

# UC Irvine

## UC Irvine Previously Published Works

### Title

Induction and inhibition of CPAF activity during analysis of Chlamydia-infected cells

### Permalink

<https://escholarship.org/uc/item/4bt8p6j5>

### Journal

Pathogens and Disease, 73(1)

### ISSN

0928-8244

### Authors

Johnson, Kirsten A

Lee, Jennifer K

Chen, Allan L

et al.

### Publication Date

2015-02-01

### DOI

10.1093/femspd/ftv007

Peer reviewed

## RESEARCH ARTICLE

# Induction and inhibition of CPAF activity during analysis of *Chlamydia*-infected cells

Kirsten A. Johnson<sup>1,#</sup>, Jennifer K. Lee<sup>1,#</sup>, Allan L. Chen<sup>2,4</sup>, Ming Tan<sup>2,3</sup> and Christine Sütterlin<sup>1,\*</sup>

<sup>1</sup>Department of Developmental and Cell Biology, University of California, Irvine, Irvine, CA 92697-2300, USA,

<sup>2</sup>Department of Microbiology and Molecular Genetics, University of California, Irvine, Irvine, CA 92697-4025, USA and <sup>3</sup>School of Medicine, UC Irvine, Irvine CA 92697-3950, USA

\*Corresponding author: Department of Developmental and Cell Biology, University of California, Irvine, 4242 McGaugh Hall, Irvine, CA 92697-2300, USA. Tel: +949-824-7140; E-mail: [suetterc@uci.edu](mailto:suetterc@uci.edu)

#equal contributions

<sup>4</sup>Present address: Department of Microbiology Immunology and Molecular Genetics, UCLA, Los Angeles, CA 90095, USA.

**One sentence summary:** Novel factors contributing to artifactual CPAF proteolysis are identified and recommendations are made to prevent promiscuous CPAF activity when performing any protein analysis of *Chlamydia*-infected cells.

Editor: Dr. Dagmar Heuer

## ABSTRACT

Studies of the chlamydial protease CPAF have been complicated by difficulties in distinguishing bona fide intracellular proteolysis from *in vitro* proteolysis. This confounding issue has been attributed to CPAF activity in lysates from *Chlamydia*-infected cells. We compared three methods that have been used to inhibit *in vitro* CPAF-mediated proteolysis: (1) pre-treatment of infected cells with the inhibitor clasto-lactacystin, (2) direct cell lysis in 8 M urea and (3) direct lysis in hot 1% SDS buffer. We identified a number of experimental conditions that reduce the effectiveness of each method in preventing CPAF activity during lysate preparation. The amount of *in vitro* proteolysis in a lysate was variable and depended on factors such as the specific substrate and the time in the intracellular infection. Additionally, we demonstrated for the first time that artifactual CPAF activity is induced before cell lysis by standard cell detachment methods, including trypsinization. Protein analysis of *Chlamydia*-infected cells therefore requires precautions to inhibit CPAF activity during both cell detachment and lysate preparation, followed by verification that the cell lysates do not contain residual CPAF activity. These concerns about artifactual proteolysis extend beyond studies of CPAF function because they have the potential to affect the analyses of host and chlamydial proteins from *Chlamydia*-infected cells.

**Key words:** proteolysis; substrates; proteolytic activity

## INTRODUCTION

CPAF is a conserved chlamydial protease that cleaves and degrades many host and chlamydial proteins (Paschen *et al.*, 2008; Zhong 2009). However, the significance of this proteolysis to the bacterial infection has been called into question because of ongoing CPAF proteolytic activity during the analysis of *Chlamydia*-infected cells (Chen *et al.*, 2012; Bavoil and

Byrne 2014). Specifically, lysates of *Chlamydia*-infected cells, prepared under standard conditions, contain CPAF that is active at 4°C and resistant to a standard protease inhibitor cocktail (Zhong *et al.*, 2000; Chen *et al.*, 2012). As a result, CPAF-dependent proteolysis of numerous host and chlamydial proteins appears to occur during lysate preparation, rather than in intact infected cells.

Received: 28 September 2014; Accepted: 16 January 2015

© The Author 2015. Published by Oxford University Press on behalf of FEMS. All rights reserved. For permissions, please e-mail: [journals.permissions@oup.com](mailto:journals.permissions@oup.com).

Importantly, the proteolysis of 12 published CPAF substrates was no longer detected by Western blot analysis when precautions were taken to inhibit CPAF activity during lysate preparation (Chen et al., 2012; Grieshaber and Grieshaber 2014). Methods to inhibit CPAF activity include pre-treatment of *Chlamydia*-infected cells with the CPAF inhibitor clasto-lactacystin prior to cell detachment and lysis, and direct lysis of cells in 8 M urea or hot 1% SDS buffer (Chen et al., 2012; Snaveley et al., 2014). Using the hot 1% SDS method, Snaveley et al. (2014) reported that vimentin is still cleaved and identified LAP1 as a new CPAF substrate. Vimentin cleavage was at greatly reduced levels and occurred at much later times during the intracellular infection than previously published (Kumar and Valdivia 2008). Thus, CPAF activity in infected cell lysates can misrepresent the extent and timing of proteolysis that may have occurred in an infected cell.

How then should one interpret protein cleavage or degradation that is detected in lysates when efforts are made to inhibit CPAF during lysate preparation? Depending on whether there is residual CPAF activity in a lysate, such proteolysis could represent bona fide effects on a substrate in the infected cell, or there could still be *in vitro* proteolysis during lysate preparation. To address this issue, we compared the effectiveness of three methods that can prevent CPAF-mediated proteolysis during lysate preparation. We also examined if experimental variables, such as the time in the infection, the cell collection procedure and the protein substrate being analyzed, can limit the effectiveness of these methods in inhibiting *in vitro* CPAF activity. Based on our findings, we outline an approach for preventing and checking for CPAF activity during protein analysis of *Chlamydia*-infected cells.

## MATERIALS AND METHODS

### Antibodies

The following antibodies were used in this study: mouse anti-vimentin (Sigma–Aldrich); mouse anti-Erk 1/2 (Cell Signaling Technology); mouse anti-p65/RelA (Santa Cruz Biotechnology); rabbit anti-RFX5 (Rockland Immunochemicals); rabbit anti- $\alpha$ -tubulin (Abcam); rabbit anti-HsSAS-6 (generous gift from Dr Pierre G6nczy, 6cole Polytechnique F6d6ral de Lausanne); goat anti-mouse 680LT and goat anti-rabbit 800CW (both from LICOR); goat anti-mouse HRP and goat anti-rabbit HRP (both from Jackson ImmunoResearch Laboratories). For information about CPAF protein substrates used in this study, see Table 1.

### Cell culture

HeLa cells (ATCC) were grown in 6-well dishes in Advanced DMEM (4.5 g glucose L<sup>-1</sup>) (Invitrogen) supplemented with 2% fetal bovine serum (Hyclone/Thermo Fisher) and 2 mM GlutaMAX-I (Invitrogen). All cell lines were grown in 5% CO<sub>2</sub> at 37°C and regularly screened for *Mycoplasma* contamination by PCR (Ossewaarde et al., 1996).

**Table 1.** Summary of CPAF substrates.

Reported substrate	Reported proteolysis	References
HsSAS-6	Cleavage	Johnson, Chen, S6tterlin and Tan (unpublished)
p65	Cleavage	Lad et al. (2007), Christian et al. (2010)
RFX5	Degradation	Zhong et al. (2000, 2001)
Vimentin	Cleavage	Kumar and Valdivia (2008), Snaveley et al. (2014)

## Chlamydia infections

Cell monolayers were infected with *Chlamydia trachomatis* serovar L2 (L2/434/Bu), LGV biovar, at a multiplicity of infection of 3 in sucrose-phosphate-glutamic acid (SPG). In parallel, uninfected control experiments were performed as mock infections in SPG alone. Infections were carried out by centrifugation at 700 × g in a Sorvall Legend Mach 1.6R centrifuge for 1 h at room temperature. After centrifugation, the inoculum was replaced by fresh cell culture medium without cycloheximide and monolayers were incubated at 37°C and 5% CO<sub>2</sub>. Chlamydial elementary bodies were verified to be free of *Mycoplasma* contamination by PCR (Ossewaarde et al., 1996).

## Methods for cell collection and lysate preparation

**Lysis in RIPA buffer:** cells were harvested by trypsinization (TrypLE Express, Invitrogen) for 3–5 min at 37°C and transferred to a 15 mL conical tube on ice. The dish was washed twice with 1X PBS to recover remaining cells, and the washes were added to the 15 mL conical tube. The cells were pelleted by centrifugation at 1500 rpm for 3 min at 4°C and lysed on ice for 10 min in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40] supplemented with protease inhibitors [2 mM pepstatin, 150 mM aprotinin (both from MP Biochemicals), 1 mM leupeptin (Calbiochem), 1 mM PMSF (Acros)]. The cells were resuspended by pipetting up and down in approximately 1 mL of ice-cold lysis buffer per 5 × 10<sup>6</sup> cells. Lysates were cleared by centrifugation at 13000 × g for 10 min at 4°C, and protein concentrations were determined by Bradford assay (Bio-Rad).

**Direct lysis in urea:** the monolayer of infected cells was washed with 1X PBS. A solution of 8 M urea (or 6–7 M urea where indicated) supplemented with 325 U mL<sup>-1</sup> of Benzonase Nuclease (Sigma–Aldrich) was then directly added to the cell monolayer at a volume of 1 mL per 6 wells of a 6-well dish, and left for 10 min on ice. The resulting lysates were pooled and protein concentrations were determined by the DC protein assay (Bio-Rad).

**Lysis in hot 1% SDS buffer:** cells were washed with 1X PBS. 1% SDS buffer [50mM Tris (pH 7.5), 150mM NaCl, 1% SDS] was heated to 95°C and directly added to cell monolayers at a volume of 1 mL per 6 wells of a 6-well dish. Cells were scraped from the monolayers, and the resulting lysates from individual wells were pooled and supplemented with 325 U mL<sup>-1</sup> of Benzonase Nuclease (Sigma–Aldrich). Protein concentrations were determined by the DC protein assay (Bio-Rad).

**Cell collection methods (Fig. 4):** *Chlamydia*-infected cells were collected by incubation in 500  $\mu$ l trypsin (TrypLE Express, Invitrogen) or 500  $\mu$ l accutase (Fisher Scientific) per well of a 6-well dish for 3–5 min at 37°C, or by scraping monolayers directly into 500  $\mu$ l 1X PBS per well of a 6-well dish. Cells were then transferred to a 15 mL conical tube on ice. The dish was washed twice with 1X PBS, and the washes were added to the 15 mL conical tube. The cells were pelleted by centrifugation at 1500 rpm for 3 min at 4°C and lysed on ice for 10 min in 8 M urea supplemented with 325 U mL<sup>-1</sup> of Benzonase Nuclease (Sigma–Aldrich).

### Clasto-lactacystin treatment

**Clasto-lactacystin pre-treatment:** clasto-lactacystin  $\beta$ -lactone (Cayman Chemical), dissolved in methyl acetate, was added to the cell culture medium at a final concentration of 150  $\mu$ M for 60 min prior to cell processing. For example, samples of *Chlamydia*-infected cells at 48 hours post-infection (hpi) were treated with clasto-lactacystin at 47 hpi for 60 min and then processed. In parallel control experiments, methyl acetate as the solvent was added to the culture medium. Treated cells were collected by trypsinization followed by cell lysis in RIPA buffer (as described above).

**Clasto-lactacystin in lysis buffer:** *Chlamydia*-infected cells were harvested by trypsinization, and cell pellets were lysed on ice for 10 min in RIPA buffer containing protease inhibitors (as described above) and 150  $\mu$ M of clasto-lactacystin  $\beta$ -lactone (Cayman Chemical). In parallel control experiments, methyl acetate was used instead of clasto-lactacystin in the RIPA buffer.

### Western blot analysis

Cell lysates were diluted in Laemmli sample buffer [50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.1% bromophenol blue] and incubated at 95°C for 5 min to denature proteins. Samples containing equal amounts of total protein were loaded and resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes, blocked in 5% milk PBST (5% dry powdered milk, 0.1% Tween-20, 1X PBS) and incubated in primary antibodies followed by HRP-conjugated (Jackson ImmunoResearch) or IRDye-conjugated (LI-COR) secondary antibodies. Blots were imaged by enhanced chemiluminescence or the LI-COR Odyssey SA infrared imaging system.

### In vitro CPAF activity assay

*Chlamydia*-infected HeLa cells at various times in the infection were processed by the three methods described above (trypsinization followed by lysis in RIPA buffer, direct lysis in 8 M urea, or direct lysis in hot 1% SDS). 4–8  $\mu$ g of these infected cell lysates, as the source of CPAF, were incubated with 12.5  $\mu$ g of uninfected HeLa cell lysate, as the source of host protein substrates, at 37°C for 30 min in CPAF reaction buffer [25 mM Tris (pH 8.0), 150 mM NaCl, 3 mM DTT]. Reactions were terminated by adding Laemmli sample buffer and boiling for 5 min. 16.5–20.5  $\mu$ g of protein from these samples was analyzed by Western blotting with specific primary antibodies.

## RESULTS AND DISCUSSION

We first examined the use of the CPAF inhibitor clasto-lactacystin to inhibit CPAF activity in cell lysates (Chen, et al. 2012). HeLa cells were infected with *C. trachomatis* L2 and collected at 36 hpi with a standard procedure involving trypsinization and lysis in RIPA buffer. We then tested the cell lysates for CPAF activity with an *in vitro* assay in which we incubated a small amount of each infected cell lysate, as a potential source of CPAF, with uninfected cell lysate as a source of host substrates. Without precautions, the infected cell lysate caused the complete cleavage of the host centrosomal protein HsSAS-6 in the *in vitro* activity assay, demonstrating that this lysate contained CPAF activity (Fig. 1a). However, pre-treatment of an infected cell monolayer with 150  $\mu$ M clasto-lactacystin for 60 min before cell collection inhibited all CPAF activity in the lysate, as shown by the ab-

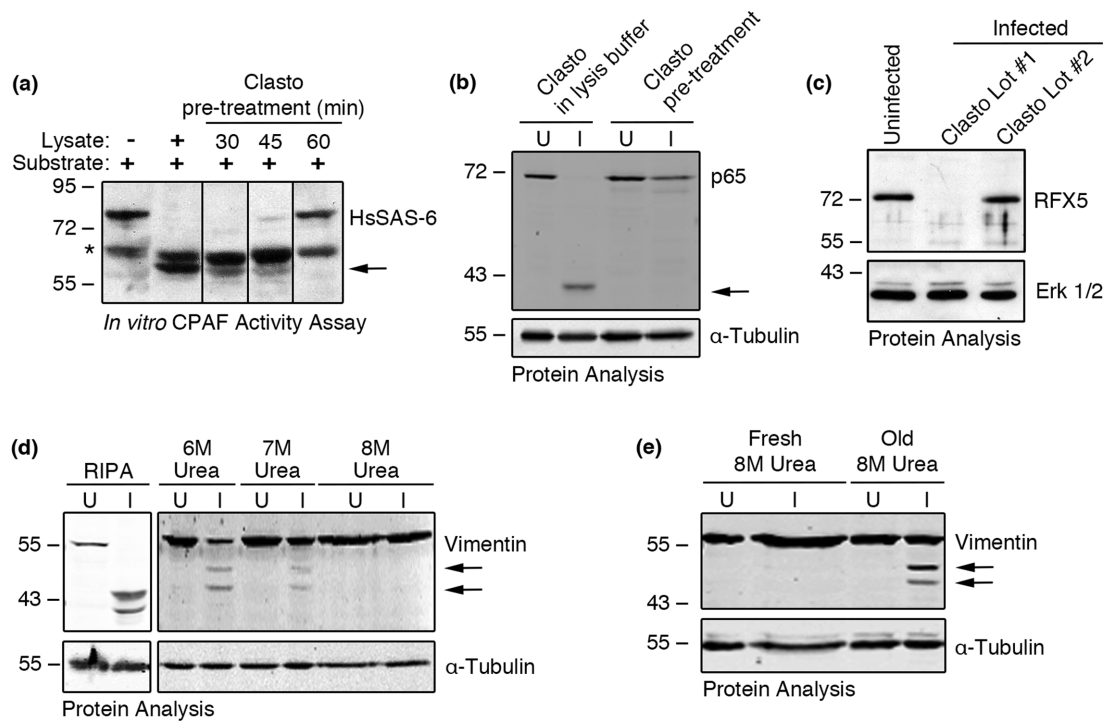
sence of a HsSAS-6 cleavage product in the *in vitro* activity assay (Fig. 1a). Shorter pre-treatment times, using the same concentration of clasto-lactacystin, did not eliminate CPAF activity in the lysate (Fig. 1a). Protease inhibitors do not typically require pre-treatment, and are usually added to the lysis buffer, but addition of 150  $\mu$ M clasto-lactacystin to the RIPA lysis buffer, without pre-treating the cells, did not prevent artifactual proteolysis of the host protein p65 (Fig. 1b).

Our experiments also confirmed the reported batch-to-batch variability of clasto-lactacystin activity against CPAF (Snively et al., 2014; Tan and Sütterlin 2014). Only some batches of clasto-lactacystin prevented degradation of the host protein RFX5 in infected cell lysates (Fig. 1c), even though we used the same concentration (150  $\mu$ M) and pre-treatment time (60 min). Clasto-lactacystin batches that were ineffective against native CPAF did not inhibit recombinant CPAF either, although they retained their anti-proteasomal activity (data not shown). Thus, clasto-lactacystin can be an effective inhibitor of CPAF, but the specific batch, concentration and treatment time has to be carefully selected and optimized.

We also tested the denaturing agent urea, which has been used to non-specifically inhibit CPAF activity during lysate preparation (Chen, et al. 2012). Direct lysis of *C. trachomatis*-infected cells at 48 hpi in 8 M urea completely prevented vimentin proteolysis. In contrast, direct lysis in 6 or 7 M urea produced lysates containing vimentin cleavage products that were larger than the cleavage products seen in RIPA lysates. These partial cleavage products have been described before (Kumar and Valdivia 2008) and are consistent with proteolysis caused by residual CPAF activity in these lysates (Fig. 1d). Lysis in an old 8 M urea solution produced a cell lysate that also contained partially cleaved vimentin (Fig. 1e). Thus, fresh 8 M urea is required to effectively inhibit *in vitro* CPAF activity, and we recommend making the 8 M urea solution on the same day it is to be used.

These studies demonstrate the importance of confirming the effectiveness of the methods used to inhibit CPAF activity during lysate preparation. Lysates of *Chlamydia*-infected cells are typically examined for evidence of CPAF-mediated cleavage or degradation of specific proteins by Western blot analysis ('Protein Analysis', Fig. 2a). We propose that each infected cell lysate should also be tested for residual CPAF activity with an 'In vitro CPAF Activity Assay' (Fig. 2a). In this assay, we incubate infected cell lysate, as a potential source of CPAF, with uninfected HeLa cell lysate as a source of host proteins and analyze the reaction products by Western blot. Loss of the host protein being studied and/or appearance of cleavage products indicate that the infected cell lysate contains residual CPAF activity. We only use small amounts of this lysate to measure residual CPAF enzymatic activity, which makes it less likely that any detected cleavage products originate from the infected cell lysate before the *in vitro* assay. To confirm the absence of carry over, we routinely check if this amount of infected cell lysate has detectable cleavage products by Western blot analysis (Fig. 2). Residual CPAF activity can also be measured by performing the *in vitro* activity assay with a GFP-tagged substrate that is not present in the infected cell lysate (Fig. S1, Supporting Information). Ideally, this *in vitro* CPAF activity assay should be performed immediately after lysate preparation because freezing and thawing can decrease residual CPAF activity (data not shown).

Using this approach, we compared the effectiveness of the three methods reported to inhibit CPAF activity. Cell lysates generated by directly adding 8 M urea to a monolayer of *Chlamydia*-infected cells from 32 to 56 hpi did not contain any detectable



**Figure 1.** Methods to inhibit CPAF activity during infected cell lysate preparation are not always effective. (a) *Chlamydia*-infected HeLa cells were pre-treated with 150  $\mu$ M clasto-lactacystin for 30, 45 or 60 min prior to collection at 36 hpi by trypsinization and lysis in RIPA buffer. Infected cell lysates were tested for CPAF activity in an *in vitro* activity assay (outlined in Fig. 2a), which was analyzed by Western blotting with antibodies to the host protein HsSAS-6. The first lane with uninfected cell lysate alone shows uncleaved HsSAS-6. A cross-reacting band is marked with '\*'. (b) Uninfected and infected cells were collected by trypsinization at 48 hpi, and lysed in RIPA buffer containing 150  $\mu$ M clasto-lactacystin. Alternatively, infected cells were pre-treated with 150  $\mu$ M clasto-lactacystin for 60 min prior to lysate preparation in RIPA buffer. Proteolysis of p65 as a substrate was monitored in the lysates by Western blot analysis with p65 antibodies, with  $\alpha$ -tubulin serving as a loading control. (c) Uninfected and infected cells were pre-treated with two different batches of clasto-lactacystin at 150  $\mu$ M for 60 min and then lysed in RIPA buffer. Lysates were assayed by Western blot for RFX5 degradation with antibodies to RFX5 or Erk 1/2 as a loading control. (d) Monolayers of uninfected and infected cells at 48 hpi were either collected by trypsinization and lysed in RIPA buffer or lysed directly in urea at the indicated concentrations. (e) Monolayers of uninfected and infected cells at 48 hpi were lysed directly in fresh or old solutions of 8 M urea. For (d) and (e), lysates were analyzed for vimentin proteolysis by Western blotting with vimentin antibodies. HsSAS-6, p65 and vimentin proteolysis products are indicated with arrows.

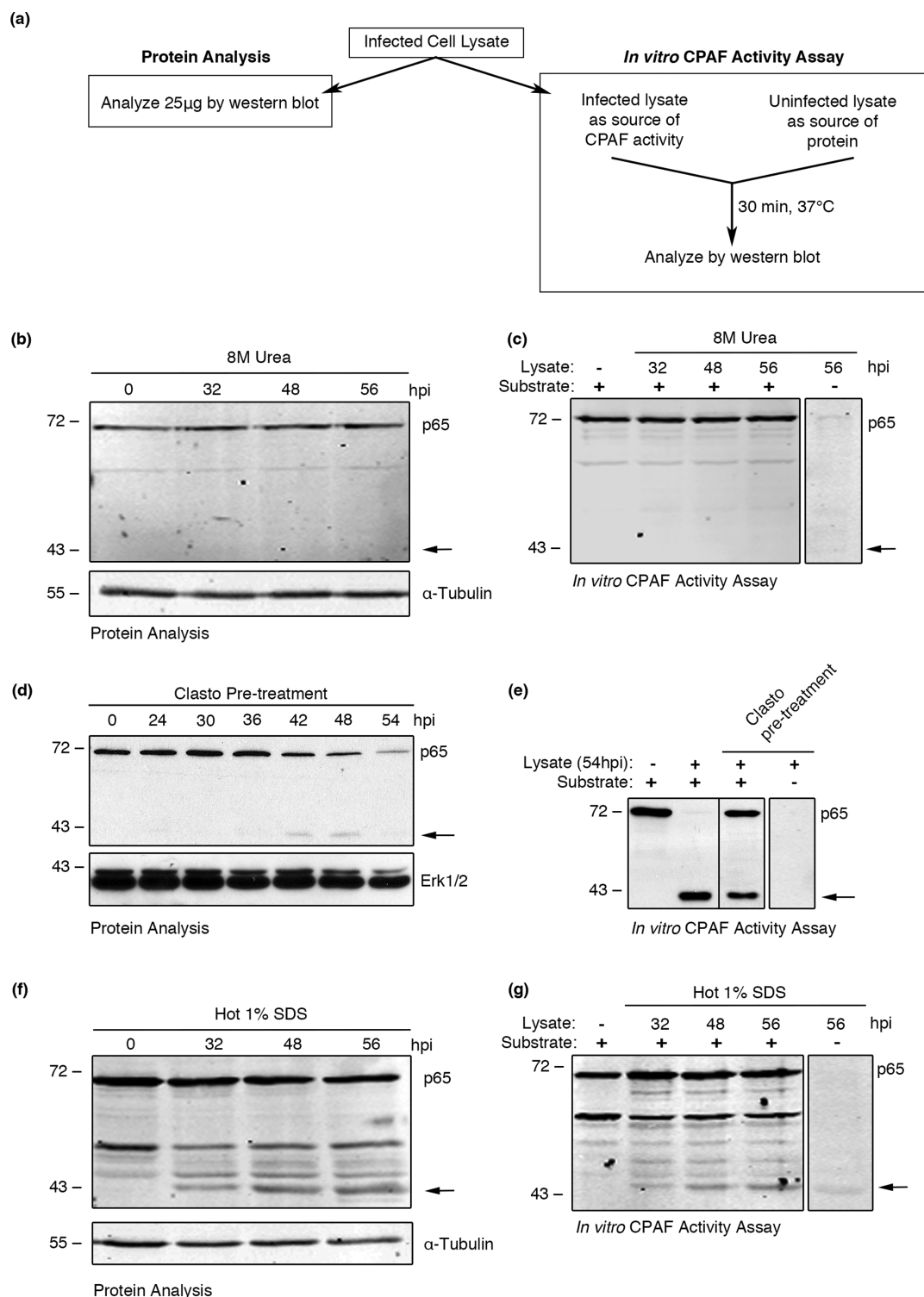
p65 cleavage product (Fig. 2b). These cell lysates also lacked CPAF activity toward p65 in the parallel *in vitro* activity assay (Fig. 2c). Thus, urea was effective at inhibiting CPAF in the lysate, and p65 does not appear to be cleaved in intact infected cells up to 56 hpi. In contrast, pre-treatment of infected cells with clasto-lactacystin for 60 min was only effective in a chlamydial infection at time points up to 36 hpi because lysates prepared at 42 hpi and later showed progressive loss of full-length p65, and appearance of an ~43 kDa cleavage product (Fig. 2d). Consistent with this observation, there was residual CPAF activity in these late lysates (shown for the 54 hpi lysate in Fig. 2e). Similarly, lysates prepared by direct lysis in hot 1% SDS buffer also showed evidence of p65 proteolysis between 32 and 56 hpi (Fig. 2f), and contained small amounts of residual CPAF activity toward p65 (Fig. 2g). We conclude that clasto-lactacystin pre-treatment and the hot SDS lysis method are less effective at inhibiting CPAF activity in lysates at late times, which may indicate that there is more CPAF in a late-stage *Chlamydia*-infected cell.

These results illustrate why the *in vitro* activity assay should be performed on each infected cell lysate. In this experiment, we had the benefit of knowing that the late proteolysis of p65 was an *in vitro* artifact because the 8 M urea lysates did not show any detectable p65 proteolysis as late as 56 hpi. Without this information, however, the protein analyses of lysates obtained with clasto-lactacystin pre-treatment or direct lysis in hot SDS would be interpreted as demonstrating CPAF-mediated proteol-

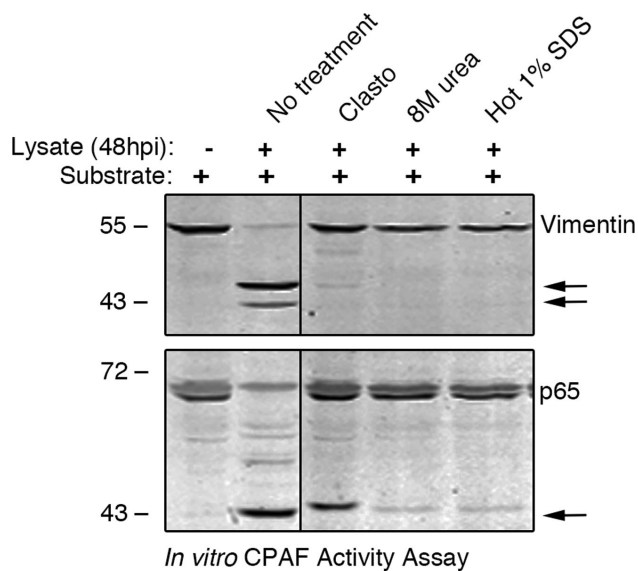
ysis of p65 at late times in the infection. The *in vitro* activity assay, however, provided an alternative explanation for this proteolysis because it showed residual CPAF activity in these late lysates. Thus, the proteolysis detected in the lysate could have occurred in intact infected cells, during cell collection and lysate preparation, or both. These studies demonstrate that proteolysis of a host or chlamydial protein cannot be correctly interpreted unless the lysate is free of *in vitro* proteolytic activity against this protein.

Snaveley et al. (2014) reported partial proteolysis of vimentin and LAP1 at late times in the intracellular *Chlamydia* infection. They took precautions to inhibit CPAF activity during lysate preparation by directly lysing *Chlamydia*-infected cells in hot 1% SDS, but the lysates were not verified to be free of CPAF activity. Interestingly, the temporal pattern of vimentin and LAP1 proteolysis resembles the p65 cleavage pattern that we observed in lysates prepared with the clasto-lactacystin pre-treatment and hot 1% SDS methods. It is therefore possible that the reported proteolysis of vimentin and LAP1 may have occurred during cell lysate preparation.

Over the course of our experiments, we noted that proteins differed in their susceptibility to CPAF-mediated proteolysis. The choice of substrates did not appear to make a difference when we examined methods to inhibit CPAF activity (data not shown), and thus we used a number of substrates in these studies. However, the specific substrate is relevant when the *in vitro* CPAF



**Figure 2.** Analysis of infected cell lysates for substrate proteolysis and CPAF activity. (a) Procedure to examine proteolysis and CPAF activity in an infected cell lysate. 'Protein Analysis' refers to Western blot analysis of infected cell lysates for cleavage or degradation of a protein of interest. The '*In vitro* CPAF Activity Assay' measures any residual CPAF activity present in the infected cell lysate. (b) At the indicated times in the *Chlamydia* infection, cells were lysed directly in 8 M urea, followed by protein analysis of the lysates by Western blotting with antibodies to p65. (c) Lysates from Fig. 2b were also assayed for CPAF activity against p65 using the *in vitro* assay. (d) Uninfected and infected cells were pre-treated with 150  $\mu$ M clasto-lactacystin for 60 min prior to lysis in RIPA buffer at the indicated times in the *Chlamydia* infection. Lysates were examined for p65 proteolysis by Western blot analysis. (e) Lysates from the 54 hpi time point of Fig. 2d were tested in the *in vitro* CPAF activity assay for p65 cleavage. (f) Cells at the indicated times in the *Chlamydia* infection were lysed in hot 1% SDS buffer, followed by protein analysis of the lysates for p65 cleavage. (g) Lysates from Fig. 2f were tested for residual CPAF activity against p65 in the *in vitro* CPAF activity assay. Expected p65 cleavage products in the Western blots are indicated with arrows. For Fig. 2c, e and g, the same amounts of representative infected cell lysates that were used in the *in vitro* assay were included to demonstrate that it does not contain detectable amounts of the p65 cleavage product. Thus, the appearance of p65 cleavage products in the *in vitro* assays testing the clasto-lactacystin pre-treated (Fig. 2e) and hot 1% SDS (Fig. 2g) infected cell lysates indicate that these lysates contain residual CPAF activity.



**Figure 3.** Protein substrates vary in their susceptibility to CPAF-mediated proteolysis. Lysates of infected cells at 48 hpi were prepared by three methods (pre-treatment with clasto-lactacystin followed by lysis in RIPA buffer, direct lysis in 8 M urea or direct lysis in hot 1% SDS) and were examined for residual CPAF activity in the *in vitro* activity assay. Reaction mixtures were examined for vimentin and p65 by Western blot analysis. Cleavage products in the Western blots are indicated with arrows.

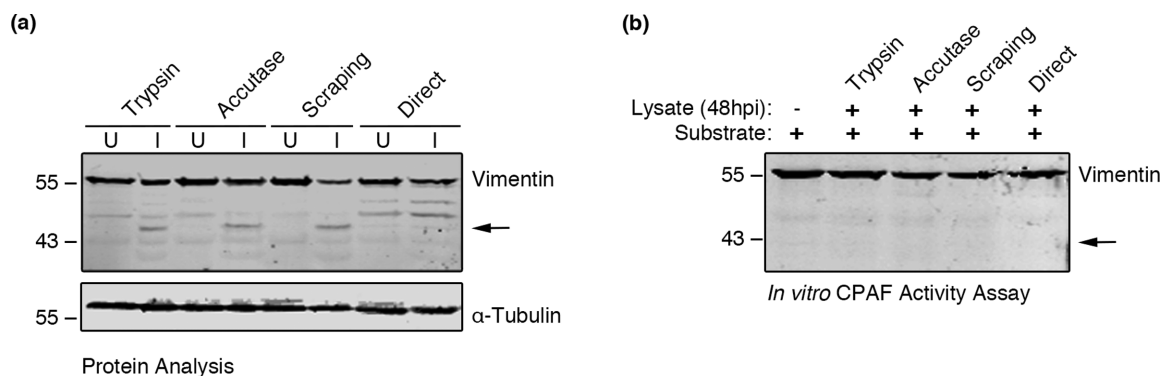
activity assay is used to help interpret the protein analysis of an infected cell lysate. To illustrate this point, we performed the *in vitro* CPAF activity assay on lysates prepared at 48 hpi with three different methods, and analyzed the reaction mixtures with antibodies to vimentin and p65. A lysate prepared with clasto-lactacystin pre-treatment caused partial cleavage of both vimentin and p65 (Fig. 3). However, lysates prepared with the 8 M urea and hot SDS methods caused some cleavage of p65, but not vimentin, indicating that the sensitivity of the *in vitro* CPAF assay depends on the substrate. We propose that the best approach is to evaluate the same substrate in both the protein analysis and the *in vitro* CPAF activity assay. Unlike in a previous experiment (Fig. 2b), 8 M urea did not fully prevent CPAF activity in this analysis (Fig. 3), showing that even this method is not always effective in inhibiting CPAF activity in a lysate.

We next tested if the method of cell detachment affects CPAF-mediated proteolysis. As an obligate intracellular bac-

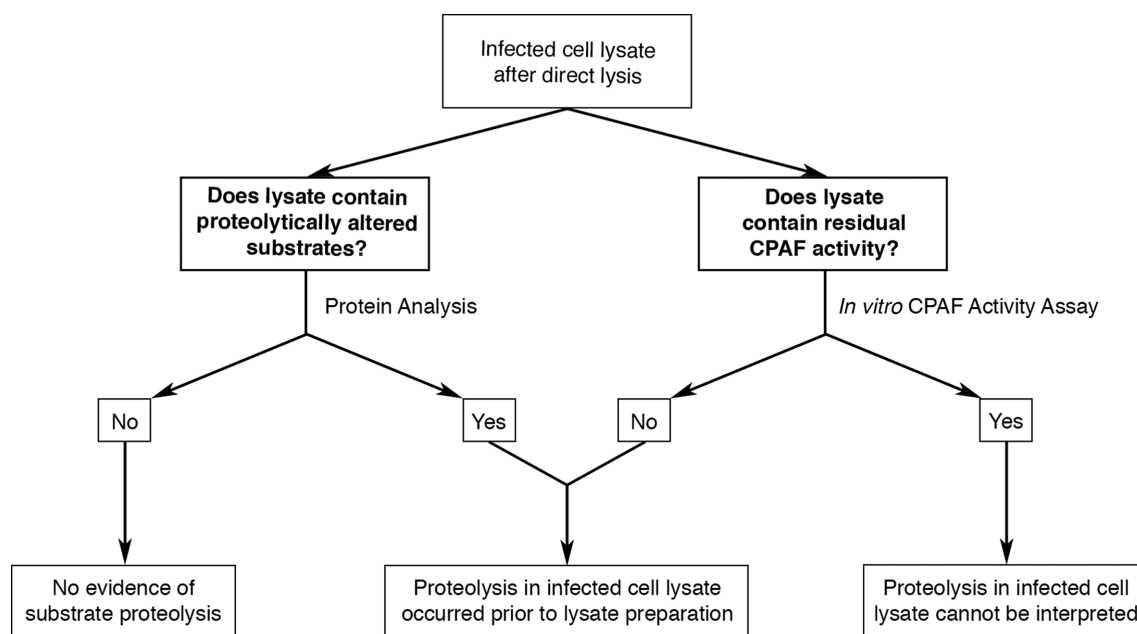
terium, *Chlamydia* is commonly cultivated in a monolayer of host cells, which is removed from the plastic surface prior to analysis. At 48 hpi, we compared direct lysis in 8 M urea with three commonly used methods of cell collection, which are trypsin treatment, accutase treatment and mechanical scraping. The detached cells were then lysed in 8 M urea to inhibit CPAF activity in the lysate. We analyzed the cell lysates for the presence of vimentin cleavage products by Western blotting, and for CPAF activity against vimentin with the *in vitro* activity assay. Lysates from cells detached with trypsin, accutase or mechanical scraping each contained small amounts of cleaved vimentin (Fig. 4a). This vimentin proteolysis was unlikely to have occurred during lysate preparation, because the cells had been exposed to urea after cell detachment and all the lysates lacked CPAF activity in our *in vitro* assay (Fig. 4b). However, the proteolysis must have occurred at some point during cell collection because a lysate prepared by direct lysis in 8 M urea contained no detectable vimentin cleavage (Fig. 4a). Putting these unexpected findings together, it appears that artifactual CPAF-mediated vimentin cleavage in these samples occurred during cell collection but *before* cell lysis. These experiments provide worrisome evidence that CPAF activity can be induced by experimental manipulations and cause proteolysis within intact infected cells. We hypothesize that standard cell detachment methods, including trypsinization, induce some degree of CPAF-mediated proteolysis in unlysed cells. Subsequent cell lysis then exaggerates this artifact by allowing *in vitro* proteolysis that can be so extensive that it produces complete loss of specific host and chlamydial proteins in the lysate (Fig. 1D, RIPA samples).

This model of CPAF activation during cell collection may explain why some but not all methods are effective in preventing artifactual CPAF-dependent proteolysis. Direct lysis with the urea or hot SDS methods is likely to inhibit CPAF activity within seconds. However, clasto-lactacystin appears to require pre-treatment for 60 min in order to reach sufficient concentrations inside an infected cell to inhibit CPAF activity induced by cell collection. Although more convenient, addition of 8 M urea or a CPAF inhibitor to a trypsinized cell pellet is unlikely to be effective because it would be too late to prevent CPAF-mediated proteolysis that has already occurred during cell collection.

In summary, protein analysis of *Chlamydia*-infected cells requires precautionary methods to inhibit CPAF activity during cell collection, and an assay to reveal whether detected proteolysis could be due to residual CPAF activity in the cell lysate (Fig. 5). The recently described CPAF null mutant (Snaveley et al., 2014) is



**Figure 4.** CPAF activity is induced by standard cell collection methods. (a) Uninfected and infected cells were detached at 48 hpi by treatment with trypsin or accutase, or by scraping, and then pelleted and lysed in 8 M urea. In parallel, cells were lysed directly in 8 M urea. Lysates were analyzed for vimentin proteolysis by Western blotting, with  $\alpha$ -tubulin serving as a loading control. (b) Infected cell lysates from Fig. 4a were tested in the *in vitro* CPAF activity assay and reactions were examined by Western blot analysis with antibodies to vimentin. Expected cleavage products in the Western blots are indicated with arrows.



**Figure 5.** Recommended procedure for analysis of proteins in lysates from *Chlamydia*-infected cells. We propose that infected cell lysates, which are analyzed for cleavage or degradation of a protein of interest ('Protein analysis'), should also be tested for the presence of residual CPAF activity ('*In vitro* CPAF Activity Assay'). This parallel analysis should examine the same substrate as the protein analysis and be performed for each lysate. If the *in vitro* CPAF assay reveals residual CPAF activity in the infected lysate, any observed proteolysis in this lysate cannot be interpreted because it will be unclear whether the proteolysis occurred in the *Chlamydia*-infected cell, during lysate preparation, or both.

an invaluable tool for determining if CPAF is necessary for the cleavage or degradation of a specific protein, but it does not address whether any CPAF-mediated proteolysis occurred in the course of the intracellular infection or during cell detachment and lysis. Of the methods that we examined, direct lysis of infected cells in 8 M urea appears to be more reliable than the other methods in its ability to inhibit CPAF activity during cell collection and lysate preparation. However, this method is not always 100% effective, and has the disadvantages that lysate samples have to be diluted for SDS-PAGE analysis and cannot be used for enzymatic assays. Clasto-lactacystin pre-treatment and direct lysis in hot 1% SDS can be effective, but not in all circumstances, especially late in the chlamydial infection. Ideally, infected cell lysates for protein analysis should be checked for residual CPAF activity toward the substrate being examined, because proteins differ in their susceptibility to CPAF.

Artificial CPAF-mediated proteolysis induced in an intact cell by standard cell detachment methods complicates protein and proteomic analyses of *Chlamydia*-infected cells. This unfortunate, and apparently unusual, property of *Chlamydia*-infected cells grown on a monolayer has broad implications for approaches such as flow cytometry, mass spectrometry, protein affinity chromatography and biochemical studies. Interpretation of past and future studies of host and chlamydial proteins will need to take into account whether the protein analysis truly reflects the situation in an infected cell prior to cell collection.

## SUPPLEMENTARY DATA

Supplementary data is available at FEMSPD online.

## ACKNOWLEDGEMENTS

We thank P. Gönczy for the HsSAS-6 antibodies and G. MacGregor for use of the LICOR OdysseySA.

**Authors' Contribution:** K.A.J. and J.K.L. contributed equally to this work

## FUNDING

This work was supported by a research scholar grant from the American Cancer Society (RSG-10-250-01-CCG) to CS. JKL was supported by a T32 training grant from NCI (T32CA009054).

**Conflict of interest statement.** None declared.

## REFERENCES

- Bavoil PM, Byrne GI. Analysis of CPAF mutants: new functions, new questions (the ins and outs of a chlamydial protease). *Pathog Dis* 2014;71:287–91.
- Chen AL, Johnson KA, Lee JK, et al., CPAF: a chlamydial protease in search of an authentic substrate. *PLoS Pathog* 2012;8:e1002842.
- Christian J, Vier J, Paschen SA, et al., Cleavage of the NF-kappaB family protein p65/RelA by the chlamydial protease-like activity factor (CPAF) impairs proinflammatory signaling in cells infected with *Chlamydiae*. *J Biol Chem* 2010;285:41320–7.
- Grieshaber SS, Grieshaber NA. The role of the chlamydial effector CPAF in the induction of genomic instability. *Pathog Dis*, 2014;72:5–6.
- Kumar Y, Valdivia RH. Actin and intermediate filaments stabilize the *Chlamydia trachomatis* vacuole by forming dynamic structural scaffolds. *Cell Host Microbe* 2008;4:159–69.
- Lad SP, Li J, da Silva Correia J, et al., Cleavage of p65/RelA of the NF-kappaB pathway by *Chlamydia*. *P Natl Acad Sci USA* 2007;104:2933–8.
- Ossewaarde JM, de Vries A, Bestebroer T, et al., Application of a mycoplasma group-specific PCR for monitoring decontamination of mycoplasma-infected *Chlamydia* sp. strains. *Appl Environ Microb* 1996;62:328–31.



- Paschen SA, Christian JG, Vier J, et al., Cytopathicity of Chlamydia is largely reproduced by expression of a single chlamydial protease. *J Cell Biol* 2008;**182**:117–27.
- Snively EA, Kokes M, Dunn JD, et al., Reassessing the role of the secreted protease CPAF in *Chlamydia trachomatis* infection through genetic approaches. *Pathog Dis* 2014;**71**:336–51.
- Tan M, Sütterlin C. The *Chlamydia* protease CPAF: caution, precautions and function. *Pathog Dis*, 2014;**72**:7–9.
- Zhong G. Killing me softly: chlamydial use of proteolysis for evading host defenses. *Trends Microbiol* 2009;**17**:467–74.
- Zhong G, Fan P, Ji H, et al., Identification of a chlamydial protease-like activity factor responsible for the degradation of host transcription factors. *J Exp Med* 2001;**193**:935–42.
- Zhong G, Liu L, Fan T, et al., Degradation of transcription factor RFX5 during the inhibition of both constitutive and interferon gamma-inducible major histocompatibility complex class I expression in *Chlamydia*-infected cells. *J Exp Med* 2000;**191**:1525–34.