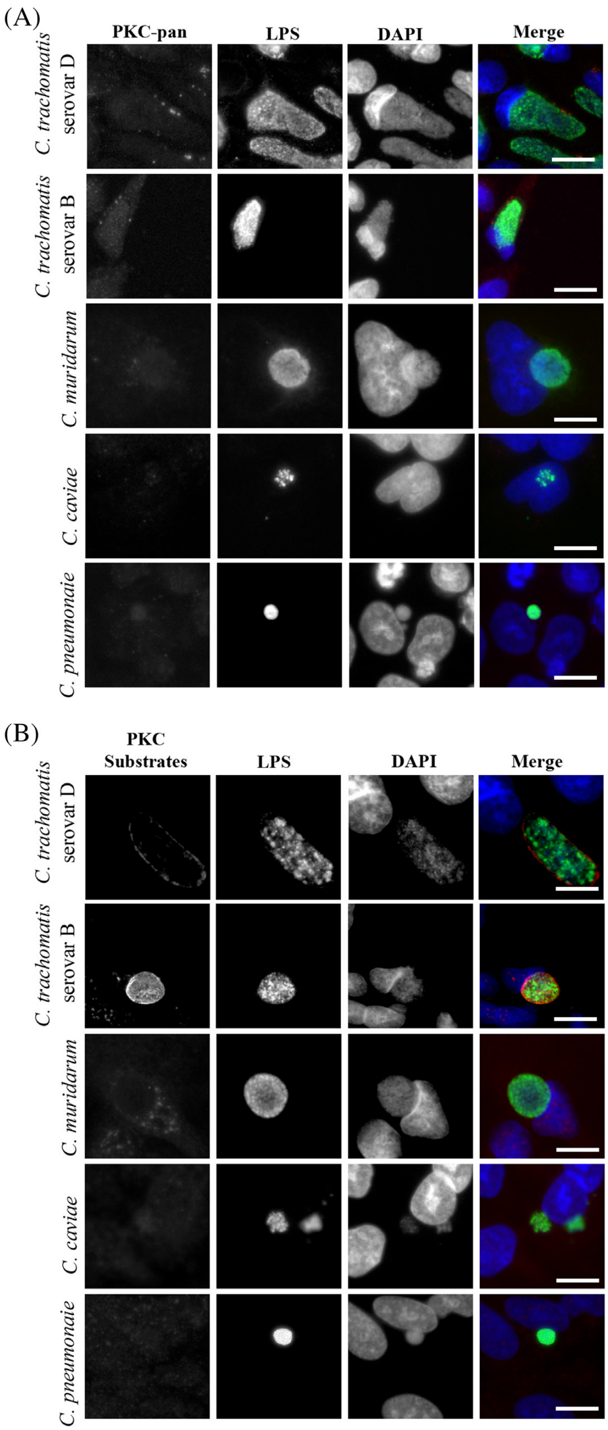

Figure 4. PKC and PKC substrate recruitment are limited to *C. trachomatis* serovars. HeLa cell monolayers were infected with *C. trachomatis* serovar D (42 hours), *C. trachomatis* serovar B, (42 hours), *C. muridarum* (18 hours), *C. caviae* (18 hours) and *C. pneumoniae* (42 hours) at an MOI of ~0.5, fixed in cold methanol and processed for immunofluorescence microscopy. **(A)** Total phospho-PKC recruitment as detected by a phospho-PKC-pan antibody (arrows indicate discrete regions of phospho-PKC recruitment) and **(B)** recruitment of phosphorylated PKC substrates are shown. All *Chlamydia* species were detected with anti-*Chlamydia* LPS antibody. Scale bar, 10 μm.

supports the notion that these regions are hubs for kinase activity on the inclusion membrane. Previous studies have demonstrated the recruitment of multiple isoforms of PKC to the vicinity of the inclusion, but the ectopic expression of fluorescently tagged PKC constructs reported in *Chlamydia*-infected cells did not elucidate the enzymatic activity or phosphorylation states nor were they shown to be localized to the inclusion microdomains (Tse *et al.* 2005). A recent phosphoproteomic study by Zadora *et al.* (2019) demonstrated that not only *C. trachomatis* affected multiple host signaling pathways including PKC signaling but also 25 chlamydial proteins, majority of which were inclusion membrane proteins, were phosphorylated at predicted PKC phosphorylation sites (Zadora *et al.* 2019). This study strongly suggested that PKC, among other host kinases, regulates *C. trachomatis* proteins via phosphorylation.

The recruitment patterns for PKCs and phosphorylated PKC substrates were found to be quite different. The PKCs all colocalized at microdomains with active Src kinases whereas the phosphorylated PKC substrates displayed substantial recruitment throughout the periphery of the inclusion. This distinction contradicts the concept that PKC is first recruited and then acts to phosphorylate proteins in the vicinity of the microdomains. Rather, our data suggest that host proteins may be recruited after PKC phosphorylation. The significant recruitment of PKC substrates to the periphery of the inclusion also suggests that there may be multiple proteins recruited during the course of infection. The variation in recruitment pattern to either microdomains or inclusion periphery has been established with multiple host proteins and chlamydial Incs. Chlamydial Incs including CT850 (Mital *et al.* 2010, 2015), CT101 (Mital *et al.* 2010; Nguyen, Lutter and Hackstadt 2018) and CT228 (Lutter *et al.* 2013; Shaw *et al.* 2018) all localize to microdomains whereas IncD (CT115) (Agaisse and Derre 2014), IncG (CT118) (Scidmore and Hackstadt 2001) and IncA (CT119) (Scidmore-Carlson *et al.* 1999) are expressed circumferentially around the periphery of the inclusion. The differential localization pattern of Incs on the inclusion membrane affords *C. trachomatis* the luxury of recruiting host proteins in an explicit manner depending on the demands of parasitism. As such, PKC substrates may be optimally positioned circumferentially whereas active PKC enzymes may need to reside within kinase-rich regions for proper activation of signaling cascades through phosphorylation events. It is also likely that many chlamydial Incs are also phosphorylated by PKC during infection (Zadora *et al.* 2019) contributing for the circumferential staining of PKC phosphorylated substrates around the chlamydial inclusion.

Species specific recruitment of PKC and phosphorylated PKC substrates to the inclusion proposes a distinctive role for *C. trachomatis* infection and putative requirements for species specific infection, which is unremarkable as recruitment of other kinases or host proteins during infection has also been reported as species specific. *Chlamydia trachomatis* serovars and *Chlamydia pneumoniae* recruit Src-family kinases to inclusion microdomains; however, there is no evidence of kinase-rich microdomains identified for *C. caviae* or *C. muridarum* (Mital and Hackstadt 2011). Myosin phosphatase, which regulates the activity of MLC2, is recruited to the inclusion by *C. trachomatis* serovars and *C. muridarum*, but not *C. pneumoniae* or *C. caviae* (Lutter *et al.* 2013). It is clear that the prerequisites for host kinases and phosphorylated host proteins vary between chlamydial species and may be key to addressing questions in chlamydial biology, host tropism and pathogenesis.

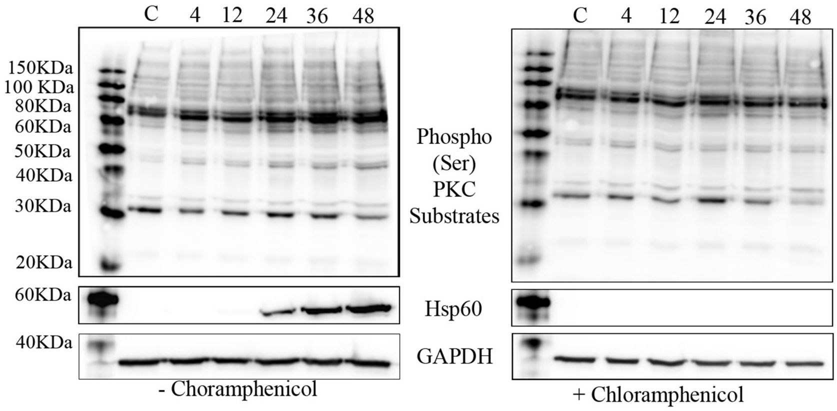

Figure 5. PKC substrates are differentially phosphorylated during *C. trachomatis* infection. *Chlamydia trachomatis* L2-infected HeLa cell lysates with and without chloramphenicol treatment were collected at 4, 12, 24, 36 and 48 hours post-infection and probed for serine-phosphorylated PKC substrates. Mock infected HeLa lysates, C, serve as a control and total protein reference. GAPDH was used as a loading control and Hsp60 was used to detect *Chlamydia*. Molecular mass is shown in kilodaltons (kD).

Given the diversity of PKC recruitment to the *C. trachomatis* inclusion, two different PKC pharmacological inhibitors were used in this study: staurosporine, which is a potent inhibitor of multiple PKC isoforms (PKCα, PKCγ and PKCη but less potent to PKCδ, PKCε and PKCζ) (Ward and O'Brian 1992), and Go 6893, a broad spectrum PKC inhibitor that targets all isoforms (PKCα, PKCβ, PKCγ, PKCδ, PKCζ and PKCμ) (Peterman *et al.* 2004). Inhibitors were used at low concentrations (0.5 μM) to limit host cell death. Both inhibitors produced similar results with no significant differences between the two different inhibitors indicating that, despite their different PKC targets, both were able to target PKC isoforms relevant to *Chlamydia* infection. Pharmacological treatment of *Chlamydia*-infected cells exhibited a modest yet significant reduction in recoverable infectious progeny, suggesting that PKC may be an important host factor during chlamydial infection. In previous studies, it was demonstrated that *C. trachomatis* inhibited apoptosis induced by staurosporine (Xiao *et al.* 2004) and that *Chlamydia*-infected HeLa cells are resistant to staurosporine induced apoptosis (Fan *et al.* 1998; Dean and Powers 2001). This supports our observation that the viability of cells infected by *C. trachomatis* were not affected by staurosporine and the reduced infectious progeny is due to inhibition of PKC rather than host cell viability.

In summary, our findings show that PKC is a host cell kinase manipulated by *C. trachomatis* during infection with multiple phosphorylated isoforms being recruited to the inclusion membrane microdomains. PKC is an integral host protein involved in multiple host signaling pathways that regulate many proteins through phosphorylation events. Given the central role PKC plays in host cell dynamics, it is not surprising to see recruitment of phospho-PKC kinases and PKC phosphorylated substrates to the periphery inclusion membrane. Future investigations to identify and characterize the recruited PKC phosphorylated substrates will provide significant insights into *Chlamydia* pathogenic mechanisms and may represent targets for future therapies designed to treat intracellular pathogens.

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Conflict of interest. None declared.

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