

**Identification and characterization of type III-secreted
chlamydial pathogenicity factors and their impact on
the infected host cell**

Dissertation

For the degree of

Dr. rer. nat.

Submitted to the examination board

of the

Department of Biology

University of Konstanz

by

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Konstanz, May 2004

Identifizierung und Charakterisierung von Typ III-sezernierten chlamydialen Pathogenizitätsfaktoren und deren Einfluss auf die infizierte Wirtszelle

Dissertation

zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften

Dr. rer. Nat.

an der Universität Konstanz

im Fachbereich Biologie

vorgelegt von

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Tag der mündlichen Prüfung: 14.07.2004

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1 SUMMARY

Chlamydophila pneumoniae is an important, obligate intracellular, gram-negative human pathogen, responsible not only for lung infections but also discussed as a risk factor for a wide range of diseases like Atherosclerosis, Coronary artery disease and Alzheimer's disease. Throughout their intracellular life cycle Chlamydia stay enclosed within a host cell membrane vacuole, termed an inclusion. The special life cycle enables this pathogen to go into a persistent state of infection, which is clinically problematic, since it cannot be eradicated with current antibiotics. The challenge for the Pharma industry is to develop specific antichlamydial antibiotics, which are potent enough to also eradicate these persistent infections. Therefore it is necessary to learn more about this pathogen, as well as about pathogen-host interactions. Since Chlamydia have been reported to possess a type III secretion system (T3SS), studying this specialized secretion device for virulence factors could result in new targets for the development of antichlamydial antibiotics.

The objective of this work was to identify putative virulence factors of *C. pneumoniae*, secreted by the T3SS, and to characterize their mode of action in the host cell.

Eight candidate genes were selected from the *C. pneumoniae* strain TW183 genome sequence. Secretion of six of the respective candidate proteins could be demonstrated with Immunocytochemistry. These were CopN, cpMip, Pkn5, CpB0736, CpB0739 and CpB0856. Secretion of all six putative chlamydial virulence factors was shown to occur into the inclusion membrane. The timely regulation of expression and secretion of these chlamydial effector proteins was shown. This implies that different virulence factors fulfil their roles during different times of the infection cycle. Furthermore it was demonstrated that individual inclusions within a host cell are autonomous with respect to secretion of the virulence factors. This indicates that regulation of secretion occurs individually for each inclusion.

A model was proposed in this work, where secretion of virulence factors into the inclusion membrane keeps these proteins within the direct surrounding of the inclusions

and thereby might create a local microenvironment, which allows Chlamydia to grow, multiply and survive within this specialized niche. Localization of secreted effector proteins within the inclusion membrane enables Chlamydia to interact with proteins in the host cell cytoplasm.

Two of the secreted effector proteins, Pkn5 and cpMip, were investigated in closer detail. Pkn5 (CpB0730), a Serine-Threonine kinase-like effector was demonstrated to possess no kinase activity itself but to influence the phosphorylation of host cell proteins. Affected host cell proteins were a Rho GDP dissociation inhibitor (RhoGDI), a protein disulfide isomerase (PDI) and a regulatory subunit of protein kinase A type I. Pkn5 and CpB0739, both being secreted at 20 hpi (hours post-infection), might influence host cell cytoskeletal remodelling. Pkn5 induced the phosphorylation of RhoGDI by host cell kinases in kinase assays. Phosphorylation of RhoGDI was described to influence Rho activity, which is involved in cytoskeletal rearrangement. CpB0739 has an adenylate cyclase domain, which might also influence the host cell cytoskeleton via cyclic AMP. Inhibition of the phosphorylation of PDI and PKA regulatory domain by Pkn5 could lead to induction of Interleukin-10 expression, which in turn inhibits host cell apoptosis. Pkn5 furthermore was shown to be posttranslationally modified with SUMO1, mediated by host cell PIASx. Localization of PIASx on the surface of chlamydial inclusions was demonstrated with Immunocytochemistry.

The herein reported results indicate that infection with *C. pneumoniae* TW183 neither affects the NF κ B nor the STAT1, STAT2 or STAT3 signal transduction pathways.

Secreted effector protein cpMip was shown to be essential for *C. pneumoniae* infection. Inhibition of its intrinsic peptidyl prolyl cis-trans isomerase activity by the immunosuppressive drug FK506 also inhibited the progress of the infection with *C. pneumoniae*. It was proposed in this work, that a substructure of the huge FK506 biomolecule could be used as lead structure for the development of specific antichlamydial antibiotics.

Further analysis of the secreted effector proteins and their intracellular functions is necessary. The herein reported identification and characterization of type III-secreted effector proteins of *C. pneumoniae* together with the investigation of their impact on

pathogen-host interaction is one step towards the development of more potent antichlamydial antibiotics.

2 ZUSAMMENFASSUNG

Chlamydophila pneumoniae ist ein wichtiges, obligat intrazelluläres, gram-negatives Humanpathogen, welches nicht nur Lungenentzündungen verursacht, sondern auch als Risikofaktor für Krankheiten wie Artherosklerose, Erkrankungen der Herzkranzgefäße und Alzheimer diskutiert wird. Während des gesamten intrazellulären Lebenszyklus sind die Chlamydien von einer Vakuole aus Wirtszellmembran, einer sogenannten Inclusion, umgeben. Der spezielle Lebenszyklus ermöglicht es dem Pathogen in einen Zustand der Persistenz überzugehen, der klinisch problematisch ist, da Chlamydien in diesem Zustand nicht antibiotisch bekämpfbar sind. Daher besteht die grosse Herausforderung für die Pharmaindustrie darin, spezifische antichlamydiale Antibiotika zu entwickeln, die auch in der Lage sind persistente Infektionen zu bekämpfen. Deshalb ist es notwendig, mehr über dieses Pathogen sowie über dessen Interaktion mit dem Wirt zu lernen. Da Chlamydien bekanntermassen ein Typ III Sekretionssystem (T3SS) besitzen, könnte man durch Studieren dieses speziellen Sekretionsapparats für Virulenzfaktoren zu neuen Targets für die Entwicklung antichlamydialer Antibiotika kommen.

Ziel dieser Arbeit war die Identifizierung von potentiellen, Typ III-sezernierten Virulenzfaktoren von *C. pneumoniae*, sowie die Charakterisierung deren Wirkmechanismen in der Wirtszelle.

Acht Kandidatengene wurden aus dem Genom von *C. pneumoniae* Stamm TW183 ausgewählt. Sekretion von sechs der ausgewählten Kandidatenproteine konnte mit Hilfe von Immunzytochemie nachgewiesen werden. Hierbei handelt es sich um CopN, cpMip, Pkn5, CpB0736, CpB0739 und CpB0856. Alle sechs Kandidaten wurden in die Inclusionsmembran hinein sezerniert. Es wurde gezeigt, dass sowohl die Expression wie auch die Sekretion dieser chlamydialen Effektorproteine zeitlich reguliert wird. Das bedeutet, dass verschiedene Virulenzfaktoren ihre jeweilige Rolle zu unterschiedlichen Zeiten des Infektionszyklus ausüben. Darüber hinaus wurde gezeigt, dass individuelle Inclusionen innerhalb einer Wirtszelle bezüglich der Sekretion der Virulenzfaktoren

autonom sind. Dies wiederum bedeutet, dass die Regulation der Sekretion in jeder Inclusion unabhängig abläuft.

In dieser Arbeit wurde ein Modell vorgeschlagen, worin die Sekretion der Virulenzfaktoren in die Inclusionsmembran diese Proteine in der direkten Umgebung der Inclusionen bindet und dadurch ein lokales Mikrokompartiment schafft, welches Wachstum, Teilung und Überleben der Chlamydien in dieser spezialisierten Nische ermöglicht. Durch die Lokalisierung der sezernierten Effektorproteine in der Inclusionsmembran sind diese in der Lage mit Proteinen im Zytoplasma der Wirtszelle zu interagieren.

Zwei der sezernierten Effektorproteine, Pkn5 und cpMip, wurden genauer charakterisiert. Es wurde gezeigt, dass Pkn5 (CpB0730), ein Serin-Threoninkinase-ähnlicher Effektor, zwar nicht selbst als Kinase aktiv ist, aber dennoch in der Lage ist, die Phosphorylierung von Wirtszellproteinen zu beeinflussen. So war die Phosphorylierung eines Rho GDP Dissoziations-Inhibitorproteins (RhoGDI), einer Proteindisulfid-Isomerase (PDI) sowie einer regulatorischen Untereinheit der Proteinkinase A TypI betroffen. Sowohl Pkn5 als auch CpB0739, die beide 20 hpi (Stunden nach Infektion) sezerniert werden, könnten einen Einfluss auf die Remodellierung des Zytoskeletts der Wirtszelle haben. Pkn5 induzierte die Phosphorylierung von RhoGDI durch Wirtszellkinasen in Kinaseassays. Es ist bekannt, dass die Phosphorylierung von RhoGDI die Aktivität von Rho Proteinen beeinflusst, welche wiederum an der Strukturierung des Zytoskeletts der Wirtszelle beteiligt sind. CpB0739 besitzt eine Adenylatzyklase-Domäne, die über zyklisches AMP ebenfalls das Wirtszell-Zytoskelett beeinflussen könnte. Die Hemmung der Phosphorylierung der PDI und der regulatorischen Untereinheit von PKA durch Pkn5 könnte zur Induktion von Interleukin-10 führen, welches die Wirtszelle vor Apoptose schützt. Die posttranslationale Modifikation von Pkn5 mit SUMO1 Protein, verursacht durch das Wirtszellprotein PIASx, wurde hier ebenfalls gezeigt. PIASx konnte mittels Immunzytochemie auf der Oberfläche der Inclusionen lokalisiert werden.

Die hier gezeigten Ergebnisse machen deutlich, dass die Infektion mit *C. pneumoniae* TW183 weder den NFκB noch die STAT1, STAT2 oder STAT3 Signaltransduktionswege beeinflusst.

Es wurde demonstriert, dass das Effektorprotein cpMip eine essentielle Rolle in der Chlamydieninfektion spielt. Die Hemmung der peptidyl prolyl cis-trans Isomeraseaktivität von cpMip durch die immunsupprimierende Substanz FK506 hatte einen hemmenden Einfluss auf die Infektion mit *C. pneumoniae*. In dieser Arbeit wird vorgeschlagen, dass sich eine Substruktur des grossen FK506 Biomoleküls als Leadstruktur für die Entwicklung spezifischer antichlamydialer Antibiotika eignen könnte.

Weitere Untersuchungen der sezernierten Effektorproteine und deren intrazellulärer Funktion sind nötig. Die hier berichtete Identifizierung und Charakterisierung von Typ III-sezernierten Effektorproteinen von *C. pneumoniae* zusammen mit der Untersuchung ihres Einflusses auf die Pathogen-Wirt Interaktion ist ein Schritt in Richtung der Entwicklung potenterer antichlamydialer Antibiotika.

3 INTRODUCTION

3.1 Chlamydia are important human pathogens

Chlamydia are obligate intracellular, gram-negative human pathogens. They are among the most widespread bacterial pathogens worldwide and have shown to be responsible for a variety of diseases in different animal species and humans. The different species infect a variety of hosts, with a wide range of tissue tropisms and varied disease pathologies. In particular, species pathogenic for humans are *Chlamydia trachomatis* (*C. trachomatis*), an agent of chronic genital and ocular infections, *Chlamydophila pneumoniae* (*C. pneumoniae*), a prevalent cause of community-acquired pneumonia, bronchitis and pharyngitis, and *Chlamydia psittaci*, which is common in avian species and can cause rare but severe pneumonia in humans. *C. trachomatis* infection is the most commonly reported notifiable disease in the US with over 700,000 reported infections per year and mostly young people under 24 years affected (298).

C. pneumoniae was initially isolated in 1965 from a child's conjunctiva during a trachoma vaccine trial in Taiwan (99). This particular isolate was called TW-183 (Taiwan isolate number 183). In studies, which have looked specifically at adults with community-acquired pneumonia, *C. pneumoniae* was the causative agent in 3.4% to 43% of cases (90, 151, 175). In average *C. pneumoniae* is associated with 10% of all community-acquired pneumonia incidents and 5% of all bronchitis and sinusitis cases worldwide and therefore represents a major human pathogen (151). The incidence of community-acquired pneumonia in the United Kingdom is approximately 0.1% per year. Not all *C. pneumoniae* infections result in pneumonia and it has been suggested that 90% or more of *C. pneumoniae* infections are asymptomatic (156).

Many people carry an acute or a persistent infection. The acute infections are usually not fatal in western countries. Apart from acute respiratory infections, *C. pneumoniae* has also been implicated in chronic respiratory carriage and culture studies have shown that asymptomatic infection may persist for at least a year (45, 110, 135, 136). The association of *C. pneumoniae* with a number of chronic human diseases, including

atherosclerosis and coronary artery disease (CAD), has excited big interest. *C. pneumoniae* infection has also been associated with COPD (Chronic obstructive pulmonary disease) and has been discussed as an important cofactor causing chronic airway inflammation, one of the central characteristics of asthma and COPD (91, 207). In most instances, the diagnosis has been based on a significant increase in specific IgG titers. The seroprevalence of *C. pneumoniae* IgG antibodies in adults is 50% or more (301). However, antibody titers cannot distinguish between acute, chronic and past infections. Nevertheless, most people become infected with *C. pneumoniae* during their lifetime and the effects of chlamydial infections as risk factor for other diseases are not well known. It was found that 68% of Finnish subjects with myocardial infarction had at least a three-fold rise in antibody titers against genus specific chlamydial lipopolysaccharide antigen compared with only 2.4% of controls (252), which was attributed to *C. pneumoniae*. It was hypothesized that chlamydia contribute to the rupture of atherosclerotic plaques, which then leads to infarction, besides the contribution of chlamydial infection to atherosclerosis itself (104, 176). However, the link between chlamydial infection, CAD and atherosclerosis is still discussed controversially (72, 109, 120, 188, 284). Trials to treat patients suffering from CAD with an antibiotic regimen have led to mixed results (10, 262), which might be partially due to the fact, that persistent infections could not be eradicated by conventional antibiotics. If the link between infection and CAD could be proven, specific antichlamydial antimicrobials would hit a huge market with 13 million people suffering from CAD in the United States alone and with half of the cases occurring due to unexplained risk factors (*e.g.* infections) (72). Chronic, persistent infections might pose an equal or even a higher risk for the patient's health compared to acute *C. pneumoniae* infections, which often remain unnoticed. These persistent infections are impossible to treat with current antibiotics (95). This is why research on chlamydial infection is clinically relevant and important.

3.2 The chlamydial infection cycle

Chlamydia are able to infect epithelial and immune cells in the infectious form of Elementary bodies (EBs) (Figure 1). After entry into the host cell their morphology

changes and EBs develop into the non-infectious Reticulate bodies (RBs), which are able to divide by binary fission. Towards the end of an infection cycle the multiplied RBs redevelop into EBs, which leave the host cell around 72-96 hours post-infection (hpi) and are ready to attack the next target cell (211). Throughout the intracellular part of their lifecycle, Chlamydia stay enclosed within a host cell vesicle, termed inclusion. Interaction between Chlamydia and host cell is only possible across the host cell membrane that constitutes the inclusion membrane. The chlamydial infection cycle can be arrested in the persistent state. Experimentally, different methods for the induction of persistent infections have been described (4, 20, 21, 127, 228, 277). In the persistent state chlamydial morphology, expression profile and metabolism change (22). So-called aberrant bodies are formed that are structurally different from EBs and RBs (228).

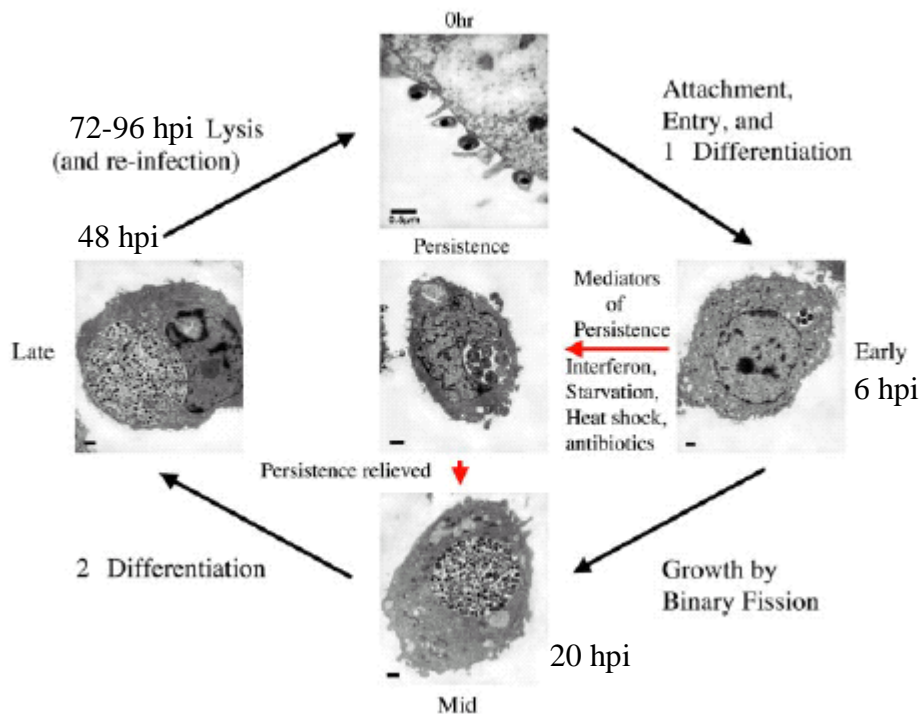


Figure 1: The chlamydial infection cycle is shown in Electron micrograph pictures with attachment phase, different intracellular phases of infection as well as with the arrest in the persistent state (derived from Byrne 2003, (35)). For better orientation time points after infection are indicated in hours post-infection for *C. pneumoniae*.

3.3 Chlamydia are resistant to genetic manipulation

So far chlamydia are resistant to genetic manipulation. Therefore alternative approaches like the heterologous expression of chlamydial genes in other bacteria (260, 276) or alternative chlamydial clone selection approaches have to be followed (96).

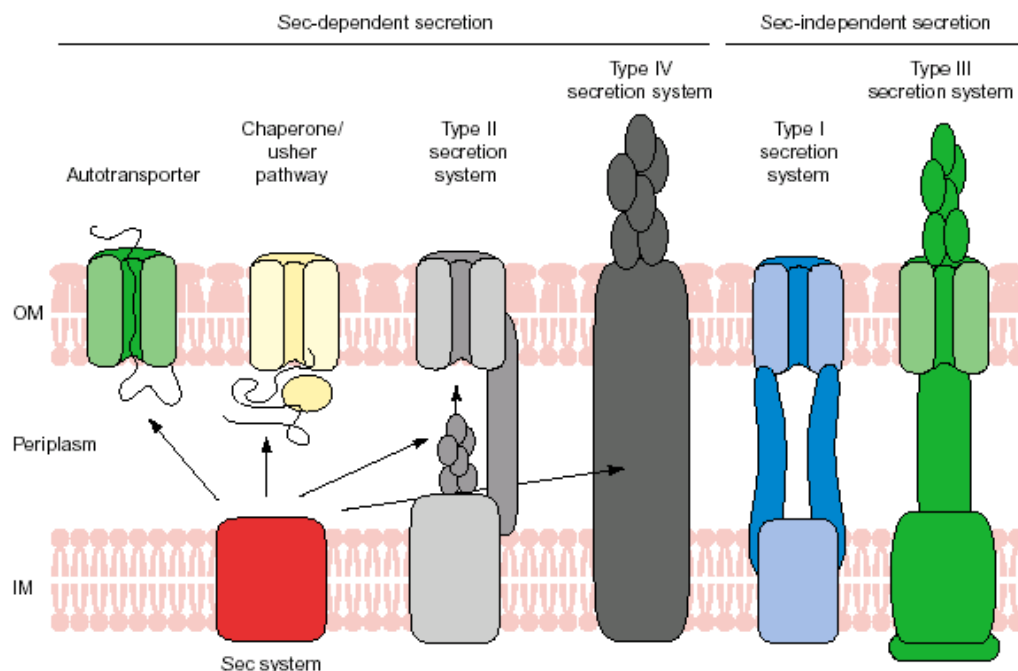


Figure 2: The different types of secretion systems in gram-negative bacteria are shown (derived from Büttner and Bonas 2002, (34)). Dependency of secretion systems from the Sec secretion system is indicated. IM = bacterial inner membrane, OM = bacterial outer membrane.

3.4 Secretion systems of gram-negative bacteria

Literature has described six different bacterial transport systems, named type I to type V secretion systems and the chaperone/usher pathway (Figure 2) (34, 133, 226). The chaperone/usher pathway consists of two components, a periplasmic chaperone plus an outer membrane pore protein. This system transports pilus subunits through the outer membrane in a Sec-dependent manner. Autotransporter proteins and two-partner secretion systems, able to promote their own translocation across bacterial membranes, have been grouped into the type V secretion system category. Type II and type IV

secretion systems are mostly Sec-dependent secretion systems (34). Proteins secreted by the Sec system possess an N-terminal signal sequence, which is cleaved off during or directly after transport through the bacterial inner membrane by signal peptidases (61, 160). Type II secretion systems have been described, for example, in *Pseudomonas aeruginosa* (*P. aeruginosa*) (15) and in *Yersinia enterocolitica* (*Y. enterocolitica*) (140). In the latter organism secretion is important for virulence. Type IV systems are a group of pilus-like secretion systems, which are able to secrete nucleoprotein complexes from donor to recipient bacterial cells (bacterial conjugation) or nucleoproteins or toxins directly into the eukaryotic host cell cytosol (41, 46, 89). Prominent examples are the transfer of oncogenic nucleoprotein T-DNA by *Agrobacterium tumefaciens* into plant cells and the secretion of *Helicobacter pylori* (*H. pylori*) virulence factor CagA into host cells. Type I and type III secretion systems are Sec-independent systems. Type I systems secrete bacterial toxins, proteases, lipases and S-layer proteins into the extracellular milieu (34). Type III secretion systems are described below in detail.

3.5 The type III secretion system

Type III secretion systems (T3SS) are key components of the virulence machinery of major human pathogens like *Yersinia*, *Shigella* and *Salmonella* (34, 54, 133, 273, 297, 299). The T3SS functions as a “molecular syringe”, enabling gram-negative bacteria to inject virulence-related proteins into the cytoplasm or into the membrane of host cells (55, 89, 280). Thereby these virulence factors are translocated across the bacterial inner and outer membranes as well as across or into the host cell membrane. The latter can either be the exterior host cell membrane or the inclusion membrane, if an intracellular bacterium uses the T3SS from within the host cell vesicle in which it resides. The T3SS of *Yersinia* is assembled from 29 subunits, the so-called *ysc*-proteins (Yersinia secretion), eleven of which are conserved amongst T3SS from other bacterial pathogens (43, 133). Yersinial outer proteins YopB, YopD and LcrV (low calcium response) are transported via the T3SS into the host cell membrane in order to establish effector translocation through this membrane (34, 212). YopB and YopD form a pore in the target cell membrane, LcrV interacts with both and is surface exposed before target cell

contact. Homologues with the same function exist in other type III-secreting pathogens (27, 137). YopN belongs to a group of regulatory proteins, which mediate the cell contact-dependent induction of *yop* gene expression and Yop protein secretion (133).

The components of the secretion apparatus are conserved, whereas type III effector proteins are not conserved between the different bacterial species as they have diverse functions to fulfill. This low level of genetic conservation makes it difficult to identify new effector proteins. Often, the open reading frames (ORFs) of effector proteins are located in T3SS gene clusters, interspersed between the ORFs encoding structural components of the T3SS (275). Secreted type III effector proteins/virulence factors can be involved in numerous pathogen-host interactions. *Yersinia*, for example, inhibit their own uptake by macrophages with the help of a type III-secreted virulence factor YopH, a tyrosine phosphatase (133). Dephosphorylation of a cytoskeleton-associated phagocyte cell protein, essential for phagocytosis, rescues *Yersinia* from uptake and destruction by these host immune cells (100, 133). Furthermore *Yersinia* blocks the oxidative burst within professional phagocytes, which saves *Yersinia* from destruction. Other type III-secreted virulence factors contribute to the successful infection of *Yersinia*, e.g. YopE, a protein, which destabilizes the host cell cytoskeleton. Further effector proteins are secreted by the yersinial T3SS, which assist in establishing the infection and preventing clearance by the host's immune system (133). The yersinial T3SS is the best investigated T3SS, but other pathogens mentioned above also possess a collection of type III-secreted effector proteins, specifically directing host cell protein targets (133). Identifying the secreted virulence factors contributed to a better understanding of pathogen-host interactions and to an approach to tackle the diseases induced by T3SS-employing pathogens (133, 149, 150).

3.5.1 Type III secretion signal sequence

It has been debated whether a signal sequence exists, which determines T3SS substrates to be secreted by this type of secretion system (240). Guttman *et al.* suggested, that a low Asp, Leu and Lys content within the first 50 amino acids is necessary for T3SS-dependent secretion of *P. syringae* type III effector proteins (105). The absence of

negatively charged amino acids in the N-terminus was reported to be a requirement for type III secretion in *P. syringae* (234, 255). Furthermore, it has been described that an aliphatic (Ile, Leu, Val) or Pro residue at amino acid position three or four, a high percentage of Ser residues and a lack of negatively charged residues within the first 12 amino acid positions is necessary for type III secreted substrates in *Pseudomonas* (255). Lloyd *et al.* reported, that an amphipatic distribution of amino acids within the first ten N-terminal amino acids might be the secretion signal for type III secretion in *Yersinia* (182). In *Salmonella* the conserved sequence WEK(I/M)XXFF has been described to be the type III secretion substrate signal (202). Another group investigated the potential secretion signal in T3SS effector proteins from *Y. enterocolitica* and came to the hypothesis that the signal lies within the mRNA sequence instead of the N-terminal amino acid sequence (6, 7, 8, 241). None of these hypotheses and rules have been shown to be generally valid. This makes it more difficult to identify type III-secreted effectors from sequenced genomes. It has been demonstrated that even a truncated form of CopN, the chlamydial YopN homologue, which lacks the first 15 aminoterminal amino acids is secreted by the yersinial T3SS. Therefore it is questionable if an aminoterminal signal sequence exists in chlamydial type III-secreted effector proteins.

3.5.2 Regulation of type III secretion

Secretion of type III effector proteins is regulated in most organisms in multiple ways. The best investigated T3SS from *Yersinia* is activated by multiple signals. *Yersinia* senses an increase in extracellular amino acids, the presence of serum proteins as well as a drop in Ca^{2+} concentration (171). The latter signals contact to the host cell. Expression of T3SS genes and assembly of the secretion apparatus is induced as soon as an increase in environmental temperature to body temperature (37°C) occurs and glutamate presence is sensed. YopN protein is required as a regulator to prevent release of the other T3SS effectors in the absence of contact with a host cell. The presence of serum albumin induces the secretion of YopN, which then starts the secretion process of the other effector proteins upon contact with the host cell (44, 171). T3SS from other pathogens are also regulated by different parameters in a sophisticated manner (83). The

P. aeruginosa T3SS is regulated by Ca^{2+} levels. *Shigella flexneri* (*S. flexneri*) senses temperature, osmolarity and pH shifts and regulates T3SS gene expression and invasiveness (133) with respect to the measured parameters. Secretion of accumulated effector proteins is also induced by host cell contact in this case. Thus a temporal and spatial regulation of effector expression and secretion is described for all type III-secreting pathogens, which have been investigated in great detail. Almost nothing is known about the regulation of the chlamydial T3SS. It has been reported that InCA secretion is restricted at lower environmental temperature (76). This might be a hint towards a temperature-regulated T3SS in chlamydia.

3.5.3 Type III secretion in Chlamydia

Genome sequencing has shown that Chlamydiae have all necessary genes for a functional T3SS (243, 260, 272). However, the genetic organisation of the T3SS in Chlamydia is unlike that of some other gram-negative pathogens, *e.g.* *Salmonella* or *Yersinia* (299) in that they are scattered across at least four distinct locations (121, 260, 272) rather than clustered in obvious "pathogenicity islands". The chlamydial YopN homologue (CopN) and the YopD homologue are encoded within these clusters. The presence of T3SS gene clusters in the chlamydial genome makes it likely that Chlamydia possess a functional T3SS, but does not prove it. The existence of a chlamydial T3SS was demonstrated on mRNA- as well as on protein-level (77, 78), by immunocytochemistry (77, 184, 190), and on the cellular level by electron microscopy (40, 192, 215). To date, not many secreted chlamydial proteins are known (77, 122, 184, 271, 276, 277, 310), the function of which often remains speculative. The first protein, secretion of which was reported to occur by a chlamydial type III secretion system, was CopN (IcrE) (77). The heterologous secretion of *C. trachomatis* CopN by the *Y. enterocolitica* T3SS proved that CopN is indeed a T3SS substrate (77). Secretion of this putative regulatory protein into the inclusion membrane has been found in *C. pneumonia* and in *C. trachomatis* (77, 184). Secretion of a chlamydial protease-like activity factor (CPAF) into the host cell cytoplasm has been demonstrated for *C. trachomatis* and *C. pneumoniae* (122, 310). CPAF has been described to degrade host

cell transcription factors involved in MHC protein expression (310, 311, 312). Lugert *et al.* observed secretion of the YopB homologue CpB0838 and of the YopD homologue CpB1058 into the host cell cytoplasm, but their putative mode of action remain unknown (184). Stenner-Liewen *et al.* described a *C. trachomatis* CADD protein (Chlamydia protein associating with death domains, CT610), which associates with host cell death domain proteins on the inclusion surface and interferes with host cell apoptosis (271). Heterologous secretion of the inclusion membrane proteins IncA, IncB and IncC by the Shigella T3SS was reported (276). It was therefore hypothesized that Inc protein secretion in Chlamydia also occurs in a type III-dependent manner (247). The identification and characterization of proteins secreted by the chlamydial type III-secretion system increases our knowledge about Chlamydia-host interaction, which could be exploited for the development of anti-chlamydial drugs.

The activity of the chlamydial T3SS might be triggered extracellularly when the EB comes into contact with the host cell membrane. Intracellular triggering might occur when Chlamydia come into contact with the inclusion membrane into or through which effector proteins are secreted (132). Potential roles of the chlamydial T3SS essential for the chlamydial survival might be amongst others the entry of EBs into the host cell, the blocking of phagolysosomal fusion (18) or the prevention of apoptosis of the host cell (2, 38, 74, 93, 230, 231, 232, 239, 303, 309). When Chlamydia are stressed by an attack of the cell-mediated immune system, the T3SS may become important for survival. Indeed, using gamma interferon to induce persistent *C. pneumoniae* infection, expression of the type III secretion protein SctN was induced (208). Thus the T3SS may help Chlamydiae to resist stress from effectors of the T helper 1 cellular immune system. The high degree of conservation of T3SS genes and clusters in both *C. trachomatis* and *C. pneumoniae* suggests that it might have been present early in chlamydial evolution and that it has been retained because it confers, as yet unknown, survival advantages. Recently it has been published (128) that T3SS gene cluster IV is conserved also in the last known common chlamydial ancestor, which dates back 700 million years ago. Conservation for such a long time without use is highly improbable.

3.6 Chlamydial influence on host cell signaling and apoptosis

Many intracellular pathogens have been described to modulate host cell apoptosis (88, 223). A summary is given in figure 3. Since Chlamydia are obligate intracellular bacteria there is an obvious need to try to control and influence their host cell environment. The fact that Chlamydia are enclosed within the inclusion membrane implies that any communication must take place across this host cell membrane. For this purpose, integral membrane proteins, membrane associated proteins or proteins secreted through this membrane seem to be feasible. The identification of such effector proteins might be more complicated than the observation of effects resulting from Chlamydia-host cell interaction. Numerous effects of such interactions like changes in the host cell phosphoproteome have been described (75). Modulation of the expression of host cell surface proteins, which are important for immune recognition of infected cells, have been reported in the literature (73, 122, 265, 310, 311, 312). Furthermore, the interaction of chlamydial proteins with proapoptotic host cell death domain proteins has been investigated (271). Chlamydial influence on the pro- and antiapoptotic balance of the host cell has also been investigated and described in detail (2, 38, 74, 93, 101, 230, 232, 239, 303, 309). These authors described blockage of both mitochondrial Cytochrome C release and caspase activation by chlamydial infection (74, 81, 239). It was further described that the expression of proteins from the IAP (inhibitor of apoptosis) family was upregulated during chlamydial infection (303) in an NF κ B-dependent manner (293). Others hypothesized that upregulated IL-10 expression in infected cells is responsible for the antiapoptotic effect (93). However, the underlying molecular mechanisms and the chlamydial effector proteins responsible for these observed phenomena mostly remain unknown.

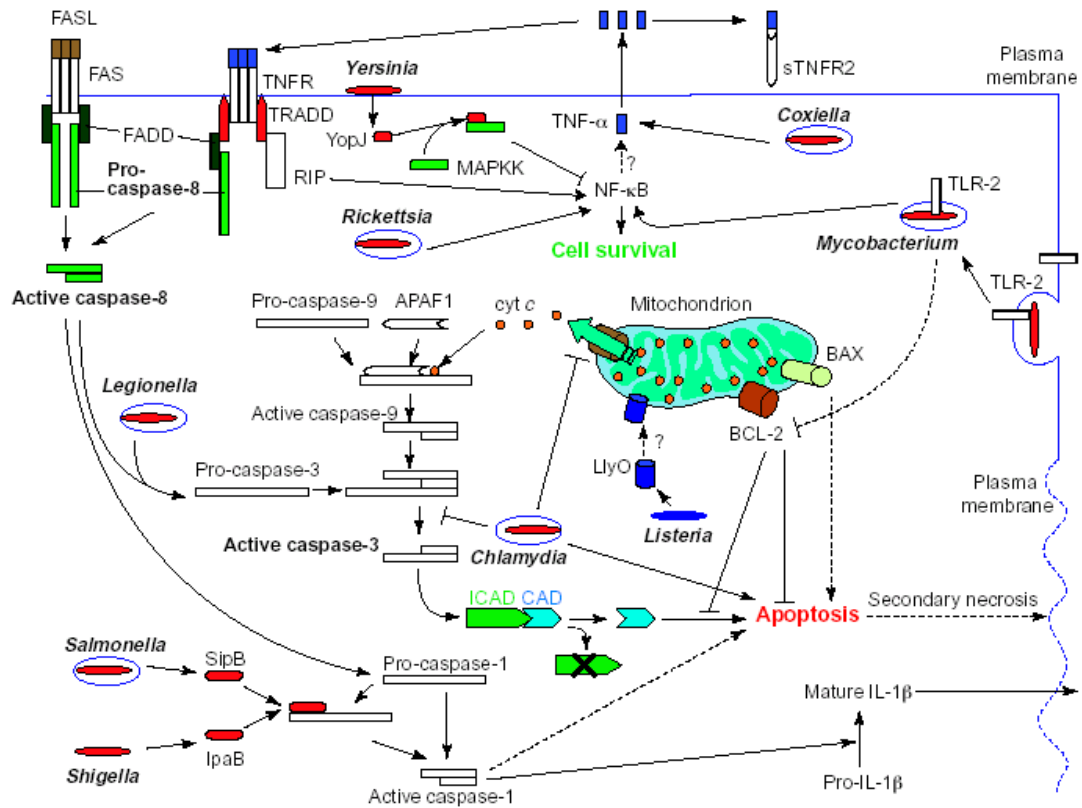


Figure 3: Modulation of host cell apoptosis by intracellular bacterial pathogens (derived from Gao 2000, (88)). Points of interference of pathogens with the apoptotic host cell signal transduction network are shown. Blue encircled bacteria live and grow enclosed within a host cell membrane vacuole (inclusion). TNF α is shown as mono or trimer as blue squares. TNFR (TNF α receptor, s = soluble), TLR (Toll-like receptor), FAS (CD95), FASL (Fas ligand), FADD (Fas-associated protein with death domain), TRADD (TNF receptor-1-associated death domain protein), MAPKK (Mitogen-activated protein kinase kinase), RIP (Receptor-interacting protein), cytC (Cytochrome C), APAF1 (Apoptotic protease activating factor 1), BAX (Bcl-associated X protein), BCL-2 (B-cell leukemia 2 protein), CAD (Caspase-activated deoxyribonuclease), ICAD (Inhibitor of CAD), IL-1 (Interleukin-1).

3.7 The NF κ B pathway as target of pathogenic bacteria

NF κ B (nuclear factor κ B) is a collective name for inducible dimeric transcription factors composed of members of the Rel family of DNA-binding proteins that recognize a common sequence motif (148). NF κ B proteins and the associated pathway are central to host cell antiapoptosis as well as to stress, immune and inflammatory responses (62). Activation of this pathway is induced by pro-inflammatory cytokines like TNF α and IL-1 as well as by LPS or TNF β . Activation then leads to phosphorylation of I κ B, which

liberates NF κ B from this inhibitory protein and ends in the translocation of NF κ B into the host cell nucleus (Figure 4).

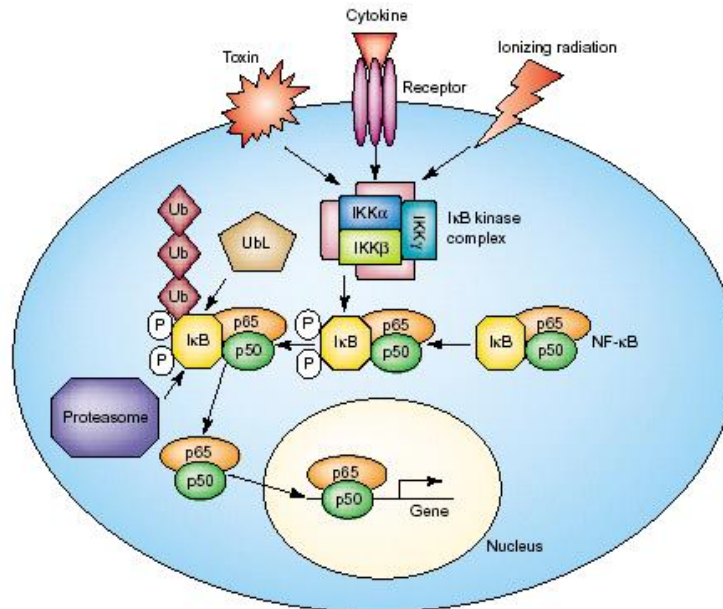


Figure 4: Pathway of NF κ B activation (108). I κ B (inhibitor-of-NF κ B), IKK (I κ B kinase complex), Ub (ubiquitin), ubiquitin ligase (UbL), p50/p65 (dimer of Rel family proteins).

The multisubunit I κ B kinase (IKK) is responsible for the inducible I κ B phosphorylation, which leads to phosphorylated I κ B being degraded by the proteasome. Unphosphorylated I κ B masks the NLS (Nuclear localization sequence) of NF κ B, thereby preventing the interaction of NF κ B with the nuclear import machinery. Within the nucleus NF κ B induces the transcription of pro-inflammatory and anti-apoptotic target genes (148, 281). NF κ B proteins are of high clinical interest as therapeutic targets (108). Furthermore, many pathogenic bacteria and viruses have adopted ways to interfere with the NF κ B signaling pathway in their favor. Yersinia type III-secreted effector protein YopP (*Yersinia enterocolitica* YopP is named YopJ in *Y. pseudotuberculosis*) acts anti-inflammatory by preventing NF κ B activation and the subsequent release of pro-inflammatory cytokines (212, 256, 307). Salmonella AvrA also blocks NF κ B activation, but at a different step of the cascade (downstream of IKK)

compared to YopP (between MKKK and IKK β) (51, 227, 307). Another type III-secreted effector of *Salmonella* with leucine-rich repeat motif has also been described to inhibit NF κ B-dependent gene expression (113). EspB, the type III-secreted effector protein of Shiga toxin-producing *Escherichia coli* (STEC), suppresses the NF κ B-dependent activation of cytokine expression (118). Thereby STEC actively counteract host defense responses, which allows them to colonize the host's intestine. In contrast, Enterohaemorrhagic *E. coli* (EHEC) actively induce the NF κ B pathway in intestinal epithelial cells by means of secreted virulence factors in order to cause acute inflammation (24). Other obligate intracellular bacteria like *Rickettsia* inhibit host cell apoptosis via NF κ B activation (50, 143). *Chlamydia* have been reported to activate the NF κ B pathway (63, 92, 294) with a maximum between 15 minutes and five hours post infection and to inhibit host cell apoptosis. Tiran *et al.* reported *C. pneumoniae* infections to be inhibited by NF κ B inhibitors (282). Therefore it seems plausible to assume that potential type III-secreted chlamydial effector proteins might also interfere with the NF κ B signaling pathway in order to prevent host cell apoptosis.

3.8 Rho proteins and the cytoskeleton as targets of bacterial toxins

The large cytotoxins A and B from *Clostridium difficile* act as glycosyltransferases on Ras-proteins (Rho, Rac and Cdc42) (32). Inhibition of these small GTP-binding proteins of the Ras superfamily influences the organization and dynamics of the actin cytoskeleton and intracellular trafficking (173, 291). In general, the small G-proteins are involved in rearrangements of the host cell cytoskeleton with Rac being responsible for lamellipodia, Cdc42 for filopodia and Rho for stress fiber formation (218). Invasive pathogenic bacteria like *S. flexneri*, *Salmonella enterica* serovar Typhimurium, *Neisseria gonorrhoeae*, *Vibrio parahaemolyticus* and *P. aeruginosa* induce cytoskeletal rearrangements associated with these small GTPases (3, 42, 79, 85, 102, 115, 117, 296). For example the *Salmonella* type III-secreted tyrosine phosphatase SptP and other type III effectors of *Salmonella* induce cytoskeletal actin changes (85, 200). *Salmonella* type III-secreted virulence factor SopE induces membrane ruffling of the host cell (115,

133). Salmonella SseF and SseG type III effectors interfere with the host cell microtubule cytoskeletal system (165). Yersinia uses type III-secreted YopE and YopO to alter the host cell cytoskeleton by activating Rho protein (17, 249, 292).

The sequenced *C. pneumoniae* genomes (146, 243, 260, 267) do not contain direct homologues of the clostridial cytotoxins. However, *C. psittaci* GPIC (guinea pig inclusion conjunctivitis) and *C. trachomatis* have been reported to contain clostridial toxin-like genes (23). Primary sequences from proteins of the glycosyltransferase superfamily as well as the chlamydial cytotoxin-like proteins all possess a conserved extended DXD-motif, which is necessary for glycosyltransferase activity (23, 33). *C. trachomatis* has been reported to show cytotoxic activity against epithelial cells with morphological and cytoskeletal changes indistinguishable from those induced by clostridium toxin B. The observed effects on host epithelial cells include cell rounding, extensive breakdown of actin-based fibers, actin recruitment to the site of EB attachment and formation of mikrospeikes extending beyond the cell periphery (23, 36). Furthermore it has been reported recently, that host cell Rac GTPase is required for successful invasion of *C. trachomatis* into the host cell. Thus, it would not be surprising if type III-secreted chlamydial effector proteins would contribute to these morphological changes of the host cell.

3.9 Cytokine-induced signaling along the JAK-STAT pathway

A wide variety of extracellular signals activate the STAT (signal transducers and activators of transcription) class of transcription factors. In mammals, the JAK/STAT signal transduction pathway mediates signaling by interferon and other cytokines. Many cytokines, lymphokines, and growth factors signal through cell surface receptor tyrosine kinases that are associated with and activate Janus kinases (JAKs) (264). Ligand-induced dimerization of the receptor induces the cross-over tyrosine phosphorylation of the associated JAKs, which in turn phosphorylates tyrosine residues on the cytoplasmic tail of the receptor (257). These phosphorylated tyrosines serve as docking sites for the Src Homology-2 (SH-2) domain of the STAT protein. JAK then catalyzes the tyrosine

phosphorylation of the receptor-bound STAT. Phosphorylation of STAT at a conserved tyrosine residue induces SH-2-mediated homo- or heterodimerization, followed by translocation of the STAT dimer to the nucleus. STAT dimers bind to specific DNA response elements in the promoter region of target genes to activate gene expression. Toll-like receptors 2 and 4, known to activate the NF κ B pathway, were also reported to activate signaling through STAT (245).

Deregulation of the negative feedback of the JAK-STAT pathway has been implicated in hematopoietic disorders, autoimmune and inflammatory diseases, interferon (IFN)-resistance, and cancer (31, 167, 229, 285). Therefore, the understanding of JAK-STAT signal transduction pathways is of high clinical importance and the development of specific inhibitors or ligands of these pathways is one goal of pharma industry. The adaptor protein c-Cbl was also reported to be involved in JAK-STAT and tyrosine kinase signaling (288). Not only since c-Cbl in connection to imatinib mesylate (Gleevec, Novartis) has been investigated, pharma industry is interested in JAK-STAT signaling (217). One of Amgen's blockbuster products, Epogen, is a nonpeptide ligand for erythropoietin-receptor (71, 237), which also signals through a JAK-STAT pathway (157). The JAK-STAT signal transduction pathway is not only important for industry, but also for bacterial and viral pathogens. Cytomegalovirus, for example, has been described to induce degradation of JAK-1, which leads to inhibition of IFN γ -induced MHC class II expression (204). Thereby cytomegalovirus attempts to evade the host cell immune system. Enterohaemorrhagic *E. coli* were reported to disrupt IFN γ signaling through STAT1 (39). *H. pylori* has also been reported to disrupt STAT1 signaling by inhibiting its phosphorylation and its translocation from cytoplasm to nucleus (205). Similar tactics have been described for intracellular pathogens like *Leishmania* (244) and *Listeria* (261).

3.10 The dual function of PIAS proteins

In vertebrates, four PIAS proteins (PIAS1, PIAS3, PIASx and PIASy) are known (179). PIAS (protein inhibitor of activated STAT) proteins negatively regulate STAT signaling

by different mechanisms. PIAS proteins were reported to inhibit binding of the phosphorylated STAT dimers to the respective DNA response element (12, 174, 177). Furthermore, PIAS proteins have the ability to act as SUMO ligases, which, like ubiquitin, covalently modify proteins with SUMO-proteins through isopeptide bonds at lysine side chains (Figure 5) (84, 141, 162, 248, 258). This modification can be reverted by isopeptidases (235).

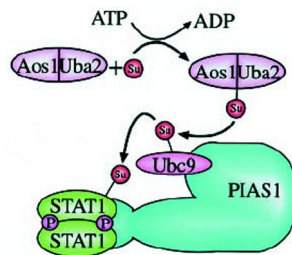


Figure 5: Mechanism of the covalent modification of target proteins with SUMO (Su) (derived from Wormald 2004; (302)). The Uba2/Aos1 heterodimer and Ubc9 protein act as E1 activating enzyme and as E2 conjugating enzyme respectively (analogous to the ubiquitin system). PIAS proteins possess E3 SUMO ligase activity.

In vertebrates three forms of SUMO exist (SUMO1, SUMO2 and SUMO3), with SUMO2 and SUMO3 being very similar (141, 253). SUMOylation occurs on core sequences with the conserved pattern Ψ KXE, where Ψ indicates a hydrophobic and X stands for any amino acid (141, 145, 263). SUMOylation of proteins (*e.g.* STAT, Mdm2, p53 etc.) can have diverse effects, ranging from changes in protein activity (145, 193, 206, 214, 216), stability (198) or changes of the localization of the modified protein within a cell (address label) (125, 199, 213, 235, 251, 302, 306, 308). SUMOylation has also been reported to participate in signal transduction events (199). In contrast to ubiquitin, SUMO stabilizes proteins. SUMO can also be an antagonist of ubiquitin (198). Usually PIAS proteins are located within the nucleus. However, there have been reports of PIAS-mediated SUMOylations in the cytoplasmic compartment (170). Additionally PIAS1 was reported to have a pro-apoptotic activity (178) as well as being able to activate and to repress p53 protein (197, 258).

3.11 Eukaryotic kinases and their importance for pathogens

Protein kinases are key enzymes of signal transduction in most organisms. In Eukaryotes protein kinases are involved in sophisticated signal transduction networks, which control and regulate many cellular functions from cell division to apoptosis. Within these networks kinases can be incorporated as domains in membrane-standing receptors or act in other compartments in an amplifying, inhibiting or otherwise modulating way. Bacteria mostly use kinases in two-component systems to sense external stimuli and regulate gene expression or other processes accordingly. Eukaryotic protein kinases differ in structure from prokaryotic two-component system kinases. It has been believed for a long time that bacteria do not possess eukaryotic-like serine/threonine kinases (STK) or tyrosine kinases. However, this assumption is not correct anymore (153, 172). Pathogenic bacteria have discovered eukaryotic-like kinases as tools for virulence. Various prokaryotes such as *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Myxococcus xanthus*, *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Yersinia* have been described to use STKs or tyrosine kinases as virulence factors or in other contexts (13, 14, 68, 133, 144, 153, 221, 238). Inhibition or manipulation of eukaryotic kinase activity is a strategy, used by many pathogenic bacteria, to promote infection (133, 195, 220, 270). Chlamydiae have also been reported to possess eukaryotic-like kinases (172, 190, 290). Activation and phosphorylation of host cell kinases has been reported to occur upon chlamydial infection (19, 26, 52, 114, 164, 190, 274). These facts might be a hint for the presence of a potentially secreted chlamydial kinase as putative virulence factor.

Eukaryotic protein kinases are enzymes that belong to a very extensive family of proteins, which share a conserved catalytic core common to both serine/threonine and tyrosine protein kinases. Many kinases are divided into a regulatory and a catalytically active domain. There are a number of conserved regions in the catalytic domain of protein kinases (111). In the N-terminal extremity of the catalytic domain there is a glycine-rich stretch of residues (Y**G**x**G**x(Phe/Tyr)Gx**V** motif, Hanks domain I), where Y is a hydrophobic and x is any amino acid. The amino acids in bold are those reported to be conserved (29, 111, 172). This domain has been shown to be involved in ATP binding with the Valine residue forming a hydrophobic pocket that sequesters the

adenine ring of ATP (172). However, the presence of this Hanks domain is not an essential prerequisite for a kinase to be functional (266). In the vicinity of this domain lies a strictly conserved Lys residue (Hanks domain II), which is directly involved in the phosphotransfer reaction. In the central part of the catalytic domain there is a conserved aspartic acid residue (part of Hanks domain VI), which is important for the catalytic activity of the enzyme (158). Altogether eleven Hanks domains exist, which are more or less conserved, some consisting of one amino acid only (111). Hanks domains VI and VIII contain amino acid residues, which are indicative for the activity of the respective kinase, being either a Serine/Threonine- or a Tyrosine-phosphorylating kinase (111).

3.12 Further discussed protein domains and motifs

3.12.1 Coiled-coil domains

The coiled-coil structural protein motif, first proposed by Crick (56), consists of right-handed amphipathic α -helices that adopt a left-handed supercoil. That way their hydrophobic faces are in continuous contact along the length of the coiled-coil. Each α -helix consists of a regularly repeating heptad sequence (abcdefg), in which positions a and d are occupied by mostly hydrophobic amino acids in the hydrophobic core of the protein fold (1). The coiled-coil motif mediates and regulates protein-protein interactions and was discussed as biophysical osmosensor (58). Most coiled-coil proteins known to date are of eukaryotic origin. Coiled-coil proteins are estimated to comprise 3-5% of the genomic complement, suggesting an involvement in a wide range of biological activities and complex cellular systems. Coiled-coil motifs also have been observed in T3SS proteins but the implication of this observation is still speculative (44, 60, 134, 139). Interestingly, processes such as phosphorylation can stabilize or destabilize coiled-coil motifs adding an additional regulatory level to the putative interaction of T3SS proteins via coiled-coil domains (169, 279).

3.12.2 The Forkhead associated (FHA) domain

FHA domains are modular protein domains that mediate protein-protein binding to targets containing phosphoserine or phosphothreonine residues (65, 67, 126, 278). Furthermore, FHA domains themselves can be phosphorylated by kinases (65). Leonard and Bakal reported the presence of genes encoding FHA domain proteins in the genomes of different microorganisms (14, 172). Molle and colleagues reported the phosphorylation of an FHA domain protein by a prokaryotic STK in *M. tuberculosis* (209). The substrate in Mycobacterium was a protein of the same species. However, FHA domain proteins are originally described as eukaryotic domains involved in multiple processes like signal transduction and transcriptional control (65). Amino acids, which are conserved amongst FHA domain proteins, have been identified and described (66, 209). The presence of genes encoding FHA domain proteins, a proteinphosphatase and kinases within the chlamydial genome suggests, that these proteins might interact in protein-phosphorylation and signal transduction events (172, 225).

3.12.3 Adenylate cyclase domains in bacterial toxins

Cyclic AMP (cAMP) is an important second messenger for eukaryotic cell signaling. It is involved in numerous signaling processes, which regulate for example host cell morphology, tissue integrity and activation of kinases like protein kinase A (53). Formation of this second messenger is catalyzed by adenylate cyclase enzymes, which produce cAMP from endogenous ATP. Bacterial pathogens like *Bacillus anthracis* (Edema Factor), *Bordetella pertussis* (CyaA) and *P. aeruginosa* (ExoY) have adopted adenylate cyclases as virulence factors in order to influence host cell signaling via increased levels of cAMP (98, 304). Edema factor and CyaA toxins enter target cells by the A-B toxin mechanism (97, 98) and are activated by host cell calmodulin. Secretion of ExoY into the host cell cytoplasm has been reported to occur by a type III-dependent mechanism (304). Accumulation of supraphysiologic levels of cAMP in the affected cells leads to changes in the host cell cytoskeleton and, as a result, in the host cell morphology. Many signaling pathways especially kinases are activated by cAMP (53).

Interestingly, an adenylate cyclase signaling pathway was shown to be involved in the cAMP-dependent regulation of the expression of T3SS genes in *P. aeruginosa* (300).

3.13 The immunosuppressants FK506, Rapamycin and Cyclosporin A

Organ transplantation often elicits immune responses to reject the foreign transplant. The most widely used drugs to deal with such graft reaction are FK506 (Tacrolimus, tradename “Prograf”), Rapamycin (Sirolimus) and Cyclosporin A (Sandimmun Neoral) (82, 86, 180, 236). Their direct target is a Ca^{2+} /calmodulin-dependent phosphatase known as calcineurin (155). Calcineurin controls the phosphorylation of specific serine residues of the transcription factor NFAT (Nuclear factor of activated T-cells) (130). In stimulated cells, an increase in calcium activates the calcineurin-dependent dephosphorylation of NFAT. Phosphorylation of this transcription factor determines, whether it translocates into the nucleus and activates the respective target genes, or not. Cyclosporin A combines with a cellular protein called cyclophilin to inhibit calcineurin, whereas FK506 combines with FKBP (FK506 binding protein) to inhibit the phosphatase (152, 180). Through inhibiting calcineurin's activity, FK506 not only suppresses the immune system but also causes side effects (11, 191, 289). In 1984 FK506 was isolated as a metabolic product of the fungus *Streptomyces tsukabaensis* at Fujisawa (Osaka, Japan) (116). FK506 is a 23-membered macrolide antibiotic. Functionally FK506 can be divided into three regions the FKBP-binding region, the calcineurin-binding region, and the northwest region (Figure 6). The northwest region of FK506 does not interact with FKBP-12 or calcineurin. It was hoped that through modifications of this region the toxicity of FK506 could be reduced.

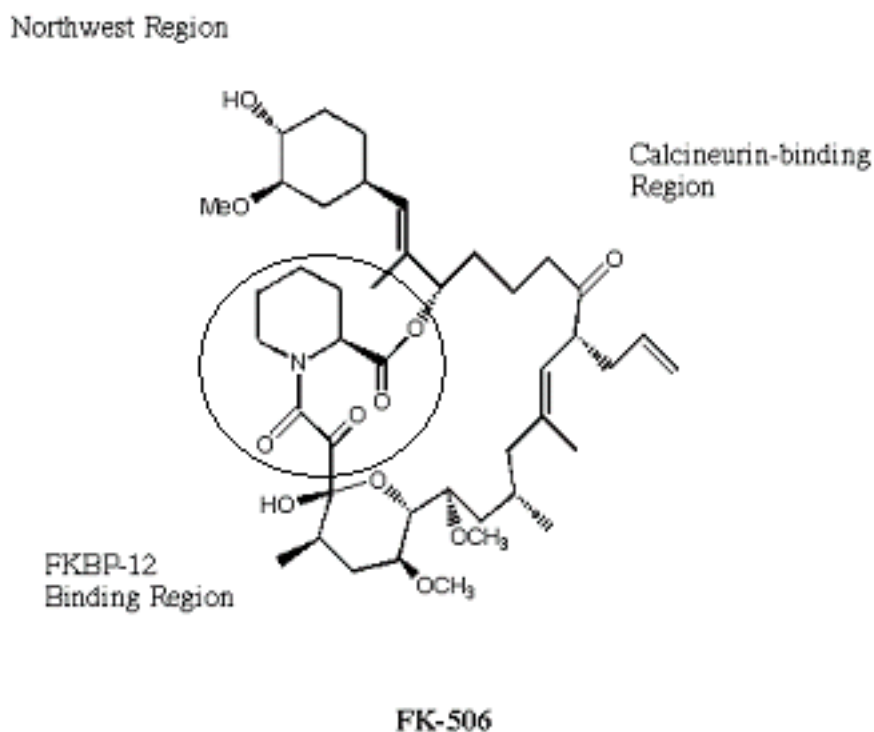


Figure 6: The chemical structure of the macrolide drug FK506 is shown. Molecular regions with known important biologic functions are indicated. The encircled region might serve as a lead structure for the development of specific anti-Chlamydia antibiotics without immunosuppressive side effects.

3.14 Inhibition of Mip PPIase activity by FK506

Human FK506-binding proteins have a peptidyl-prolyl cis-trans isomerase (PPIase) activity, which can be inhibited by FK506 (25). Besides their described role in immunomodulation these proteins fulfil basic cellular processes such as protein folding and trafficking. Human FKBP-12 interacts with several intracellular signal transduction proteins including type I TGF-beta receptor (295). This binding could be antagonized by FK506. FK506-binding proteins were also found in a number of pathogenic bacteria, such as *Legionella pneumophila*, *C. trachomatis*, *E. coli* and *Neisseria meningitides* (106, 129, 183). Conserved amino acids, which are important for binding of FK506, have been identified in FKBP's (106, 246). Macrophage infectivity potentiator, an FKBP protein of *L. pneumophila* (IpMip), is a virulence factor that plays an essential role in

the ability to survive and multiply in phagocytic host cells (47, 48, 49). LpMip as well as Mip protein from *C. trachomatis* (ctMip) were shown to play an important role in infection initiation and to possess PPIase activity (47, 48, 49, 185, 186). This PPIase activity, which was shown to be essential for successful Legionella infection (119), can be inhibited by FK506 and Rapamycin, which also leads to reduced infectivity of *C. trachomatis* (185). Chlamydial infections were not able to develop properly upon treatment with FK506. Therefore Mip proteins as virulence factors with known inhibitable enzymatic activity are attractive targets for antimicrobial research.

3.15 Aims of the study

Since Chlamydia have been described to possess a functional T3SS and to interfere with host cell signaling and morphology in a similar way as described for other type III-secreting pathogens, the objective of this work was to identify and characterize type III-secreted effector proteins of *C. pneumoniae*. Some of the identified, secreted effector proteins should be further investigated in order to elucidate their contribution to chlamydial virulence as well as their point(s) of interference with host cell signal transduction. The overall ratio of this concept was to identify new points of interference with chlamydial virulence, which could be used for the development of specific antichlamydial antibiotics. Such antibiotics, which are able to eradicate also persistent chlamydial infections are currently not available and would be of great clinical interest.

4 MATERIALS and METHODS

4.1 Materials

4.1.1 Chemicals

Roche (Mannheim, Germany), Roth (Karlsruhe, Germany), Sigma (Munich, Germany) and Serva (Heidelberg, Germany) supplied chemicals. All chemicals were ordered at “ACS” or “p.A.” purity grade. Radio chemicals and ampholytes were ordered from Amersham Biosciences (Freiburg, Germany).

4.1.2 Buffers and Solutions

Buffer/Solution	Composition
DNA loading buffer	20% Ficoll 400, 100 mM EDTA, 0.25% Xylen-Cynol, 0.25% bromophenolblue, 0.25% OrangeG
1x PBS	140 mM NaCl, 6.5 mM Na ₂ HPO ₄ , 2.5 mM KCl, 1.5 mM KH ₂ PO ₄ [pH 7.25]
1x TBE	100 mM Tris-HCl, 100 mM boric acid, 2.5 mM EDTA
1x TBS	20 mM Tris-HCl, 100 mM NaCl [pH 7.5]
1x TBS-T	1x TBS + 0,05% Tween 20
Stacking gel buffer (4x)	0.5 M Tris-HCl [pH 6.8]
Separating gel buffer (4x)	1.5 M Tris-HCl [pH 8.8]
Laemmli running buffer	25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS [w/vol]
5 x SDS sample buffer	50 mM Tris-HCl [pH 6.8], 4% [w/vol] SDS, 12% glycerol, 0.01% [w/vol] Coomassie Brilliant Blue G-250, 4% [w/vol] β-ME
Cell lysis buffer (SDS-PAGE)	50 mM Tris-HCl [pH 7.5], 1% Triton, 1 mM Pefabloc
10 x blotting buffer	250 mM Tris-HCl [pH 8.3], 1.92 M glycine, 10% methanol, 0.01% SDS

Silver stain fixation solution	10% [vol/vol] acetic acid, 30% [vol/vol] ethanol
Silver stain sensitizer	30% [vol/vol] ethanol, 0.5M sodiumacetate, 0.5% [vol/vol] glutaraldehyde, 0.2% [w/vol] sodiumthiosulfate
Silver nitrate solution	0.1% AgNO ₃ , 0.02% formaldehyde (37%)
Silver stain developer	2.5% [w/vol] Na ₂ CO ₃ , 0.01% [vol/vol] formaldehyde (37%)
Silver stain stop solution	1% [vol/vol] acetic acid
Western blot stripping buffer	5 mM NaPO ₄ [pH 7.5], 2 mM β-ME, 2% SDS
RIPA buffer	120 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 0,5% DOC, 0,1% SDS, 200 μM NaVO ₄ , 20 mM NaF, 1 mM PMSF
IP wash buffer	50 mM Tris-HCl [pH 7.5]
Buffer I (protein purification)	50 mM NaH ₂ PO ₄ [pH 7.5], 300 mM NaCl
10 x kinase assay buffer	250 mM Tris-HCl [pH 7.5], 13.2 mM CaCl ₂ , 50 mM MgCl ₂ , 10 mM EDTA, 12.5 mM EGTA
IEF buffer	7 M urea, 2 M thiourea, 4% [w/vol] CHAPS, 2,5% [w/vol] DTE, 5% [vol/vol] glycerol, 10% [vol/vol] isopropanol, 2% [vol/vol] ampholytes, 2% [w/vol] bromophenol blue
IPG strip reducing buffer	50 mM Tris-HCl, 8 M urea, 30% glycerol, 2% [w/vol] SDS, 2% [w/vol] DTE
IPG strip alkylating buffer	50 mM Tris-HCl, 8 M urea, 30% glycerol, 2% [w/vol] SDS, 2.5% JAA, 0.00015% bromophenol blue

Table 1: Buffer and solution compositions with final pH indicated in brackets

4.1.3 Chromatography column material

Superose 6 (Amersham Biosciences)

Ni-NTA Superflow (Qiagen)

4.1.4 Transfection reagents

Lipofectamine 2000 (Invitrogen)

Fugene 6 (Roche)

4.1.5 Antibodies

4.1.5.1 Primary antibodies

Mouse monoclonal anti His₄ BSA free antibody (Qiagen)

Mouse monoclonal anti β -actin antibody (Sigma)

Mouse monoclonal anti c-myc antibody (clone 9E10) (Roche)

Mouse monoclonal anti SUMO-1 (GMP-1) antibody (Zytomed GmbH, Berlin, Germany)

Rabbit anti SUMO-3 (Sentrin-2) antibody (also recognizes SUMO-2 (Sentrin-3), Zytomed)

Rabbit anti PIASx (1/2) antibody (Abcam)

Rabbit anti phospho-Threonine antibody (Cell signaling)

Mouse monoclonal anti phospho-Serine/anti phospho-Threonine antibodies (reporter antibodies, Qiagen) and biotinylated anti-mouse antibody (secondary antibody, Qiagen) for Luminex kinase assay

Mouse monoclonal anti Caspase 7 antibody (capture antibody, US Biological, Swampscott, MA, USA), rabbit anti active Caspase 7 antibody (reporter antibody, Biocat, Heidelberg, Germany) and biotinylated goat anti-rabbit antibody (secondary antibody, DPC Biermann, Bad Nauheim, Germany) for Luminex apoptosis assay

Polyclonal rabbit antibody directed against *C. pneumoniae* IncA protein was kindly provided by Prof. U. Gross (University of Göttingen, Göttingen, Germany).

The polyclonal rabbit antibody H15Snew08, directed against *C. pneumoniae* CpB0730 potential myristoylation site, was made against the synthetic peptide antigen HGGIQRQHGSNSPS, corresponding to amino acid residues H⁴⁷⁹ to S⁴⁹³ of CpB0730 (peptide number SP012125). The peptide N-terminus was coupled to Ovalbumin. This antigen was used for the immunization of rabbits at Neosystem (Strasbourg, France).

The generation of polyclonal rabbit antisera directed against chlamydial candidate proteins is explained below in detail.

4.1.5.2 Secondary antibodies

Peroxidase-conjugated AffiniPure Goat Anti Mouse IgG (H+L)

Peroxidase-conjugated AffiniPure Goat Anti Rabbit IgG (H+L)

(Jackson ImmunoResearch Laboratories, provided by Dianova, Hamburg, Germany)

Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes, Leiden, The Netherlands)

Alexa Fluor 568-conjugated goat anti-rabbit antibody (Molecular Probes)

Direct immunolabeling of primary antisera the was performed with the Alexa Fluor 568 Zenon Rabbit IgG labelling kit (Molecular Probes)

4.1.6 Kits

Dneasy Tissue Kit	Qiagen
Expand High Fidelity PCR System	Roche
Lumi Light Plus Western Blotting Substrate	Roche
Qiagen plasmid isolation kits	Qiagen
QIAquick PCR purification/gel extraction/nucl. removal Kit	Qiagen
Qiagen DyeEx spin kit 2.0	Qiagen
Quick Change Site XL directed mutagenesis Kit	Stratagene
VenorGeM Mycoplasma PCR detection kit	Minerva Biolabs
NFκB, STAT1, STAT2, STAT3 activation hit kits	Cellomics
Pathfinder Chlamydia culture confirmation system	Bio-Rad
IP Starter Pack	Amersham Pharmacia
BCA protein assay	Perbio Science
Alexa Fluor 568 Zenon Rabbit IgG labelling kit	Molecular Probes

4.1.7 Enzymes

The enzymes used were purchased from Roche, Stratagene (La Jolla, CA, USA), Promega (Mannheim, Germany), and New England Biolabs (Frankfurt a. M., Germany).

4.1.8 Media

Luria-Bertani broth and agar was purchased from Gibco BRL.

SOC medium was provided by Invitrogen (Karlsruhe, Germany).

NZA medium: 10 g NZ-amine A (Quest International, Bussum, The Netherlands), 5 g yeast extract, 7.5 g NaCl per liter

Eukaryotic cell culture media and components were supplied by Gibco-BRL

HEp-2 cell culture medium: MEM supplemented with 10% heat-inactivated FCS (Eurobio, Les Ulis, France), 2 mM L-glutamine, 1% [vol/vol] NEAA, 1 mM sodium pyruvate, 0.08% sodium bicarbonate

Infection medium: MEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1% [vol/vol] NEAA, 10 mM HEPES, 0.1% glucose

HEK 293 EBNA cell culture medium: DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine

4.1.9 Antibiotics

Ampicillin: 50 mg/ml stock in H₂O (aliquots stored at -20°C), used at 50-100 µg/mL for the cultivation and selection of *E. coli* in liquid culture and on agar plates.

4.1.10 Bacterial strains

Chlamydophila pneumoniae TWAR strain TW183 (ATCC: VR-2282)

The *E. coli* strains used in this work are listed in the table below:

Strain	Genotype	Source
One Shot Top 10 Chemically competent <i>E.coli</i>	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Δ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
XL10-Gold ultracompetent cells	Tet ^R Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1</i> <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA96</i> <i>relA1</i> <i>lac</i> <i>Hte</i> [F' <i>proAB</i> <i>lacI</i> ^q Δ <i>M15</i> <i>Tn10</i> (Tet ^R) <i>Amy</i> <i>Cam</i> ^R] ^a	Stratagene
BL21(DE3)	F ⁻ <i>ompT</i> <i>hsdS</i> (rB ⁻ mB ⁻) <i>dcm</i> ⁺ Tet ^r <i>gal</i> λ(DE3) <i>endA</i> <i>Hte</i> (pLysS <i>Cam</i> ^r)	Stratagene

Table 2: *E. coli* strains used in this work.

4.1.11 Eukaryotic cell lines

HEp-2 (ATCC: CCL-21) HeLa contaminant cell line

HEK 293 EBNA (ATCC: CRL-1573) kidney epithelial cell line

4.1.12 Vectors and plasmids

Name	Description	Reference
pCR2.1-TOPO	Cloning vector for cloning of Taq-amplified PCR products prior to subcloning of these constructs into the final target vectors. Ampicillin and Kanamycin resistance	Invitrogen
pET15b	<i>E.coli</i> expression vector for IPTG-inducible overexpression of His ₆ -tagged fusion proteins. Ampicillin resistance	Novagen
pET32a	<i>E.coli</i> expression vector for IPTG-inducible overexpression of His ₆ -Trx-tagged fusion proteins. Ampicillin resistance	Novagen
pGEX-2T	<i>E.coli</i> medium copy number expression vector (pBR322-derived) with ampicillin resistance gene. Expression is induced by IPTG. Proteins are expressed as N-terminal GST fusions. A thrombin cleavage site allows for the removal of the GST tag. Ampicillin resistance	Amersham Biosciences
pCS3+ MT	Multipurpose pBluescript II KS ⁺ -derived vector for the high-level transient expression of N-terminal myc ₆ -tagged fusion proteins in eukaryotic cells from an sCMV IE94 promoter. Ampicillin resistance	(250, 286)
pCS3+ MTNLS	pCS3+MT with additional nuclear localization sequence in front of the myc tag sequences. Ampicillin resistance	(250, 286)

Table 3: Plasmids that were used to express the different constructs and candidate proteins in *E.coli* or in human cell culture.

4.1.13 Oligonucleotides

ORF	fusion protein N to C terminus		primers (5' to 3')	Restriction sites
CpB0334 (CopN)	GST-CopN	fp	ATTACCCGGGAATGGCAGCATCAGGAG GCA	SmaI/SmaI
		rp	ATTTCCCGGGTTATGACCAAGGATAGGG TTTAG	
CpB0687 (cpMip)	GST-CpMip	fp	CGCGGGATCCATGAACAGACGGTGGAA TTTAGTT	BamHI/ EcoR1
		rp	AACCGGAATTCACCCGCCGGATTCTTTT TAAGCTAATGAGCG	
CpB0730 (Pkn5)	GST-CpB0730	fp	ATTAGGATCCATGGATTGTCGTGGTGGC ATC	BamHI/ EcoR1
		rp	AACCGGAATTCACCCGCCGGATTCTTTT TAAGCTAATGAGCG	
CpB0733	His ₆ -CpB0733	fp	TTCCATGGTACCCCATATGGTGGCAAAA TATCCACTAGAGCC	NdeI/XmaI
		rp	AACCCGGGTCGACATTAGCTTCCCCCTG ATTCACG	
CpB0736	His ₆ -CpB0736	fp	TTCCATGGTACCCCTCGAGGCAGATTTG GAAGTATTTCAAGC	XhoI/ BamHI
		rp	AAGGATCCGTCGACATTAGCCCAAAGGA CCTGCACTTTTTG	
CpB0739	His ₆ - TrxA - E ¹¹⁴ -R ³⁶⁷	fp	TTCCATGGTACCCCATATGGCAGTACGA TTAATTGTTGATGAAGG	NcoI/Sall
		rp	AACCCGGGTCGACATTATTTATTGTAGT CTATTTTATATTTCAACCCTTC	
CpB0837	M ¹ -L ¹³⁵ -GST- G ¹⁵⁷ -L ²⁰⁴	fp1	TTCCATGGTACCCCATATGACATCAGGA GTTAGTGGAAAGTTC	NdeI/XmaI
		rp1	CCAATAACCTAGTATAGGGGAAAGTACA ATACCATTGATGG	
		fp2	CCATCAATGGTATTGTACTTTCCCCTATA CTAGGTTATTGG	
		rp2	GAAGCTTTCCAAGACTCTCCGGATCCAC GCGGAACCAG	
		fp3	CTGGTTCCGCGTGGATCCGGAGAGTCTT GGAAAGCTTC	
		rp3	AACCCGGGTCGACATTACAAGGACTGTT TTGCCTG	
CpB0856	His ₆ -CpB0856	fp	TTCCATGGTACCCCATATGACTGCCAAC ACTTTTGGAACCTC	NdeI/XmaI
		rp	AACCCGGGTCGACATTATTTCACTAAGA CTTTCATGCATTGTTC	

Table 4: Forward (fp) and reverse (rp) oligonucleotide primers used for cloning of effector candidate genes from *C. pneumoniae* TW183 into bacterial expression vectors pET15b, pET32a or pGEX-2T. The composition of the fusion protein antigens and the restriction sites used for cloning are indicated.

4.1.14 Devices and software

Cellomics Array Scan	Cellomics
Luminex 100 IS LiquiChip workstation	Luminex
Luminex 100 IS 2.2 software	Luminex
Confocal microscope:	Leica
DMRE microscope	Leica
TCS SP2 True confocal scanner	Leica
LAS-1000 Luminescence Image analyzer	Fuji Film (Düsseldorf, Germany)
FLA-5000 scanner	Raytest
DMLB fluorescence microscope	Leica (Bensheim, Germany)
IM1000 image software	Leica
Biologic HR FPLC workstation	Bio-Rad
Agilent 2100 Bioanalyzer	Agilent
Branson sonifier 150	Heinemann (Schwäbisch Gmünd, Germany)
ABI Prism 310 Genetic analyzer	Applied Biosystems
Adobe Photoshop 7.0	Adobe Systems Inc. (San Jose, CA, USA)
Aida Image analyzer Version 3.22	Raytest

4.2 Methods

4.2.1 Microbiological methods

4.2.1.1 Cultivation of *E.coli*

E. coli was grown under aerobic conditions at 37°C on LB agar plates or in LB broth containing the appropriate antibiotics. Liquid cultures were incubated on a shaker at 220rpm.

Glycerol stocks were prepared by mixing liquid cultures 1:1 with 40% glycerol [vol/vol] , freezing the mixture in liquid nitrogen and keeping the stocks at -80°C for long-term storage.

4.2.1.2 Transformation of *E.coli*

For transformation 3-5 µl ligation reaction or 1 µl of prepared plasmid DNA were added to the chemically competent *E.coli* and cells were incubated on ice for 5 to 10 min. Thereafter suppliers' protocols for chemical transformation were followed. Finally, after addition of 250 µl SOC cells were grown at 37°C at 220 rpm for 60 min before the whole reaction was plated on selective agar plates.

4.2.1.3 Chlamydia propagation

HEp-2 cells were used as host cell line for *Chlamydomphila pneumoniae* TWAR strain TW183 throughout this work. The infection of approximately 70% confluent HEp-2 monolayers was carried out at an MOI of 5 or 25 on coverslips of Sterilins (Bibby Sterilin, Staffordshire, UK) or in 25 cm² cell culture flasks (Greiner bio-one, Frickenhausen, Germany) as reported previously (260). Chlamydia, resuspended in infection medium, were centrifuged for 1 h at 37°C onto the HEp-2 cell culture at 1600 x g in a Hettich Rotanta 46RC centrifuge. After centrifugation the chlamydial suspension was replaced by fresh infection medium. For the generation of chlamydial stocks infected cell cultures were scraped off the plastic flasks with plastic scrapers, resuspended in 6 mL infection medium per 25 cm² flask and transferred to a 50 mL Falcon tube. Sterile glass beads were added and the cell material was homogenized by

vortexing for 2 min. The resulting homogenate was frozen at -80°C as chlamydial stock or aliquots were directly used for propagation of Chlamydia in a new round of infection. In the latter case, 4 mL of infectious homogenate were added to a 25 cm² flask of almost confluent HEp-2 cells. Two mL of fresh infection medium were added and the centrifugation procedure was carried out as described. By this method up to eight consecutive rounds of infection were carried out in order to increase the chlamydial yield.

4.2.2 Eukaryotic cell culture

4.2.2.1 Transfection of eukaryotic cell cultures

Cationic lipid transfection agents are widely used tools for the transfection of eukaryotic cells in cell culture. These lipid agents are able to form complexes with negatively charged DNA and to translocate as such complexes through the host cell membrane. Transfections were carried out using Lipofectamine 2000 (Invitrogen) or Fugene 6 (Roche) as transfection agents according to the supplier's manual. 24µg DNA was used per cell culture petri dish with 10 cm diameter. Medium was changed after 6 h and transfected cells were harvested for downstream applications 48 h post transfection.

4.2.2.2 Incubation of chlamydia-infected cell cultures with FK506

HEp-2 cells were infected on coverslips as described. Infection medium with different concentrations of FK506 (Biomol GmbH, Hamburg, Germany) dissolved in sterile DMSO was added to infected and uninfected cells directly after the centrifugation step. The cells were incubated under these conditions until fixation with methanol followed by ICC with the Pathfinder Chlamydia culture confirmation kit as indicated by the supplier. Additionally, nuclei were stained with Hoechst 33342.

4.2.2.3 Generation of cell lysates

After two washings with ice-cold PBS infected and uninfected HEp2 cells were scraped off from 25 cm² cell culture flasks with a plastic scraper at different time points of the infection cycle. Cells were resuspended in 200 µL/25 cm² ice-cold cell lysis buffer and

lysed by ultrasonication for 15 sec at setting two. Lysates were stored frozen at -20°C until analysis by SDS-PAGE and Western blotting.

4.2.3 Molecular biological methods

4.2.3.1 Isolation of genomic DNA from *C. pneumoniae*

C. pneumoniae TW183 DNA was prepared by isoamylalcohol/chloroform extraction from isolated elementary bodies (EBs) as follows. A detailed description can be found in reference (260). In brief purified chlamydial EBs were pelleted for 60 min at 30000 x g at 4°C . After resuspending the EBs in TE-buffer cells were lysed with SDS and proteins were degraded by addition of proteinase K. Under high NaCl conditions and by addition of CTAB denatured proteins, cell membrane fragments and polysaccharides were precipitated. In a two-step phase extraction with an isoamylalcohol/chloroform mixture CTAB-protein and CTAB-polysaccharide complexes were removed and DNA was isolated with the aqueous supernatant. DNA was precipitated with isopropanol, washed with 70% ethanol and the pellet was dried. DNA was redissolved in 10 mM Tris-HCl buffer with pH 8.5.

4.2.3.2 Standard PCR

Standard PCR reactions were carried out in 100 μl volumes using the GeneAmp PCR System 9700 from Applied Biosystems. The following reagents were mixed: Expand High Fidelity PCR System Polymerase (2.6 Units) with the supplied buffer 2, 100 ng template DNA, 30 pmol of each primer, 10 mM dNTPs each. The cycling protocol was started with a denaturation step for 3 min at 94°C followed by 30 cycles with 30 sec at 94°C denaturation, 30 sec 50°C primer annealing and 3 min at 72°C polymerization, followed by a final polymerization step at 72°C for 7 min.

For analysis PCR samples were mixed with SYBR Green according to the supplier's manual. Fluorescent PCR fragments were separated on 0.8-1.5% agarose gels prepared with 1x TBE and visualized with the GeneGenius Bio Imaging System from Syngene. Alternatively linear DNA could be analyzed with the Agilent 2100 Bioanalyzer

according to the manufacturer's protocol using the DNA 1000, DNA 7500 or DNA 12000 chips. For further use PCR fragments were purified using the Qiaquick PCR Purification kit (Qiagen). Gel extraction was used if various products were generated.

4.2.3.3 Cloning procedures

PCR fragments and plasmids were digested with the appropriate restriction endonucleases using 10 U/ μ g DNA following the supplier's manual. After purification or gel extraction (QIAquick Gel extraction Kit) ligation of DNA fragments was performed using T4 DNA ligase (New England Biolabs). *E. coli* Top 10 (Invitrogen) were used for plasmid transformation according to instructions.

Plasmid isolation from 2 ml of an overnight culture was performed using Qiaprep Spin Kit and sequences were verified by restriction endonuclease cleavage, gel analysis and sequencing. Larger amounts of plasmid DNA were prepared with the Qiagen Plasmid Maxi Kit.

4.2.3.4 Cloning of candidate sequences into bacterial expression vectors

Effector candidate sequences were cloned in frame with tags (GST, His₆) into the expression vectors pET15b, pET32a (Novagen) or pGEX-2T (Amersham Biosciences). Therefore candidate ORFs were PCR-amplified. Primers and fusion protein compositions are listed in table 4. Purified chlamydial chromosomal DNA was used as a template. The resulting PCR products were purified by gel extraction, ligated into the respective vectors and verified by sequencing.

Two hydrophilic domains of CpB0837 were cloned as a fusion, with GST being in the middle of the two CpB0837 sequences (nt 1-80 and nt 469-508). This was done by fusion PCR with overlapping primer sequences. Primers and fusion protein compositions are listed in table 4. The two hydrophilic domains and the GST ORF were first amplified separately by PCR using the indicated primers. Then CpB0837 domain 1 fragment was joined with the GST fragment by fusion PCR before the resulting fragment finally was joined with the domain 2 fragment of CpB0837. The resulting construct with the GST tag separating the two candidate gene fragments was cloned into the respective expression vector and verified by sequencing.

4.2.3.5 Cloning of wt CpB0730 and gain-of-function mutants into eukaryotic expression vectors

The CpB0730 ORF was amplified in PCR reactions with primers listed in table 5.

Oligonucleotide	sequence 5' to 3'	restriction site
Wild 730 pCS3+	<u>GGCGGATCCCCGCCAT</u> G GATTGTCGTGGTGG CATCCC	BamH1
Funky 730 V1 pCS3+	<u>GGCGGATCCCCGCCAT</u> G GATGGTCGTGGTAT CCCTGGCCTTCCTGAACCTCAGGTG	BamH1
Funky 730 V2 pCS3+	<u>GGCGGATCCCCGCCAT</u> G GGTTGTGGTCGTAT CGGTCCTCCTTCCTGAACCTCAGGTGATTGGC	BamH1
Funky 730 Anti pCS3+	GGTGATGATGGTGATG <u>AGGCCTCCG</u> CCGGAT TCTTTTAAAGC	StuI

Table 5: Oligonucleotides used for cloning of wt CpB0730 and gain-of-function mutants thereof into eukaryotic expression vectors. Restriction sites are underlined and the start codon is indicated in bold letters.

For mutant generation the primers covering the 5' region of CpB0730 carried mutations in order to restore the missing GXGXXG motif. All reactions were carried out at 50°C annealing temperature. The resulting PCR products were purified by gel extraction and ligated into the vectors pCS3+ MT and pCS3+ MTNLS (Figure 7). The resulting vectors Wild pCS3+ MT, V1 pCS3+ MT, V2 pCS3+ MT, Wild pCS3+ MTNLS, V1 pCS3+ MTNLS and V2 pCS3+ MTNLS were verified by sequencing (Table 6).

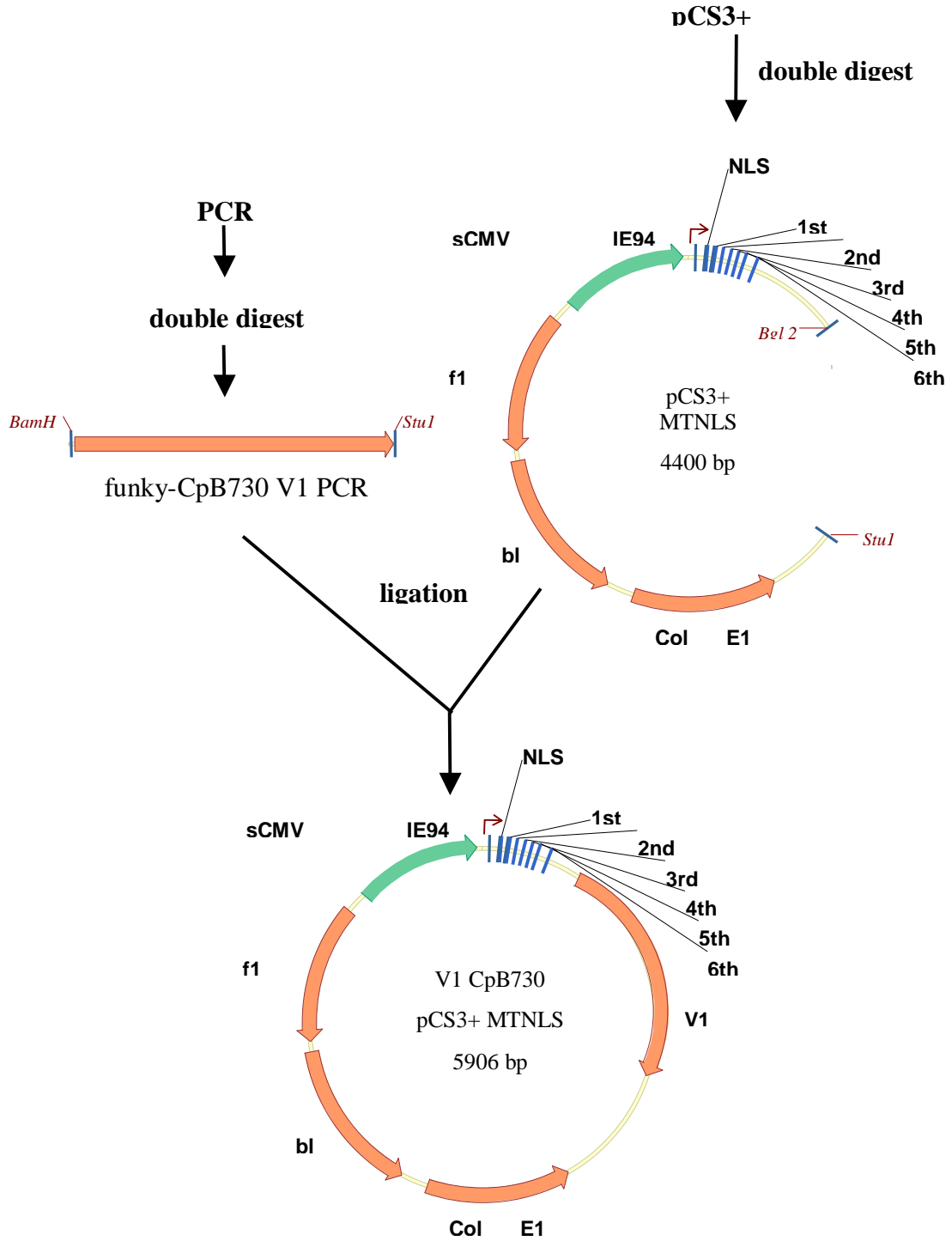


Figure 7: Cloning strategy: wt and gain-of-function mutants were PCR amplified and both PCR products and vectors were double digested as described. Dephosphorylated vector was ligated with inserts as described above.

Name	Description	cloning sites
Wild pCS3+ MT	CpB730 wt cloned into pCS3+ MT for transient, strong and ubiquitous expression of the myc-tagged protein in mammalian cells	Bgl2/Stu1
V1 pCS3+ MT	CpB730 functional mutant V1 cloned into pCS3+ MT accordingly	Bgl2/Stu1
V2 pCS3+ MT	CpB730 functional mutant V2 cloned into pCS3+ MT accordingly	Bgl2/Stu1
Wild pCS3+ MTNLS	CpB730 wt cloned into pCS3+ MTNLS for transient, strong expression of the myc-tagged protein in the nuclei of mammalian cells	Bgl2/Stu1
V1 pCS3+ MTNLS	CpB730 functional mutant V1 cloned into pCS3+ MTNLS accordingly	Bgl2/Stu1
V2 pCS3+ MTNLS	CpB730 functional mutant V2 cloned into pCS3+ MTNLS accordingly	Bgl2/Stu1

Table 6: Plasmids that were constructed for the transient expression of myc-tagged wildtype CpB0730 and functional mutants thereof in transfected cultured mammalian cells with cytoplasmic or nuclear localization.

4.2.3.6 Site-directed mutagenesis

Dominant-negative kinase mutants (K^{47} to R^{47}) were created by site-directed mutagenesis with the respective CpB0730 pCS3+ constructs as templates together with primers CpB0730 K-R fp and CpB0730 K-R rp (Table 7).

Oligonucleotide	sequence 5' to 3'	T _{anneal}
CpB730 K-R fp	ccattctacagtcataagag <u>ttttt</u> ctcc	60
CpB730 K-R rp	ggagaaaaaact <u>ctt</u> atgactgtagaatgg	60

Table 7: Site-directed mutagenesis primer sequences and annealing temperatures are shown. The mutated codon is underlined.

The procedure was carried out using the Quik Change XL site-directed mutagenesis kit (Stratagene) as described in the manufacturer's manual. The cycling protocol was started with a denaturation step for 1 min at 95°C followed by 18 cycles with 50 sec at 95°C denaturation, 50 sec 60°C primer annealing and 11 min 50 sec at 68°C polymerization, followed by a final polymerization step at 68°C for 7 min. Mutated plasmids were transformed into the XL10-Gold ultracompetent cells as described in the manual and transformed clones were selected on tetracycline containing agar plates. Mutations were verified by plasmid preparation from these clones followed by sequence analysis.

4.2.3.7 DNA Sequencing

Sequencing of plasmid DNA was carried out with the Applied Biosystems BigDye terminator sequencing ready reaction kit, basically as described in the manufacturer's manual. 2µL BigDye mix, 1 µL 5x buffer, 5 pmoles of sequencing primer and 250 ng plasmid DNA were brought to a total volume of 10 µL with HPLC grade H₂O (Merck) and mixed in a PCR tube. The PCR protocol started with a denaturation step for 3 min at 96°C followed by 25 cycles with 10 sec at 96°C denaturation, 5 sec 50°C primer annealing and 4 min at 60°C polymerization, followed by a final polymerization step at 60°C for 1 min. The product DNA was purified with the Qiagen DyeEx spin kit 2.0 as described by the supplier, except that columns were additionally washed with 700 µL HPLC grade H₂O prior to loading the PCR reaction onto the column. 10 µL of HPLC grade H₂O were added to the loaded columns prior to centrifugation. 10 µL of purified DNA were mixed with another 10µL of HPLC grade H₂O in 0.5 mL sample tubes and were then ready for sequencing. DNA was sequenced in an ABI Prism 310 Genetic analyzer (Applied Biosystems) following the manufacturer's protocol. Sequences were analyzed with Chromas 1.45 (shareware) and VectorNTI software.

4.2.3.8 Mycoplasma test

Cell cultures and chlamydial stocks were continuously tested for Mycoplasma contamination with the VenorGEM PCR detection kit (Minerva, Berlin, Germany) according to the supplier's manual. DNA extracted with the Dneasy Tissue Kit from

400 μ L cell culture supernatant or from chlamydial stocks was used in the PCR reactions. The resulting PCR samples were analyzed by agarose gel electrophoresis as described in the manual. Agarose gels were photographed with the GeneGenius Bio Imaging System (Syngene) (Figure 8).

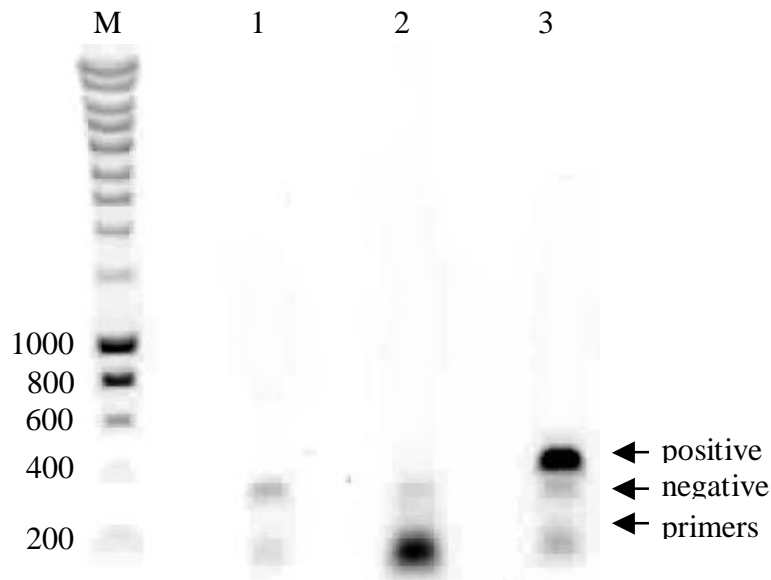


Figure 8: VenorGEM Mycoplasma PCR analyzed on 0.8% agarose gel. Lane 1: negative control, lane 2: Hep-2 + TW183 cell culture supernatant, lane 3: positive control, M: Smart ladder (Eurogentec).

4.2.4 Biochemical methods

4.2.4.1 Expression and purification of candidate protein antigens for immunization

Protein expression was induced in transformed *E. coli* (strain BL21(DE3), Stratagene) at an OD_{600} of 0.5 and a growth temperature of 21°C overnight or at 37°C for 4h with 1 mM IPTG. Cells were harvested by centrifugation. The pellet of His₆-tagged protein was resuspended in 20 ml buffer I, ruptured in a French pressure cell at 16,000 p.s.i. and centrifuged for 30 min at 17300 x g. In case of the protein being soluble, the supernatant

was loaded onto a Ni-NTA superflow column from Qiagen (Hilden, Germany) equilibrated with buffer I. The column was washed with 20 mM imidazole and eluted with 250 mM imidazole, both in the same buffer. GST-tagged proteins were loaded onto Glutathione-Sepharose columns (Amersham Biosciences) equilibrated in buffer I. After washing with buffer I, the protein was eluted with 10 mM glutathione. If the protein was insoluble in inclusion bodies, 6M Gua was used to solubilize the protein. After an additional centrifugation step at 17300 x g, the supernatant fraction was loaded onto the respective column. 8M urea was added in this case to buffer I for all following purification steps. The eluates from either column type were run on preparative 20x14 cm SDS-PAGE gels, which were stained with Coomassie Brilliant blue in water. Following the destaining of the gels with water, the protein bands of interest were cut out. Elution of the protein was carried out, by placing the gel slice into a dialysis membrane bag in SDS running buffer and by applying an electric field in an SDS-PAGE chamber.

4.2.4.2 SDS-PAGE

10 to 15% polyacrylamide gels were prepared according to Laemmli *et al.* (168). General recipes are described in the table below (Table 8). Full Range Rainbow molecular weight marker (Amersham Biosciences) was used as a protein standard. Samples were mixed with 5 x SDS sample buffer and boiled for 5 min at 95°C. After brief centrifugation samples were loaded onto SDS-PAGE gels. Minigels were run at 100 V to 200 V in a Mini-Protean II electrophoresis cell (Bio-Rad).

4.2.4.3 Gel Code Blue staining and SYPRO Ruby staining

Protein bands/spots in wet SDS-PAGE gels were stained with Gel Code Blue stain reagent (Pierce) or SYPRO Ruby protein gel stain (Molecular probes) according to the suppliers' manuals.

	Stacking gel (2 gels) 4%	Separating gel (2 gels) 10%	Separating gel (2 gels) 15%
H₂O	3 ml	2.79 ml	1.14 ml
Stacking gel buffer (4x)	1.25 ml	-	-
Separating gel Buffer (4x)	-	2.5 ml	2.5 ml
acrylamide/bis (30%/ 0.8%)	0.67 ml	3.33 ml	5 ml
SDS (20%)	0.1 ml	0.1 ml	0.1 ml
glycerol	-	1.2 ml	1.2 ml
TEMED	3.8 µl	7.5 µl	7.5 µl
APS	37.5 µl	75 µl	75 µl

Table 8: Recipes for SDS-PAGE stacking and separating gel mixtures.

4.2.4.4 Silver staining

Silver staining of SDS-PAGE protein gels was performed according to a modified protocol of Heukeshoven (123). In brief, gels were fixed for 20 min in fixation solution, incubated for 30 min in sensitizer and then washed three times for 20 min in MQ. After incubating the sensitized gel in fresh silver nitrate solution for 30 min, the addition of developer led to visualization of protein bands/spots on the gel. This process was stopped by soaking the gel for 5 min in stop solution. After four consecutive washing steps in MQ for 5 min the gels could be scanned using the FLA-5000 scanner (Raytest). For long term storage or for autoradiography gels were dried in between two sheets of cellophane in a GelAir Dryer (Bio-Rad) for 2 h.

4.2.4.5 2D SDS-PAGE, 1st dimension: IEF

IEF was performed on an Ettan IPG Phor system (Amersham Biosciences) with 18 cm pH 3-10 (non-linear gradient) or pH 4-7 (linear) Immobiline DryStrips from Amersham Biosciences. Rehydration of the strips was carried out as described in the supplier's

manual. Protein samples were precipitated with the 8-fold volume of ice-cold acetone for 30 min. By this precipitation method sample volumes could be reduced and the samples could be desalted for better IEF performance. Precipitated protein was pelleted by centrifugation at 12000 x g for 20 min at 4°C. The pellet was air dried and re-dissolved in 340 µL IEF buffer. Four µL 1% immobiline buffer as well as 4 µL 2% [w/vol] bromophenol blue were added. The sample was mixed by vortexing for 1 min and then distributed within the IEF coffins. Pre-swollen IPG strips were inserted upside-down into the coffin and overlaid with paraffin oil. Coffins were put onto the IPG Phor at 50 µA per strip at RT for 80 to 90 kWh o/n with the program described in the table below (Table 9). The ends of the strips were removed and strips were rinsed with MQ. Strips were equilibrated 12 to 15 min in IPG strip reducing buffer and then alkylated for 5 min with JAA in IPG strip alkylating buffer.

Step	Voltage	time [h]	Mode
1	30 V	8	Step'n'hold
2	200 V	0.1	Gradient
3	200 V	0.3	Step'n'hold
4	500 V	0.3	Gradient
5	500 V	0.3	Step'n'hold
6	2000 V	2.3	Gradient
7	2000 V	1	Step'n'hold
8	8000 V	2.3	Gradient
9	8000 V	99	Step'n'hold

Table 9: IEF program for IPG Phor

4.2.4.6 2D SDS-PAGE , 2nd dimension : SDS-PAGE

The second dimension consisted of a 0.75 mm 10% SDS-PAGE and was performed in a ProteanII cell (Bio-Rad) o/n at 400 V and 60 mA with watercooling. IPG strips were placed on top of the SDS-PAGE gel and covered with melted agarose in Laemmli buffer.

4.2.4.7 Immobilized metal affinity chromatography (IMAC) with rHis₆-CpB0730

Solubilization buffer: 100 mM NaH₂PO₄, 10 mM Tris-HCl, 6M Gua, 0.1% Triton,

20 mM β-ME, pH 8.5

Wash buffer: 100 mM NaH₂PO₄, 300 mM NaCl, 6M Gua, 0.1% Triton,

20 mM β-ME, pH 8.0

Elution buffer: 100 mM NaH₂PO₄, 300 mM NaCl, 6M Gua, 0.1% Triton,

20 mM β-ME, pH 3.5

Column material: Ni-NTA Superflow (Qiagen)

Workstation: Biologic HR FPLC workstation (Bio-Rad),

UV detection at 280 nm

All purification steps were carried out at 4°C. 1 g rHis₆-CpB0730 inclusion bodies were solubilized o/n in 10 mL solubilization buffer. Insoluble particles were removed by centrifugation at 14000 x g and 4°C for 5 min. The supernatant was used for batch load with 10 mL Ni-NTA Superflow resin, which was previously washed with 5 mL solubilization buffer. 10 mL Ni-NTA Superflow was mixed with 10 mL soluble supernatant and stirred for 1.5 h at RT. The protein-loaded Ni-NTA Superflow material was packed into an FPLC column (10mL) and attached to the FPLC workstation. After equilibrating the column with 20mL solubilization buffer and washing with 30mL wash buffer, rHis₆-CpB0730 was eluted at pH 3.5 with 20mL elution buffer at a flow rate of 0.5 mL/min. Eluted protein was collected in 1 mL fractions.

4.2.4.8 Size exclusion chromatography with rHis₆-CpB0730 peak fractions

Buffer: 100 mM NaH₂PO₄, 100 mM NaCl, 4M Gua, pH 8.0

Column material: Superose 6, 6% cross-linked agarose, 13 μm diameter (Amersham Biosciences)

Workstation: Biologic HR FPLC workstation (Bio-Rad), UV detection at

280 nm

One mL of the unified peak fraction from the IMAC column was loaded onto the equilibrated Superose 6 size exclusion column. 1.5 mL fractions were collected at an isocratic flow rate of 0.5 mL/min. The resulting purified rHis₆-CpB0730 protein was identified by peptide mass fingerprint with MALDI-TOF analysis.

4.2.4.9 Renaturation of FPLC-purified rHis₆-CpB0730

Dialysis buffer 1: 10 mM Tris-HCl, 150 mM NaCl, 2M Gua, [pH 7.5]

Dialysis buffer 2: 10 mM Tris-HCl, 150 mM NaCl, 5 mM β-ME, [pH 7.5]

Dialysis cups: Slide-a-lyzer MINI dialysis units (Pierce)

To remove the Gua contained in FPLC-purified rHis₆-CpB0730 stepwise dialysis was carried out. This protocol should enable proper refolding of rHis₆-CpB0730 protein. Dialysis was carried out o/n at 4°C in dialysis buffer 1 followed by dialysis buffer 2 respectively. Purified and dialyzed rHis₆-CpB0730 (0.13 μg/μL) was frozen at -20°C in 30% glycerol for kinase assays.

4.2.4.10 Conventional kinase assays

Cell lysates and purified proteins were phosphorylated in *in vitro* kinase assays and analyzed in SDS-PAGE gels or on Western blots by autoradiography. For 1D SDS-PAGE 20 μL reactions were composed of 2 μL 10 x kinase assay buffer, 2 μL 10 mM DTT, approximately 14 ng wt or mutant CpB0730 (IP or FPLC-purified dialyzed protein), 0.8 μL (8 μCi) [γ^{32} P]-ATP and of 0.1 to 1.5 μL of the respective substrate. HEp-2 cell lysate (10.5 μg/μL), MBP (2 μg/μL, Sigma) and histones (1 μg/μL, Sigma) were used as substrates. Non-radioactive kinase assays were performed with 5 μM non-radioactive ATP. As a positive control 0.1 μg recombinant PKCδ (personal gift from S. Dammeier) was used. For 2D SDS-PAGE analysis the volume of the kinase assays was increased to 100 μL. The reactions were started by addition of ATP or [γ^{32} P]-ATP and incubated for 12 min at 31°C. Twenty μL reactions were stopped by addition of 5 μL 5 x SDS sample buffer and boiling for 5 min at 95°C prior to application of the samples to SDS-PAGE analysis. 100 μL reactions intended for 2D SDS-PAGE analysis were stopped by the addition of 900 μL ice-cold acetone, which led to protein precipitation. If

protein bound to IP beads should be included in 2D SDS-PAGE analysis, the supernatant was submitted to acetone precipitation as described, while the IP beads were incubated in IEF urea buffer. After acetone precipitation the IP urea buffer fraction supernatant and the precipitated re-dissolved protein fraction were combined again. SDS-PAGE gels of radioactive kinase assays were dried as described and analyzed by autoradiography with the FLA-5000 scanner (Raytest).

4.2.4.11 Luminex kinase assays

Luminex is a flexible, bead-based solid-phase ELISA-like system for non-wash bioassays, mostly protein assays. With this system several ELISA measurements can be carried out in a single well with antibodies coupled to styrol beads. All bead types carry identification codes that enable the detection unit to identify and count the measured beads, which give a positive signal. Thereby many analytes can be measured simultaneously with high numbers of beads and signals per analyte, which results in statistically significant data. Multiplexing of assays offers the potential for the simultaneous detection and quantification of up to 100 different analytes within a single sample. For non-radioactive Luminex kinase assays the LiquiChip Ser/Thr kinase kit (Qiagen) was used according to the manual. Simultaneously MBP (bead code 18), Histone H1 (bead code 20), and Qiagen peptide substrates 3 (bead code 25) and 4 (bead code 27) were used as bead-coupled kinase substrates in a multiplex assay. Approximately 2500 beads from each substrate were present in each well. For autophosphorylation assays additionally bead-coupled wt CpB0730 (bead-code 77) was added as potential kinase substrate. Approximately 15 ng wt, mutant or DN CpB0730 were used per well. IP Sepharose beads were ruptured by gentle ultrasonication (5 sec, setting 2, Branson sonifier) prior to addition to the assay in order not to block the Luminex needle. PKC theta (250 ng, Panvera) was used as a positive control and DN CpB0730 mutant IP and IP from cells transfected with empty vector only were used as negative controls. Kinase assays were performed with 8 mM MgCl₂ and 5 mM ATP in either 1 x kinase assay buffer or in the assay buffer 1, which was provided with the kit. Additionally 50 µg/mL phosphatidylserine (Sigma) and 5 µg/mL diglycerol (Fluka) were added as PKC activators to 100 µL Luminex kinase assay reactions. Detection

and secondary antibodies were provided in the Qiagen kit. Streptavidin-coupled Phycoerythrin (SAPE) for Luminex assays was supplied by Prozyme (San Leandro, CA, USA) and Qiagen (Hilden, Germany). Kinase reactions were stopped after 60 min by the addition of stop solution as described in the manual. After stopping the reactions, sepharose bead particles sedimented during further 15 min incubation and supernatants were transferred to fresh 96-well plates for measurement. This step was included to prevent clogging of the Luminex needle by sepharose bead particles. For autophosphorylation assays wt CpB0730 substrate beads were produced by immunoprecipitating CpB0730 with styrol anti-myc beads. Monoclonal anti c-myc antibody (Roche) was coupled to Luminex styrol beads as described (59). These anti c-myc antibody beads were used in IP reactions.

4.2.5 Immunological methods

4.2.5.1 Immunization of rabbits and generation of antisera

The protein from the dialysis bag was used together with Ras-Ribi (Sigma) as adjuvant for the immunization of Chinchilla Bastard rabbits. Rabbits were boosted three more times before they were sacrificed after 8 weeks. Antisera were affinity purified using CNBr-activated Sepharose 4B according to the method described by Amersham Biosciences (“Affinity Chromatography Handbook, Principles and Methods”, 1993).

4.2.5.2 Immunoprecipitation

In order to precipitate wt and mutant CpB0730 from a cell lysate with a specific monoclonal anti-myc antibody (Roche), transformed cell cultures in 10 cm tissue culture petri dishes were harvested in RIPA buffer. First, cells were washed twice with ice-cold PBS. Thereafter cells were scraped off with a plastic scraper, resuspended in 1 mL of ice-cold RIPA buffer containing protease inhibitors (1 mM Pefabloc SC (Serva) or PMSF (Roche)) and optionally phosphatase inhibitors (20 mM NaF, 10 mM β -glycerophosphate, 1 mM sodium pyrophosphate, 200 μ M NaVO₄). Lysis was performed by ultrasonication with the Branson sonifier at level 3. Cell debris was

removed by centrifugation at 12000 x g for 20 min at 4°C. A 50% slurry of Protein A : Protein G (1:1) Sepharose beads with RIPA buffer was prepared with the IP Starter Pack (Amersham Biosciences) as described in the manual. Supernatants of the cell lysates were mixed 50:1 with antibody. In order to capture the antibody protein complexes 30µL slurry per mL lysate were added. The IP mixtures were incubated o/n at 4°C in an overhead shaker. The next morning IP beads were pelleted by centrifugation with 12000 x g at 4°C for 30 sec. The supernatant was discarded and the beads were washed three times with 1 mL ice-cold RIPA buffer and once with IP wash buffer. Thereafter IP beads were ready for downstream kinase assays or SDS-PAGE analysis. For short term storage beads were frozen in 30% glycerol at -20°C.

4.2.5.3 Western blotting

Proteins from SDS-PAGE gels were transferred to Protran Nitrocellulose transfer membranes (Schleicher & Schuell, Dassel, Germany) using a wet blotting system (TE series Transphor Electrophoresis unit by Hoefer) with 1 x blotting buffer at 950 mA for 45 min. Blots were blocked with 5% BSA in 1 x TBS by slight shaking for 2h at room temperature. Afterwards blots were incubated with antibodies, diluted in TBS + 0.05% BSA, overnight at 4°C. Antisera directed against candidate effector proteins were used at a 1:100 dilution, IncA antiserum was used at a 1:50 dilution whereas commercial antibodies were used according to the suppliers' manuals. After three consecutive washing steps with TBS-T for 10 min, bound antibody was detected using a 1:50000 dilution of horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany) for 2 h at room temperature. After three further washing steps with TBS-T, Western blots were developed with Lumi Light plus Western blotting substrate (Roche Diagnostics, Mannheim, Germany) and photographed with a LAS-1000 Luminescence Image analyzer (Fuji Film). Mouse anti β -actin antibody (Sigma) was used for normalization of the blots as indicated by the supplier.

4.2.5.4 Stripping of Western blots

Bound antibody was removed from Western blots by incubation for 30 min at 60°C in western blot stripping buffer. After washing the Western blots three times for 10 min with TBS-T, Western blots were ready for another immunodetection of proteins.

4.2.5.5 Detection of Chlamydia by MIF test

C. pneumoniae infected cell cultures were fixed in -20°C methanol for 5 min at -20°C and then subjected to immunodetection with the commercial MIF tests Pathfinder Chlamydia culture confirmation kit or with the *C. pneumoniae*-antigen-IFT kit as indicated by the suppliers.

4.2.5.6 Preparation and immunolabeling of samples for ICC

HEp-2 cell monolayers were grown on sterile glass coverslips and infected with *C. pneumoniae* TW183. Chlamydia were applied to the cell monolayers by centrifugation as described and medium was changed directly after centrifugation. Infected and uninfected cell monolayers were fixed at different time points of the infection cycle with 3.7% formaldehyde in PBS for 10 min at room temperature. The fixed cells were permeabilized with 0.1% Tween20 in PBS for 90 sec before the cells were washed twice with PBS. Samples were blocked with 1% Human Normal Serum (Dianova, Hamburg, Germany) for 1 h. All antisera against candidate proteins were used in 1:200 dilutions in PBS, and fixed cell samples were incubated therein for 2 h or o/n. After incubation with the candidate antiserum, unspecifically bound antibody was removed by 15 min treatment with PBS + 0.01% Tween20. After two washing steps with PBS an Alexa Fluor 488-conjugated secondary anti-rabbit antibody (Molecular Probes) was used at a concentration of 10 µg/mL for 2 h in PBS. Washing steps were carried out again in the same manner, before coverslips were mounted onto microscope slides. For double immunostaining of the chlamydial inclusions samples were additionally incubated with the primary antibody directed against chlamydial MOMP protein (medac, Wedel, Germany) in combination with an anti-mouse Alexa Fluor 568 antibody (Molecular probes). Hoechst 33342 was used at 10 µg/mL to stain eukaryotic as well as chlamydial DNA. Rabbit anti-IncA primary antibody (a kind gift of Professor U. Gross,

University of Göttingen) was used as a marker for the inclusion membrane at a 1:50 dilution.

Co-immunodetection with two different rabbit antisera (candidate protein and IncA inclusion membrane reference protein) was carried out in two steps. First, ICC for IncA was performed as described with Alexa Fluor 488-conjugated secondary anti-rabbit antibody. In a second step, directly Alexa Fluor 568-labeled anti-candidate protein antiserum was used. The direct labeling of candidate antisera was performed with the Alexa Fluor 568 Zenon labeling kit (Molecular Probes) according to the supplier's manual.

4.2.5.7 Labelling of host cell membranes with DilC16₍₃₎

A DilC16₍₃₎ stock solution was prepared by dissolving the delivered DilC16₍₃₎ in DMSO to a final concentration of 2.5 mg/mL. Methanol or formaldehyde fixed cell samples were washed with PBS and stained with a 1:10000 dilution (0.25 µg/mL) of DilC16₍₃₎ (Molecular Probes) in PBS for 30 min at RT. Samples then were mounted onto microscope slides as described.

4.2.5.8 Conventional fluorescence microscopy

ICC samples were investigated using a DMLB fluorescence microscope from Leica. Fluorescent pictures were recorded and overlaid with the IM1000 software from Leica.

4.2.5.9 Confocal fluorescence microscopy

The same ICC samples could be analyzed with a confocal microscope, which consisted of a DMRE microscope (Leica) and a TCS SP2 True confocal scanner (Leica) together with Argon/Krypton Ion (458 nm, 476 nm, 488 nm, 514 nm excitation, Omnichrome), UV (351 nm) and He/Ne Ion (543 nm, 633 nm) laser systems (Innova). The Leica TCS software was used for acquiring confocal pictures.

4.2.6 Mass spectrometric methods

4.2.6.1 Sample preparation for MALDI-TOF analysis

Protein bands/spots from silver stained SDS-PAGE (1D or 2D) were cut out from the gel. If spots were cut out from dried gels, samples were swollen in MQ o/n and cellophane was removed. In a silanized Eppendorf cup 1D SDS-PAGE bands were reduced with 10 mM DTT and afterwards alkylated with 55 mM JAA. 2D SDS-PAGE samples already were reduced and alkylated prior to running the second dimension. After washing the gel pieces three times for 15 min with MQ, dehydration of the gel slice was started by the addition of 50% MeCN/50 mM NH_4HCO_3 for 10 to 20 min. After removal of all liquid from the gel piece, 50 mM NH_4HCO_3 with 10 ng/ μL porcine modified Trypsin (Promega) was added until the gel piece was covered. Tryptic digest took place o/n at 37°C. The resulting peptides were dried in a SpeedVac centrifuge. The gel piece was washed with 30 μL MQ and the extract was added to the SpeedVac sample. The gel piece then was washed with 25 μL 70% MeCN and this wash also was added to the SpeedVac. NH_4HCO_3 evaporates as CO_2 and NH_3 . The dried digest was dissolved in 1% TFA. Two μL thereof were desalted with a C18 μZiptip (Millipore) and washed three times with 10 μL 0.1% TFA within the Ziptip. The sample was eluted from the Ziptip with 0.8 μL HCCA solution and directly spotted onto the 600/384 MALDI anchor target (Bruker Daltonics) according to the dried-droplet method. HCCA solution consisted of 0.5 μg HCCA/mL dissolved in a 2:1 mix of MeCN and 0.1% TFA.

4.2.6.2 MALDI-TOF and MS/MS analysis

MALDI-TOF mass spectra were measured with an Ultraflex TOF/TOF mass spectrometer from Bruker Daltonics in reflector and positive ion mode. Ions were accelerated in an electric field of 20 kV. With this method the exact and sensitive identification of tryptic fragments of proteins of interest was possible. The mass spectrometer was externally calibrated with the peptide standard Pepmix from Bruker Daltonics. Additionally an internal calibration was performed using keratin peaks, which further increased the mass accuracy to 50 ppm. The collection of detected peak masses, also called peptide mass fingerprint, was used for data base searches in order to

identify the sample protein. The Mascot search engine was used together with NCBI (non-redundant) and MSDB databases to identify proteins that fit to the measured peptide mass fingerprints. For even more precise identification peptide ions of single mass peaks were fragmented and fragments were analyzed in MS/MS mode. The resulting peptide fragment amino acid sequences were again used for database searches in order to identify the respective proteins.

4.2.6.3 SELDI analysis

SELDI analysis allows the combination of MALDI-TOF analysis with protein enrichment procedures. Peptides with specific characteristics can be extracted from complex peptide mixtures by this method. SELDI chips with different surfaces allow the enrichment of, for example, hydrophobic, hydrophilic or phosphorylated peptides of a sample. Since low amount of peptide analyte is often a problem in proteomics, this approach increases the amount of analyte in a defined target spot area. With a gallium-IMAC chip, phosphorylated peptide fragments can be enriched and analyzed with the MALDI-TOF device. SELDI analysis was carried out in a ProteinChip Reader (Model PBS IIC) from Ciperger with the ProteinChip Software version 3.1. H4, IMAC 30 and IMAC 40 chips were used. H4 ProteinChip Arrays mimic reversed phase chromatography with C16 functionality and are used for general investigation of complex peptide fragment mixtures. IMAC30 and IMAC40 ProteinChip Arrays are immobilized metal affinity capture array with a nitriloacetic acid (NTA) surface. After complexation of gallium ions on these NTA surfaces phosphopeptides, which bind to gallium, can be enriched. Application of peptide samples onto the chip and subsequent washing steps were carried out according to the Ciperger protocol.

4.2.7 Bioinformatics

4.2.7.1 Bioinformatic analysis

The genomes of different type III-employing pathogens were compared with the Phylosopher™ software (Genedata, Basel, Switzerland) in order to identify homologous operon structures and type III-relevant genes. PSI-BLAST and PROSITE algorithms

(5) were applied for domain and homology searches. Candidate proteins were analyzed with the VectorNTI software (Infor-Max Inc., Oxford, UK) for their alpha-helicity, polarity and hydrophobicity in order to predict their putative cytoplasmic solubility or (inclusion-) membrane localization and to unveil antigenic areas. Candidate open reading frames (ORFs) were selected from the complete genome sequence of *C. pneumoniae* TW183 (accession NC_005043). This genome was sequenced for ALTANA Pharma AG by GATC Biotech AG (Konstanz, Germany).

4.2.7.2 Data management

Microscopic pictures were overlaid with the Leica IM1000 software and graphs were assembled with Adobe Photoshop 7.0. Western blots and autoradiographies were analyzed with the Aida Image Analyzer version 3.22. MALDI-TOF and MS/MS data recording was controlled with the Flex Control and annotation was done with the Flex Analysis software from Bruker Daltonics. For further MALDI and MS/MS data analysis the BioTools software (Bruker Daltonics, version 2.2) was used. SELDI data were collected and analyzed with the Ciphergen ProteinChip Software version 3.1. Cellomics data were analyzed with the Cellomics Data Viewer software. Luminex data were acquired and managed with the Luminex 100 IS 2.2 software.

5 RESULTS

5.1 Identification of candidate chlamydial outer (Cop) proteins

5.1.1 Bioinformatic identification and analysis of candidate Cops

Type III secretion genes are located on four clusters in the *C. pneumoniae* genome (Figure 9). Based on the genomic sequence of *C. pneumoniae* strain TW183, effector gene candidates were selected according to their homologies with known type III effector proteins, according to the presence of eukaryotic protein domains and due to bioinformatic parameters (Table 10).

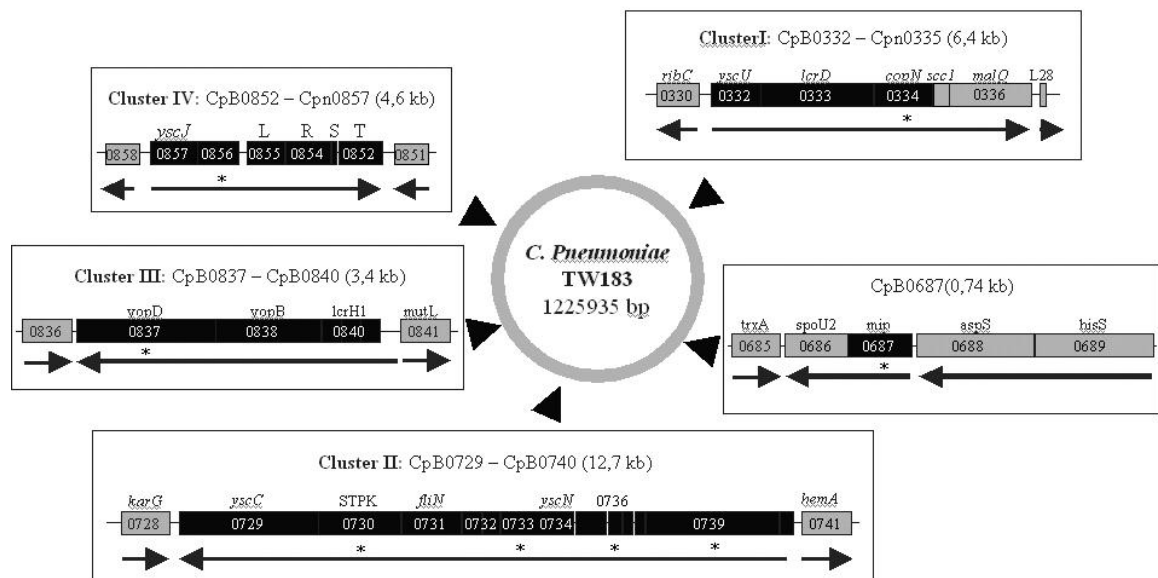


Figure 9: Clusters of type III secretion genes within the *C. pneumoniae* TW183 genome are shown (derived from Schuhmacher 2001, (260)). Type III-relevant genes are displayed in dark grey, surrounding genes are displayed in slight grey. Candidate genes used in this work are indicated by asterisks. Annotations refer to homologous genes or indicate putative protein function (STPK, serine threonine protein kinase). Arrows indicate operon structure as well as the direction of transcription as reported by Schuhmacher.

Candidate protein	homologue	protein motif
CopN (CpB0334)	lcrE (YopN)	SUMOylation core sequence
cpMip (CpB0687)	lpMip, ctMip	PPIase motif, signal peptidase II cleavage site
Pkn5 (CpB0730)	YopO/YpkA	S/T protein kinase domains, POLO box domain, Leucine rich repeat domains, SUMOylation core sequence
CpB0733	-	Ubiquitin Carboxyl-terminal hydrolase 8 motif, SUMOylation core sequence, high probability for coiled-coil domain
CpB0736	-	Growth factor/Cytokine receptor signature motif, high probability for coiled-coil domain
CpB0739	-	extended DxD motif, FHA domain, SUMOylation core sequence, homology to eukaryotic adenylate cyclases
CpB0837	YopD	hydropathy plot similar to yersinia YopD
CpB0856	-	hypothetical protein

Table 10: Candidate proteins are listed together with homologies to known T3SS-relevant proteins from *Yersinia*. “Lp” and “ct” indicate the origin of Mip proteins from *L.pneumophila* or *C. trachomatis*, respectively. Interesting protein domains identified by bioinformatic analysis are also displayed.

Candidate ORFs CpB0334, CpB0687, CpB0730, CpB0733, CpB0736, CpB0739, CpB0837 and CpB0856 were selected due to these criteria and will be discussed in the following paragraphs. Each of the four clusters was represented by at least one candidate gene.

CpB0334 (CopN)

CopN (CpB0334) was selected as representative for gene cluster I and was used as a control Cop, since its T3SS-dependent secretion by *Chlamydia* already has been reported (77, 184). Hence, secretion of CopN into the inclusion membrane was

expected. The CopN homologue in *Yersinia*, YopN (LcrE), was reported to be a T3SS-secreted secretion regulator plug, opening the secretion channel upon contact with the host cell. As some other candidate Cop proteins, CopN possesses a predicted SUMOylation sequence (LKAE).

CpB0687 (cpMip)

The cpMip gene locus is separated from the other T3SS genes in the *C. pneumoniae* genome. Nevertheless it was selected as candidate Cop due to its homology to the known *Legionella* virulence factor lpMip (48, 49, 186) and due to its described homology to and activity as PPIase. The amino acid sequence alignment in figure 10 shows the domain, described to be responsible for FK506 binding and PPIase activity. This indicates that this FK506 binding domain is conserved from bacteria to man, since human FKBP1a displays the same conserved features. Conserved amino acids, which were described to be important for PPIase activity and/or responsible for FK506 binding (106, 246), are indicated by asterisks and crossbars respectively. All aligned sequences share 17.1 % identical and 77.6 % similar amino acids in the here displayed domain. Pairwise homologies between Mip proteins are listed in table 11.

Table 11: Amino acid sequence identities of Mip and Mip-like proteins from *C. pneumoniae* (cp), *C. trachomatis* (ct), *C. psittaci* (cpsi) and *Legionella pneumophila* (lp) in %.

[%] identity	cpMip	ctMip	cpsiMip	lpMip
cpMip	-	57	71	26
ctMip	57	-	57	29
cpsiMip	71	57	-	25
lpMip	26	29	25	-

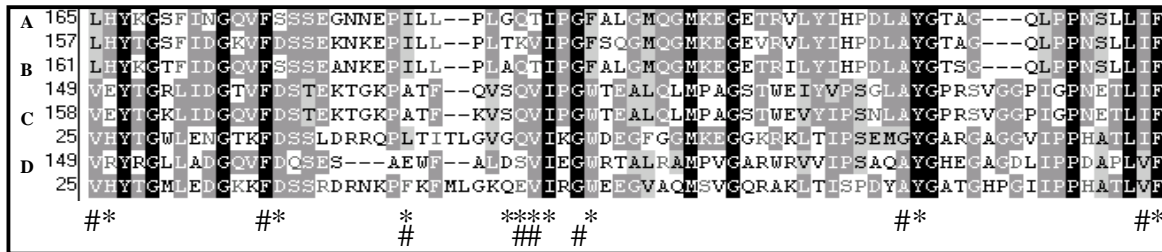


Figure 10: Amino acid alignment of the conserved PPIase region of Mip/PPIase proteins from different organisms. Amino acids conserved in Mip/PPIase proteins according to Hacker *et al.* (106) are indicated with asterisks (*). Crossbars (#) mark amino acid residues, important for FK506 binding according to Rockey *et al.* (246) **A:** cpMip, Mip *C. pneumoniae*, NP_876959 **B:** ctMip, Mip *C. trachomatis*, NP_220056 **C:** cpsitMip, Mip *C. psittaci*, NP_828952 **D:** lpMip, Mip *L. pneumophila*, CAD42886 **E:** lmMip, Mip *Legionella micdadei*, A43596 **F:** nmPPIase, *Neisseria meningitidis* serogroup A PPIase, P56989, **G:** paPPIase, *Pseudomonas aeruginosa* PPIase, P30417; **H:** FKBP1a, human FK506-binding protein 1A, A43596. All aligned sequences share 17.1 % identical and 77.6 % similar amino acids in the here displayed domain.

Signal peptidase II leader sequences and detection of cleaved and uncleaved forms on Western blots have been reported for Mip proteins (183). Signal peptidases II usually cleave off the signal peptide of proteins secreted by the Sec system. Analysis of lpMip, ctMip and cpMip primary sequences with the LipoP 1.0 signal peptide prediction server from the Center for Biological Sequence Analysis of the Technical University of Denmark (www.cbs.dtu.dk/services/LipoP) resulted in the prediction of signal peptides for all Mip protein sequences.

CpB0730 (Pkn5):

CpB0730 (Pkn5) was of major interest due to its sequence homology to eukaryotic Serine-Threonine kinases (STK) and its potential to interact with the host cell signal transduction network. The Pkn5 gene is part of gene cluster II. In the Pkn5 amino acid sequence all Hanks domains are present, except Hanks domain I (figure 11). The Hanks I (YGxGx(Phe/Tyr)GxVal) motif, which is necessary for ATP binding, is absent in this putative chlamydial kinase. However, although the conserved glycines are missing, the hydrophobic (Y) residue is a leucine in CpB0730 and the conserved Val is also present. The conserved Lys residue of subdomain II (which interacts with α and β phosphoryl groups of ATP and helps to anchor and orient ATP for catalysis) and the invariant Glu residue of subdomain III are present.

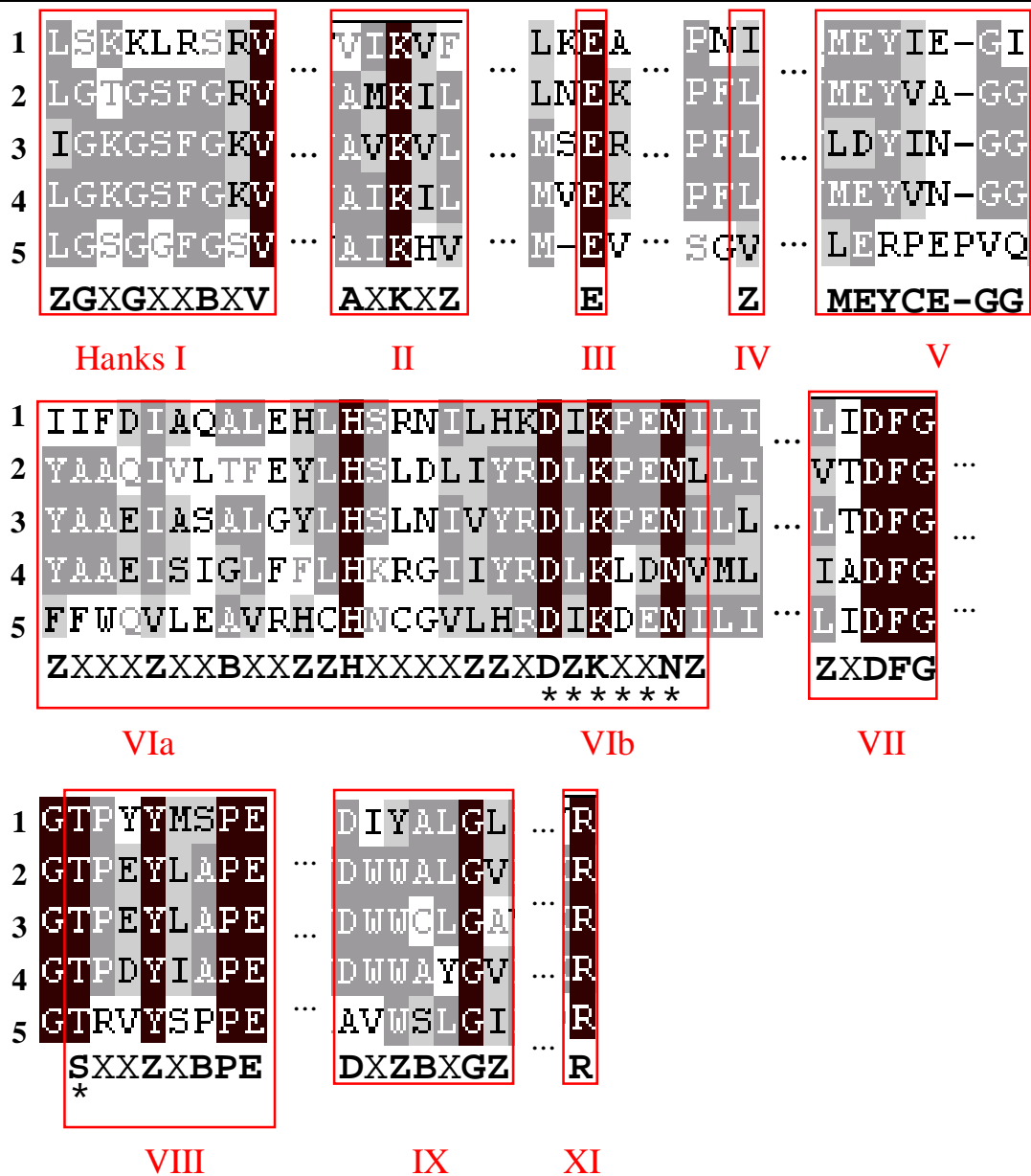


Figure 11: Alignment of CpB730 with different eukaryotic STKs. Hanks domains are indicated. **1** Putative S/T kinase CpB730 (*C. pneumoniae*), **2** PKA-C- α catalytic subunit (mouse), **3** SGK (serum/glucocorticoid regulated kinase) (human), **4** PKC-C- α catalytic subunit (mouse), **5** PIM-1 proto-oncogene S/T kinase (mouse); amino acid abbreviation key: **B** = small amino acid, **Z** = hydrophobic amino acid, **X** = any amino acid, * = Indicator amino acids for S/T kinases (in contrast to Tyrosine-kinases)

Subdomain VIb is the catalytic loop and contains a highly conserved motif, Z-X-D-Z-K-X-X-N-Z, where Z is a hydrophobic and X any amino acid. According to Hanks *et al.* the amino acid sequence D-L-K-P-E-N is a strong indicator of Serine/Threonine over Tyrosine specificity of a kinase (111). This is a strong hint that Pkn5 (D-I-K-P-E-N) is a

STK. Pkn5 furthermore possesses a conserved subdomain VII (D-F-G). The A-P-E motif in subdomain VIII is called the P+1 loop because it is the docking site for the P+1 residue of the substrate. In some kinases, the Ala is exchanged for another small amino acid, like Ser in the case of Pkn5. The Asp residue in subdomain IX and the Arg residue in subdomain XI are conserved in all prokaryotic kinases. Pkn5 contains the conserved Asp residue in subdomain IX. Interestingly, the conserved Arg (R²⁵⁷), which was reported to be absent in CT673, the *C. trachomatis* homologue of CpB0730, is present in CpB0730 (cpPkn5). Subdomain XI stabilizes the protein substrate binding during phosphorylation. Additionally to its kinase characteristics, CpB0730 possesses three Leucine-rich repeat domains (L²⁰⁸-L²²², I³⁷⁷-I³⁸⁴, L⁴⁵⁵-L⁴⁶³), one POLO box domain (Phospho-Ser/Phospho-Thr binding domain) and a SUMOylation core sequence (I¹⁴¹-E¹⁴⁴) (145, 263).

CpB0733

PSI-BLAST analysis revealed a regional similarity between CpB0733 (Cluster II) and ubiquitin carboxyl-terminal hydrolase 8 of 29.3 %. In the TW183 genome CpB0733 was annotated as hypothetical protein. CpB0733 displays a SUMOylation core sequence (L¹³⁶-E¹³⁹). Furthermore, a high probability for coiled-coil domains was predicted with the COILS program (187) (Figure 12).

CpB0736

PROSITE analysis of CpB0736 (Cluster II) amino acid sequence displayed a growth factor or cytokine receptor signature motif. The amino acid stretch L⁴⁹-L⁵⁶ was identified as potential Leucine-rich repeat. As for CpB0733, a high probability of coiled-coil formation was predicted.

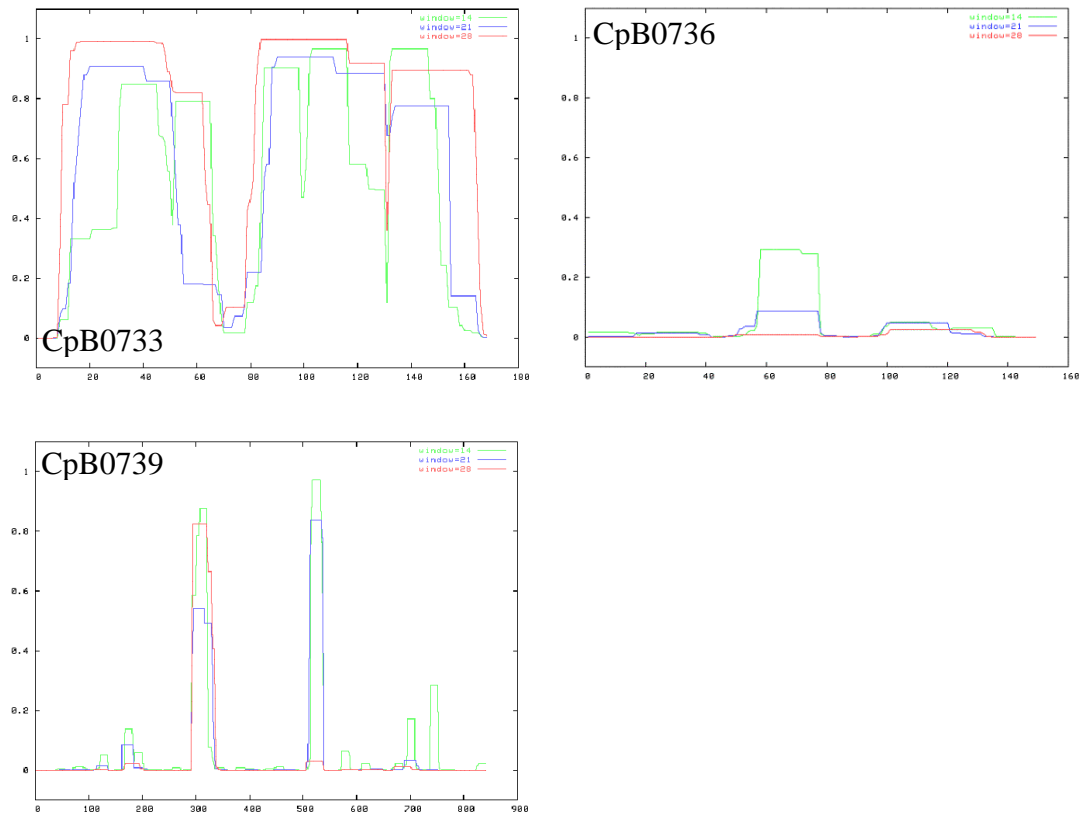


Figure 12: Probability prediction for the presence of coiled-coils in candidate protein amino acid sequences. Candidate Cop amino acid sequences were screened for putative coiled-coil domains with the COILS program ((187), www.ch.embnet.org/software/COILS_form.html). Peaks indicate areas of high probability for the formation of coiled coils by the respective amino acid stretches. Search algorithms with different windows of 14 (green), 21 (blue) or 28 (red) amino acids were used. X-axes indicate the amino acid position, Y-axes display the probability score in arbitrary units.

CpB0739

Candidate protein CpB0739 (Cluster II) was annotated as Forkhead associated (FHA) domain protein (226) with sequence homology to adenylate cyclases. Two of the three amino acids conserved amongst Forkhead associated (FHA) domain proteins are present in CpB0739 (S⁴³⁶ and N⁴⁵⁹). The neighbouring gene, CpB0740, has T3SS chaperone homology. With L⁸¹KNE⁸⁴, A¹⁷⁷KAE¹⁸⁰, P²¹²KGE²¹⁵ and V⁶⁷²KTE⁶⁷⁵ multiple potential SUMOylation sequences with the conserved pattern ΨKXE are present (Ψ = hydrophobic aa, X = any aa, (263)). Also for this candidate protein a high probability for the presence of coiled-coil domains was predicted. An extended DXD motif, found

in the superfamily of glycosyltransferases and chlamydial toxin-like ORFs, is also present in CpB0739 (Figure 13).

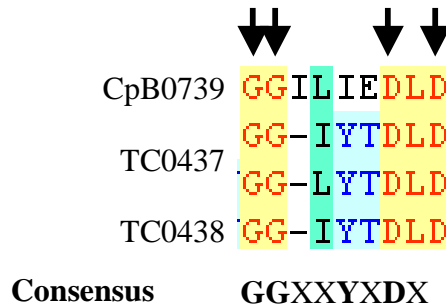


Figure 13: Alignment of the conserved “extended DXD motif” of CpB0739 with cytotoxin-like genes from *C. trachomatis* serovar MoPn. Conserved amino acid residues are indicated by arrows (23, 33).

CpB0837

The hydrophobicity profile of CpB0837 resembles that of the yersinial type III-secreted protein YopD (Figure 14). Like in *Yersinia*, CpB0837 (YopD) is located within a triple gene cluster (cluster III Figure 9) together with the yersinial homologues YopB (CpB0838) and lcrH (sycD, CpB0840). It is questionable whether CpB0839 is a real ORF. The YopB/D lcrH triple gene cluster is very conserved in other T3SS and CpB0839 is a very short sequence, which also might just be unspecific linker DNA. The CpB annotation was done automatically and therefore not necessarily means that every annotated ORF must be an expressed coding sequence.

CpB0856

CpB0856 represents the one candidate from T3SS gene cluster IV and is located in between the genes homologous to the yersinial genes YscJ and YscL, which encode structural proteins of the T3SS machinery. No homologies to other eukaryotic or prokaryotic proteins are known. As published recently, this T3SS gene cluster is conserved in *Chlamydia* and chlamydial ancestors since 700 million years (128) and therefore is thought to play an important role.

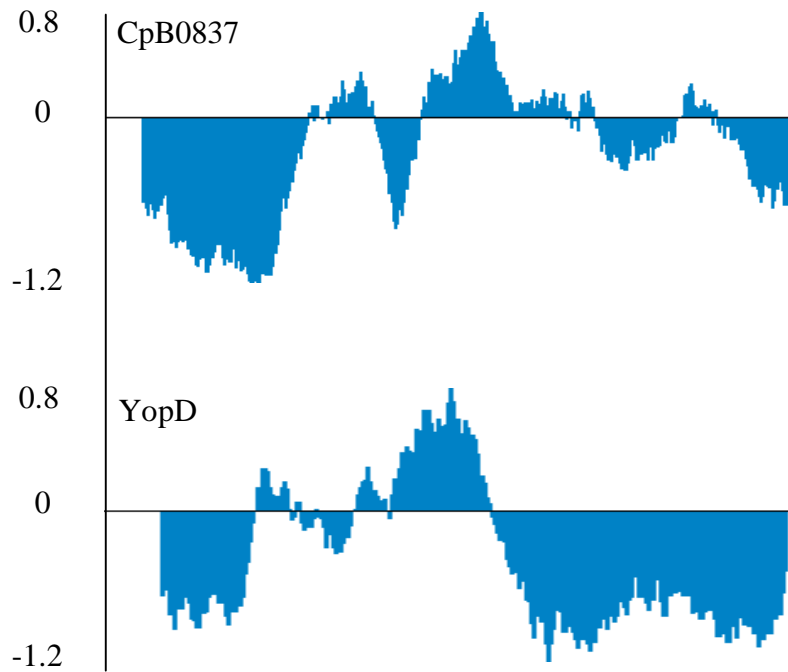


Figure 14: Comparison of hydropathicity plots of CpB0837 and YopD according to Kyte and Doolittle (166).

5.1.2 Chlamydial Cop candidates lack a conserved T3SS secretion signal

Analysis of N-terminal amino acid sequences of candidate Cops for signal sequences revealed no conserved motifs (Figure 15). Only CpB0837 has a higher than average Serine content within the first 50 amino acids. Only CopN and CpB0837 display low Asp, Leu and Lys contents within this stretch, which, according to Guttman *et al.* would indicate a T3SS secretion signal (105). All candidate Cops but CopN (CpB0334) and cpMip (CpB0687) have negatively charged amino acids amongst the first 12 amino acids in the N-terminus. The absence of negatively charged amino acids was reported to be a requirement for type III secretion in *Pseudomonas syringae* (255). Furthermore, the reportedly necessary aliphatic (Ile, Leu, Val) or proline residues at amino acid position three or four could be found in none of the candidate Cops. Only CpB0687 and CpB0739 have an amphipathic distribution within the first ten amino acids, as was reported for *Yersinia Yops* (182), whereas all other candidate Cops do not. The WEK(I/M)XXFF conserved sequence, present in many *Salmonella* type III-secreted effectors, is also not present in the potential chlamydial type III effectors (202). No

homologies in chlamydial Cop candidate DNA sequences could be found, which implies that there are also no conserved signals in the mRNA sequence. Therefore, hypothetical conserved T3SS signal sequences in *Chlamydia* differ from those of *Yersinia*, *Salmonella* and *P. syringae* (105, 255) or are non-existent.

Figure 15: (A) Alignment of candidate Cop N-terminal amino acids 1 to 50. Aliphatic or proline residues at position three or four are indicated in bold letters. Negatively charged amino acids within the first 12 amino acid stretch are underlined. (B) Evaluation of amino acid composition within this sequence stretch. Percentages and total numbers of specified amino acids within the first 50 amino acids of each candidate Cop are listed.

A

Cop	1	amino acid sequence	50
CpB0334	MAASGGT	GGLGGTQGVNLAAVEAAAAKADAAE	VVASQEGSEMNIQQSQD
CpB0687	MNRRWNL	VLATVALALSVASCDVRSKDKDKDQ	GSLSVEYKDNKDTNDIELS
CpB0730	MDCRGGI	PLPEPQVIGGYHVKKILSKKLR	SRVVHGLHPETRHSTVIKVS
CpB0733	MAKYPLE	PVLAIKKDRVDRAEKVVKEKRRLLE	IEQEKLREKEAERDKVKN
CpB0736	MADLE	VFQADFALLFEAGLLAIKQGEDS	SARKLQSLHILNPNHYGHDLG
CpB0739	MAVRLIV	DEGPLSGVIFVLEDGISWSIGRDSS	ANDIPIEDPKLGASQAI I
CpB0837	MTSGVSG	SSSQDPTLAAQLAQSSQKAGNAQ	SGHDTKNVTKQGAQAEVAAG
CpB0856	MTANTFG	TLDILMKHSKEDDL	SRFLPKNLLVESPHPEEIPKLSLSFTMSW

B

CpB	% of aa (number of aa) within aa 1-50 of putative effector proteins				
	Ser	Asp	Leu	Lys	Asn
0334	8% (4)	4% (2)	4% (2)	2% (1)	4% (2)
0687	10% (5)	14% (7)	12% (6)	10% (5)	8% (4)
0730	8% (4)	2% (1)	8% (4)	10% (5)	0
0733	0	6% (3)	10% (5)	20% (10)	2% (1)
0736	4% (2)	10% (5)	18% (9)	4% (2)	4% (2)
0739	12% (6)	10% (5)	8% (4)	2% (1)	2% (1)
0837	16% (8)	4% (2)	4% (2)	6% (3)	4% (2)
0856	12% (6)	6% (3)	16% (8)	8% (4)	4% (2)

5.2 Generation of antisera

5.2.1 Heterologous overexpression in *E.coli* and purification of candidate Cop proteins

Effector candidate sequences were cloned as GST- or His₆-fusion proteins into bacterial expression vectors as described. Plasmids were sequenced and inducible expression of the respective candidate proteins in *E.coli* was tested by SDS-PAGE analysis. With these expression vectors candidate Cop proteins or fragments thereof were overexpressed in *E.coli* strain BL21(DE3) (Stratagene) after induction by IPTG. Candidate proteins CpB0730 and CpB0856 were expressed as inclusion bodies whereas all other candidate proteins were soluble. Tagged proteins were purified by Ni-NTA- or GST-affinity chromatography. Since TrxA-His₆-CpB0739(E¹¹⁴-R³⁶⁷) displayed a higher molecular weight (63 kD) than assumed (45 kD) on SDS-PAGE (Figure 16), the identity of this antigen was corroborated by mass spectrometric analysis (data not shown). This cloning, expression and purification work was done at Trenzyme (Konstanz, Germany) and therefore will not be reported in further detail.

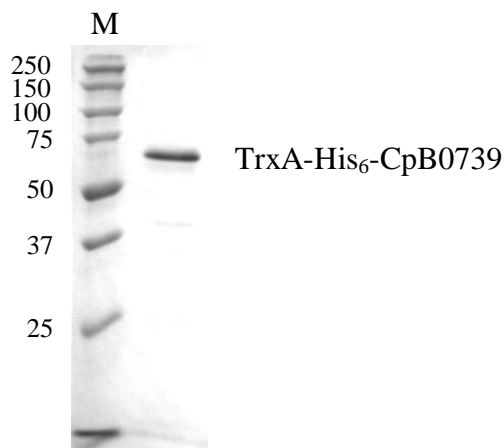
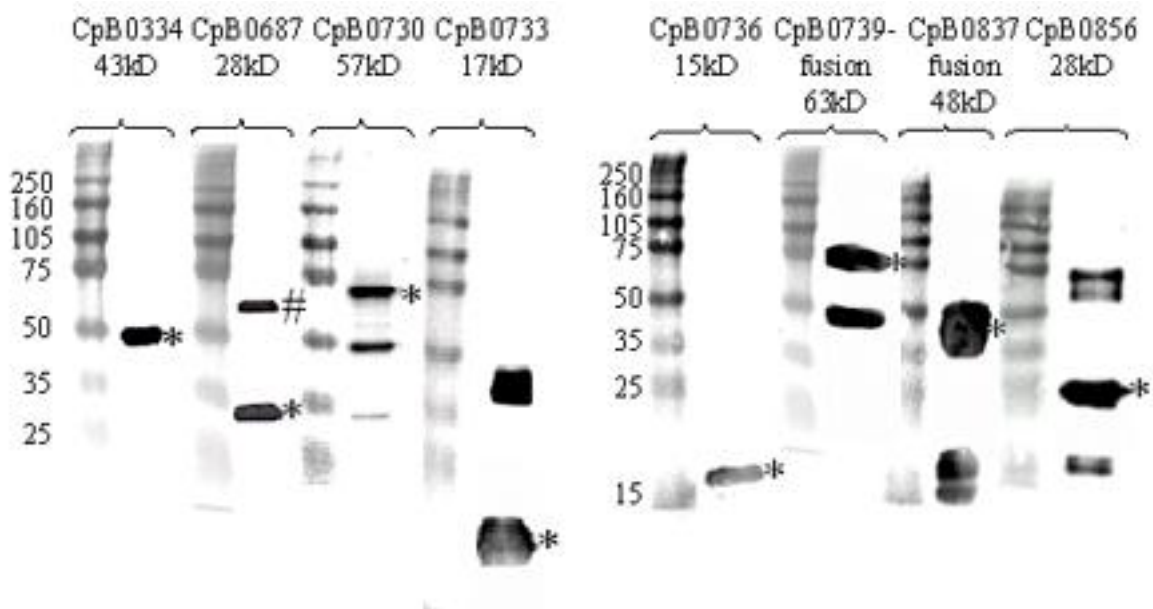


Figure 16: Western blot with anti-His antibody. Candidate fusion protein TrxA-His₆-CpB0739 displays with approximately 63 kD a higher molecular weight than expected (45 kD). M = prestained broad range molecular weight protein standard (Bio-Rad).

5.2.2 Generation of antisera against potential Cops

The purified candidate proteins were used for the multiple immunization of rabbits in combination with Ras-Ribi adjuvant (Sigma). Antisera were gained at Trenzyme after 8 weeks, affinity purified as described and were stored in PBS with 2 % [w/vol] BSA. The quality of the antisera was controlled by Western blot analysis with the purified proteins or fusion-proteins as samples and 1:1000 dilutions of antisera. All antisera recognized their epitopes specifically (Figure 17). GST-CpB0687 was detectable both as fusion protein and as cleaved CpB0687. CpB0739 and CpB0837 fusion proteins differ in size from the chlamydial proteins, since only fragments of the chlamydial proteins were used as tagged epitopes for immunization (Table 4). CpB0733 and CpB0856 antisera recognized an additional band with higher molecular weight of unknown identity. Some antisera also recognized degradation products of the purified protein samples.

Figure 17: Western blot quality control of Trenzyme antisera directed against candidate Cop proteins or fusion proteins thereof. Purified candidate proteins were applied to SDS-PAGE and Western blotting with the respective Trenzyme antisera used for detection as described. For each Trenzyme antiserum to test one marker lane (left) and one lane with the purified protein (right) is presented. Molecular weights of the marker lanes are indicated once on the very left. Candidate protein bands are indicated by asterisks, GST-candidate fusion is indicated by crossbar.



5.2.3 Generation of antisera against a synthetic CpB0730 peptide

In order to have an additional antiserum at hand, that directly recognizes CpB0730 without tags, an antiserum, directed against a synthetic peptide sequence derived from the CpB0730 primary sequence, was generated. The peptide sequence chosen was H⁴⁷⁹ to S⁴⁹³, which is close to the C-terminus of CpB0730 (Figure 18).

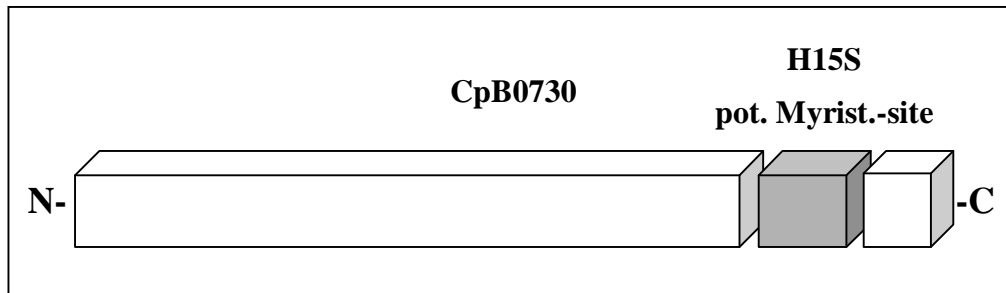
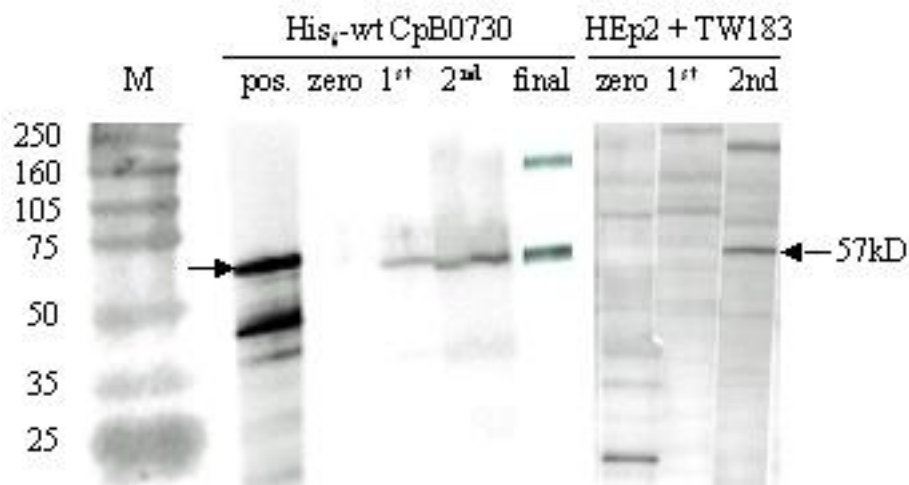


Figure 18: Relative localization of the peptide epitope used for the generation of peptide specific antibodies against CpB0730 in the CpB0730 amino acid sequence. The name of the used peptide sequence „H15S“ is used as part of the name of the resulting antibody.

This epitope sequence covers the predicted myristoylation site of CpB0730. Peptide synthesis as well as immunization of rabbits, harvesting and purification of the antisera was done at Neosystems (Strasbourg, France). The resulting antiserum H15Snew08 recognized CpB0730 on western blots at a 1:100 dilution (Figure 19).

Figure 19: Neosystem „H15Snew08“ antiserum was tested with His₆-tagged wt CpB0730 and infected cell lysates on Western blot strips. Anti-His₆-antibody (Qiagen) was used as positive control antibody (pos.). Zero, 1st, 2nd and final describe the serum gained before immunization (zero), after first boost immunization (1st), after 2nd boost immunization (2nd) and the final antiserum gained after the rabbit has been sacrificed (final). Arrows indicate the bands corresponding to CpB0730.



5.3 Candidate Cop expression and secretion

5.3.1 Expression of candidate Cops during the chlamydial infection cycle

The generated antisera were used, to detect the expression of all candidate Cop proteins during the *C. pneumoniae* expression cycle by SDS-PAGE and western blotting. Therefore, lysates of infected and uninfected cells were prepared as described. Cell lysates of infected cells were prepared at different stages of the infection cycle: 0.5 hpi when the Chlamydia are still externally attached to the host cell, 6 hpi when the EBs already have developed into RBs, 20 hpi when the RBs are dividing, 48 hpi when the RB population has maximally multiplied itself by binary fission and starts to redevelop into EBs and finally 72 hpi when the inclusions have grown to full size and the release of the newly formed EBs starts (figure 1 from Introduction).

All candidate proteins were detected in infected, but not in uninfected cell lysates by Western blot analysis (Figure 20). All Western blots were normalized with anti β -actin antibody. Figure 20 shows that CpB0736 and cpMip were expressed throughout the infection cycle to a similar extent whereas the other candidate proteins were differently expressed during different periods of the infection cycle. CpB0739 and CpB0856 were only strongly expressed during the late phase of the infection cycle (48-72 hpi). Small amounts of CpB0739 were also present at 30 min post-infection, and even less at 6 and 24 hpi. CpB0733 showed an expression pattern similar to CpB0739 but with a clear signal at 6 and 24 hpi. CopN protein was always detectable exhibiting a slightly increasing signal towards the end of the infection cycle. Expression of CpB0837 started with strong expression in the early infection cycle with declining signal towards the end. The putative chlamydial kinase Pkn5 was detected from the beginning of the infection reaching a maximum between 6-24 hpi, followed by a decline to zero signal at 72 hpi. In summary, different groups of putatively T3SS-secreted effector proteins exist, which are expressed during different periods of the infection cycle.

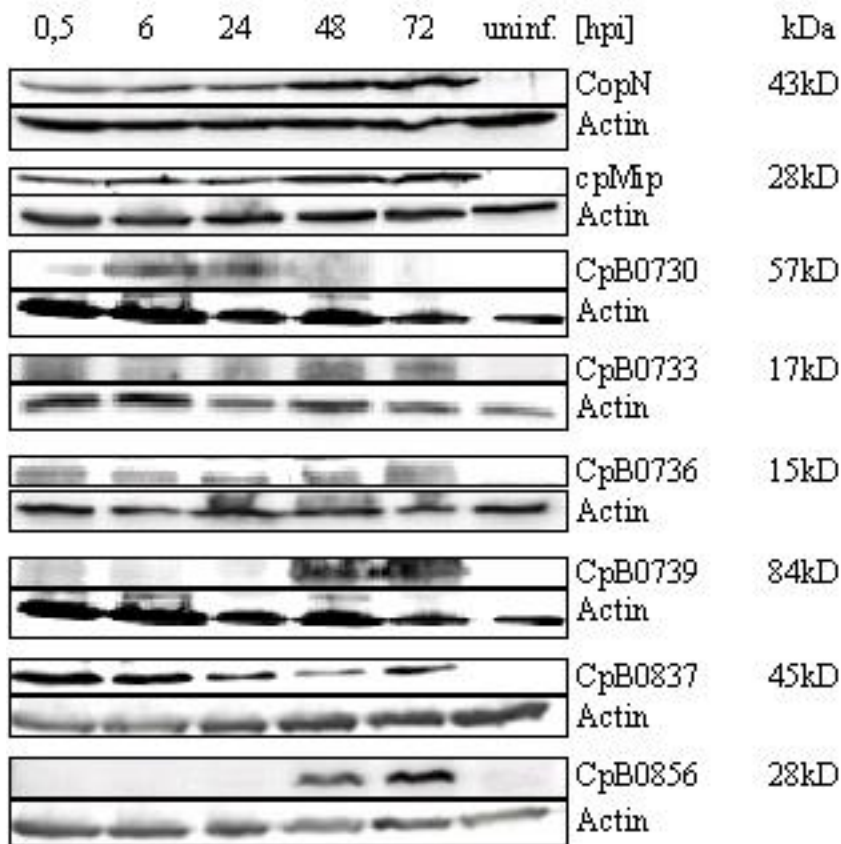


Figure 20: Expression of effector candidate proteins over 72 hpi. Infected HEp-2 cells were lysed at different times of the infection cycle and proteins were separated on 10 or 15% SDS-PAGE. Lysate of uninfected cells was used as a control. Proteins were transferred onto Nitrocellulose membranes by electroblotting. Immunoblots were probed with the generated antisera and normalized with anti- β -actin antibody for equal loading. Approximate molecular masses are indicated in Kilodalton.

5.3.2 Detection of secretion of candidate proteins

5.3.2.1 Detection of secretion by ICC

Antisera raised against candidate Cop proteins allowed the visualization of the corresponding epitopes in the developing chlamydial inclusions within host cells. Co-localization of the commercial anti-MOMP (chlamydial major outer membrane protein) signal with the respective antiserum indicated the localization of the investigated proteins inside the chlamydial inclusions (Figure 21).

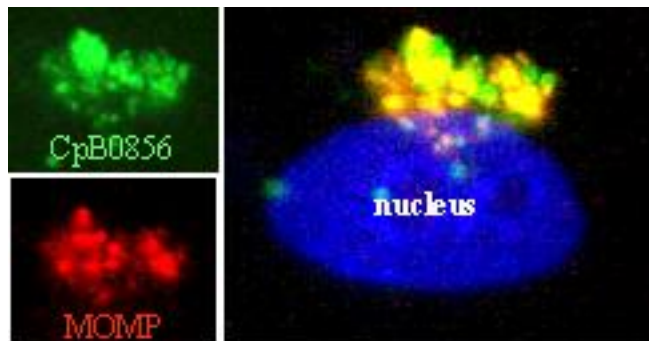


Figure 21: Co-localization ICC of candidate protein CpB0856 with chlamydial MOMP protein within the chlamydial inclusion at 72 hpi. Infected HEp-2 cells were fixed and immunolabeled as described. Candidate protein was detected with the respective antibody together with an Alexa Fluor 488-conjugated secondary antibody (green). Chlamydia within the inclusion were visualized with a commercial antibody directed against chlamydial MOMP protein together with an Alexa Fluor 568-conjugated secondary antibody (red). Nuclei were stained with Hoechst (blue) and ICC pictures were overlaid. Thereby green and red overlay resulted in a yellow signal.

Although the two candidate proteins CpB0733 and CpB0837 could be visualized within the inclusions throughout the infection cycle, neither secretion into the inclusion membrane nor into the host cell cytoplasm could be detected (data not shown). CpB0733 and CpB0837 were annotated as hypothetical proteins in the *C. pneumoniae* genome.

The proteins CpB0736 and CpB0739 were detectable by immunocytochemistry (ICC) throughout the infection cycle (at 0.5 hpi, 6 hpi, 20 hpi, 48 hpi and 72 hpi) in the inclusions of *C. pneumoniae* infected HEp-2 cells (data not shown). The same was valid for CpB0856 with the exception that it was undetectable at 6 hpi. Secretion of CpB0736, CpB0739 and CpB0856 was only found at 20 hpi (Figures 22 and 23). ICC with the respective antisera displayed a rim-like staining of the inclusion membrane, whereas the lumen of the inclusion was not stained at 20 hpi. Secretion of CpB0739 and CpB0856 does not overlap with the time window of maximal expression (Figure 20). These proteins were found inside the chlamydial inclusions at later time points, indicating continuous synthesis of these proteins with secretion only occurring during a certain period of time within the infection cycle.

The CpB0334 (CopN) protein was detectable inside Chlamydia at all investigated time points. Secretion, however, was only detected at the late stage of the infection cycle at 72 hpi (Figure 22). In contrast to other candidate proteins, CopN was not completely secreted into the inclusion membrane since CopN still could be detected within the inclusion lumen.

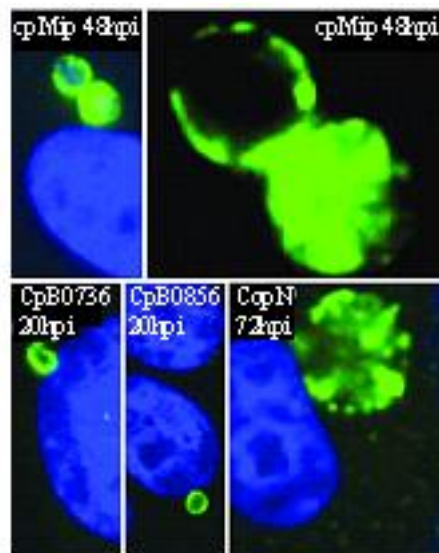


Figure 22: Immunofluorescence images of HEp-2 cells infected with *C. pneumoniae* TW183. Antisera raised against effector candidate proteins show the localization of the respective proteins within the inclusion membrane and relative to the host cell nucleus. Blue: host cell nuclei, green: candidate protein. Annotation and time point of secretion are indicated.

CpMip was detectable in the inclusion membrane from 20 hpi until 72 hpi, showing the strongest signal between 48 and 72 hpi. The polyclonal cpMip antiserum visualized the rim-like structure of the inclusion membrane (Figures 22 and 23).

CpB0730 (Pkn5) was detected inside the chlamydial inclusion at most time intervals investigated (0.5 hpi, 6 hpi, 48 hpi, 72 hpi). At 20 hpi however, the inclusion membranes surrounding the still small inclusions were stained by ICC with anti-Pkn5 antiserum, which indicated secretion of this kinase (Figure 23).

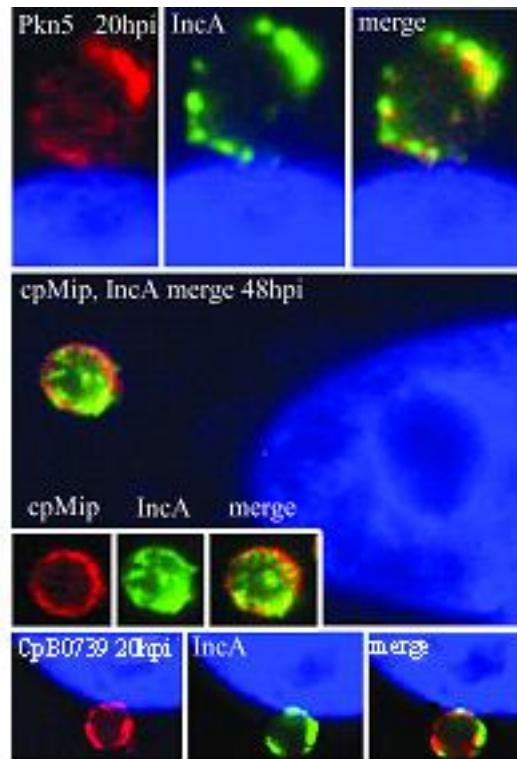


Figure 23: Double ICC images of HEp-2 cells infected with *C.pneumoniae* TW183. Antisera raised against effector candidate proteins show the localization of the respective proteins within the inclusion membrane (red). Polyclonal anti-IncA antiserum (green) was used as a marker for the inclusion membrane. Blue: host cell nucleus. Annotation and time point of secretion are indicated.

5.3.2.2 Proof of the localization of Cops within the inclusion membrane

The observed rim-like fluorescence in candidate Cop ICCs was a strong indicator for the respective Cops being localized within the inclusion membrane compartment. This localization had to be proven however. The first attempt was to label all host cell membranes with DilC16₍₃₎ (Molecular Probes), which is a lipophilic fluorescent membrane dye. This dye stained all membranes within the host cells, as expected, but was a too intense and ubiquitous signal to be used for specific localization studies (data not shown). Therefore the next attempt was to perform double immunostaining of the samples with candidate Cop antiserum and with anti IncA antiserum. The latter was reported to detect secreted IncA protein within the inclusion membrane. The problem to perform double ICC with two antisera both derived from rabbit within one single

sample could be solved by direct labelling of one antiserum with the Zenon Rabbit IgG labelling kit (Molecular Probes) as described. Finally, the localization of all secreted proteins within the inclusion membrane compartment could be confirmed by double-ICC with IncA antiserum together with the respective candidate protein antiserum (Figure 23).

5.3.2.3 Detection of candidate proteins by confocal microscopy

The localization of protein CpB0837 was investigated by confocal microscopy (Figure 24). No secretion of CpB0837 but a dotted staining pattern of the inclusions was observed. This was different from the usually more uniformly labeled inclusions after ICC with other candidate Cop antisera (data not shown). One possible explanation would be that CpB0837 is expressed more in EBs than in RBs or vice versa. Since the Western blotting experiments showed CpB0837 to be more expressed in early infection cycle it was concluded, that the here observed dots must probably be mostly EBs.

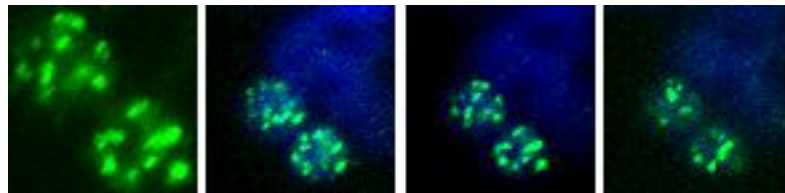


Figure 24: Confocal images of different layers of an infected HEp-2 cell at 72 hpi. Chlamydia were immunolabelled with polyclonal α CpB0837 antibody and FITC-conjugated secondary antibody (green). The host cell nucleus was stained with Hoechst (blue). The upper left picture shows the FITC channel only.

5.3.3 Regulation and timing of secretion of chlamydial Cops

The investigated chlamydial effector proteins are secreted at different time points of the infection cycle. Interestingly expression and secretion of candidate proteins are not always synchron. Secretion of CpB0739 and CpB0856, for example, does not overlap with the time window of maximal expression (Figure 20). These proteins were found inside the chlamydial inclusions at the later time points investigated, indicating continuous synthesis of these proteins with secretion only occurring during a certain period of time within the infection cycle. The CopN protein was detectable inside

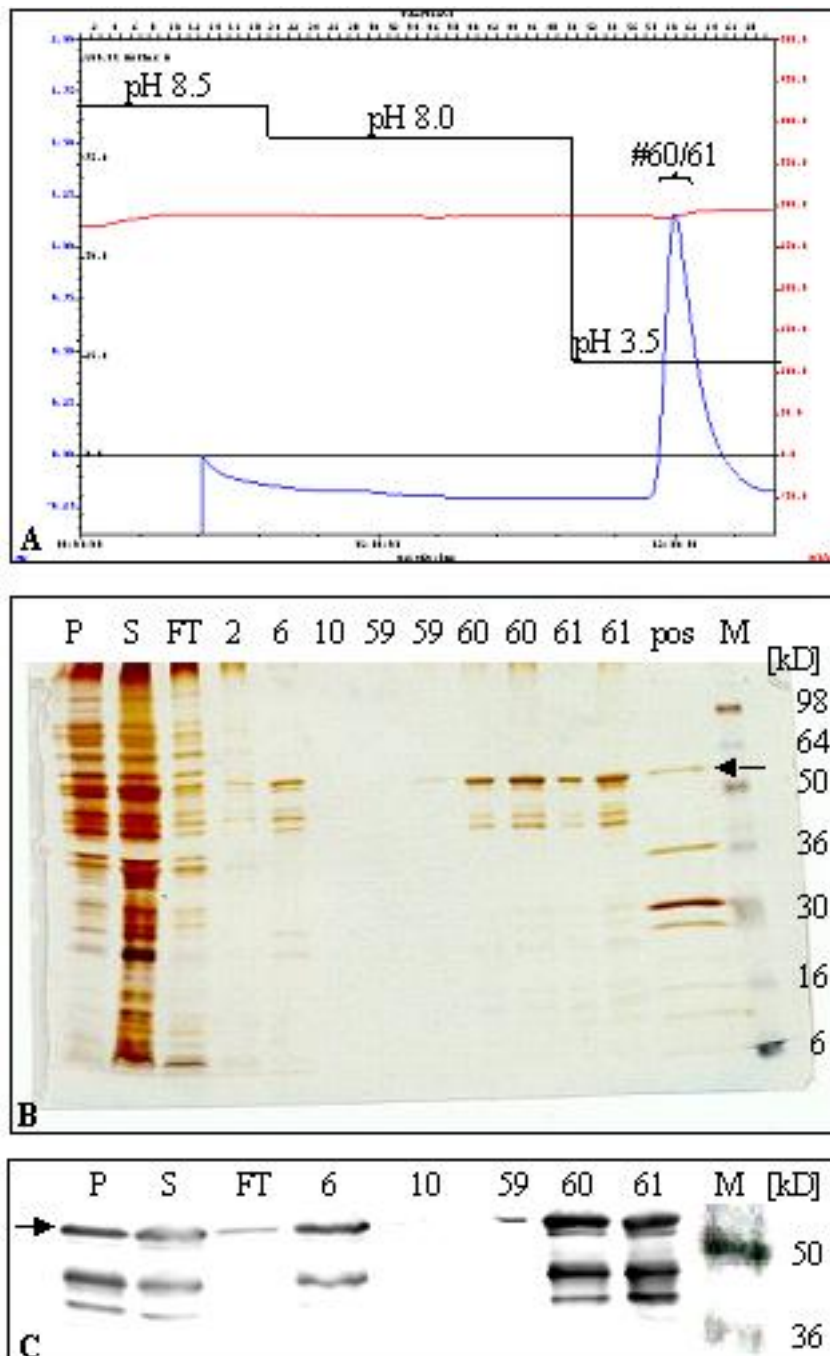
Chlamydia at all investigated time points. Secretion, however, was only detected at the late stage of the infection cycle at 72 hpi (Figure 22). Interestingly, timing of candidate secretion seems not to be regulated on an inter-inclusional level. Sometimes secretion-active and secretion-inactive inclusions were seen in direct neighbourhood within one single host cell (Figure 22, cpMip).

5.4 Is CpB0730 a functional S/T kinase?

5.4.1 Purification and refolding of rHis₆-CpB0730 from inclusion bodies

Inclusion bodies of rHis₆-CpB0730 expressed in *E.coli* BL21(DE3) after IPTG induction were used as basis for the purification of CpB0730 protein under denaturing conditions by IMAC. 1 g inclusion bodies were solubilized as described and the supernatant was used for batch loading with 10mL Ni-NTA resin. 10 mL Ni-NTA resin and 10 mL rHis₆-CpB0730 supernatant were stirred for 1.5 h at RT and finally loaded to the FPLC column. After equilibrating the column with 20mL solubilization buffer and washing with 30mL wash buffer (pH 8.0), rHis₆-CpB0730 was eluted at pH 3.5 with 20mL elution buffer at a flow rate of 0.5 mL/min. Eluted protein was collected in fractions of 1 mL. The rHis₆-CpB0730 eluted as a distinct peak mainly in two fractions (#60 and 61) (Figure 25). 50 µL from each fraction were precipitated with 200µL Acetone, washed with 70% Acetone and samples were analyzed by 12.5% SDS-PAGE analysis. Protein was detected by silver staining of SDS-PAGE gels as well as by Western blot analysis with anti His₄ antibody (Figure 25). Not only full-length rHis₆-CpB0730 protein but also degradation products thereof were purified with this chromatography step. This can be seen on the Western blot, where also the degradation fragments reacted with the anti His₄ antibody. Unified peak fractions (2mL) contained approximately 800 µg of purified rHis₆-CpB0730.

Figure 25: Purification of rHis₆-CpB0730 by Ni-NTA affinity chromatography from solubilized inclusion bodies. **A:** Chromatogram with peak fractions #60/61. Eluted protein was detected with a UV detector (blue). Buffer pH steps are indicated. **B:** Silver stained 12.5% SDS-PAGE of chromatography fractions, **C:** Detection of rHis₆-CpB0730 by Western blot with anti His antibody. P: pellet, S: supernatant of solubilized rHis₆-CpB0730, FT: flow through, numbers: fraction numbers, pos: CpB0730 positive control, M: See Blue Marker. The arrows indicate the position of the rHis₆-CpB0730 bands at 57 kD.



Since degradation products of rHis₆-CpB0730 were co-purified, it was subsequently tried to separate these from full-length rHis₆-CpB0730 by size exclusion chromatography with Superose 6 resin. Therefore 1mL of the unified peak fractions from the IMAC column was purified with the Superose 6 size exclusion column FPLC. The elution profile and the analysis of the eluted fractions (10 µL samples) by silver stained SDS-PAGE gels can be seen in figure 26.

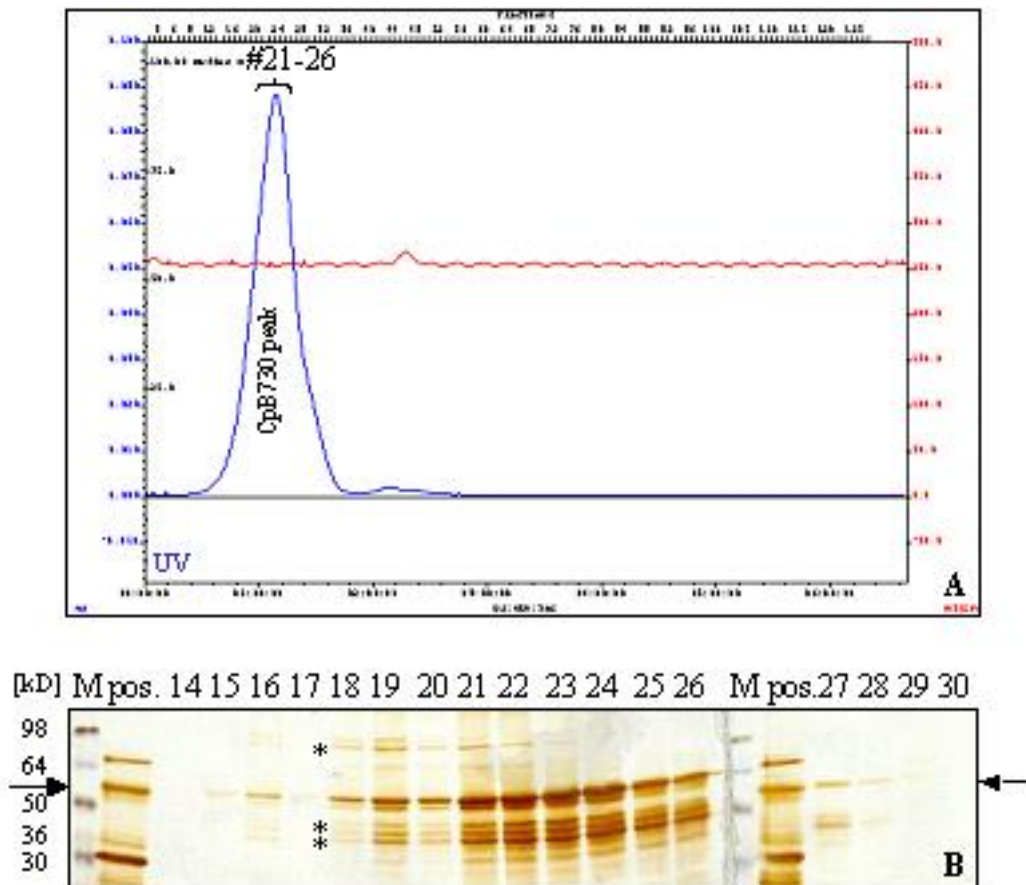


Figure 26: Purification of rHis₆-CpB0730 by size exclusion chromatography. **A:** Chromatogramm with peak fractions #21-26. Eluted protein was detected with a UV detector (blue). **B:** Silver stained 12.5% SDS-PAGE of chromatography fractions, pos: rHis₆-CpB0730 positive control, numbers: fraction numbers, M: See Blue Marker. The arrows indicate the position of the rHis₆-CpB0730 bands at 57 kD. Asterisks mark the positions of the degradation product bands as well as of the 90 kD CpB0730 band as revealed by Western blot and mass spectrometric analysis (data not shown).

The 40 to 45 kD degradation products of rHis₆-CpB0730 could not be removed completely by this chromatography. The protein was diluted over several fractions by this step. Fractions 21 to 26 (9 mL total volume) contained 0.2 mg/mL of the purified

protein. Purified rHis₆-CpB0730 protein was identified by peptide mass fingerprint with MALDI-TOF analysis combined with MS/MS analysis (Figure 27). Interestingly, rHis₆-CpB0730 protein was not only identified from the 57 kD main (arrows) and the 40 to 45kD degradation product bands (asterisks), but also from the approximately 90 kD band (asterisk) visible in fractions 18 to 23. The purified protein was still in a denatured state in 4M Guanidinium hydrochloride. Therefore 100 µL of the purified protein were stepwise dialyzed against 2M and 0M Gua as described. Thereby tiny amounts of protein precipitated, so that finally 0.13 µg/µL of purified rHis₆-CpB0730 were soluble in buffer.

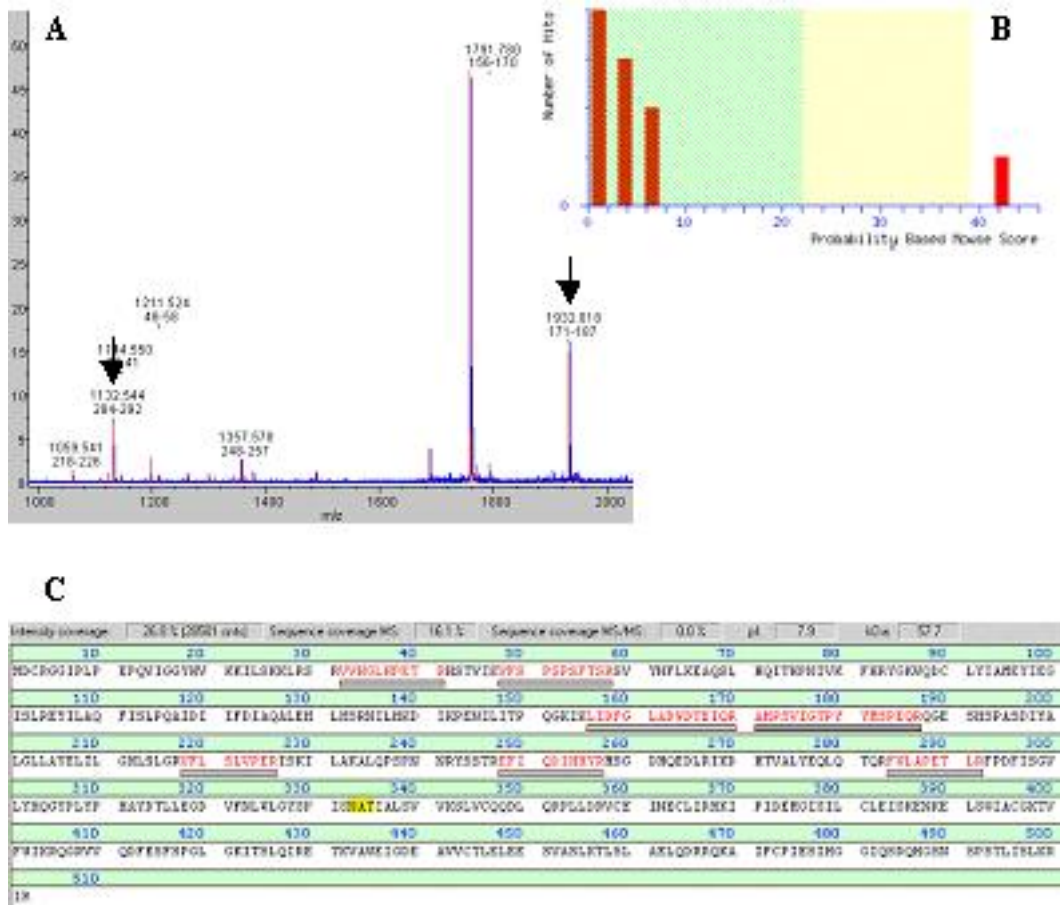


Figure 27: MALDI-TOF analysis of FPLC-purified rHis₆-CpB0730. In **A** the MALDI-TOF peak spectrum is shown and two peaks are indicated, which were used for MS/MS analysis. **B** shows the result of the combined Mascot search, which clearly identified chlamydial CpB0730 from the MALDI-TOF peak list. In **C** the sequence coverage of 16.1% is shown graphically. Grey bars and red letters indicate identified mass peaks.

5.4.2 Kinase assays with renatured CpB0730

Kinase assays with the FPLC-purified dialyzed protein were carried out as described. HEp-2 lysate as well as MBP and Histones were used as substrates. Kinase assay reactions were separated on 1D and 2D SDS-PAGE gels and analyzed by autoradiography. However, no activity of purified refolded CpB0730 could be detected (data not shown). Since refolded CpB0730 was inactive, a different attempt was started to prepare active CpB0730 protein for functional assays. Therefore the strategy was chosen to express wt CpB0730 and its gain-of-function mutants in eukaryotic cell culture in order to purify this protein by IP.

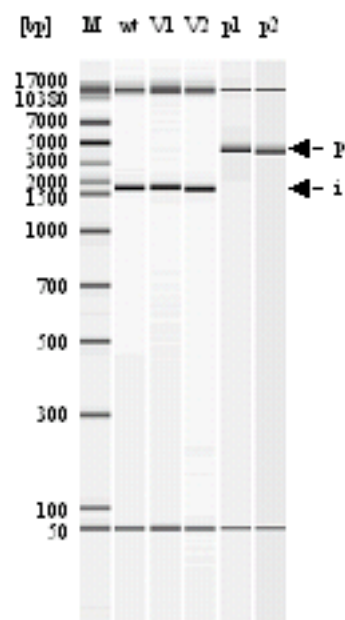


Figure 28: DNA analysis of double-digested wildtype (wt) CpB0730, gain-of-function mutant V1 and V2 PCR fragments as well as of double-digested, dephosphorylated plasmids pCS3+ MT (p1) and pCS3+ MTNLS (p2). Plasmid (p) and insert (i) bands are indicated by arrows. Analysis was carried out with the Agilent 2100 Bioanalyzer.

5.4.3 Cloning of wt CpB0730 and gain-of-function mutants into eukaryotic expression vectors

For heterologous expression of our target proteins in eukaryotic cells, the pCS3+ vector system (250, 286) was chosen. With these vectors a strong and constitutive expression from a strong CMV promoter is possible. The pCS3+ MT and pCS3+ MTNLS variants used, express the cloned proteins as 6 x myc tagged fusion proteins with (+NLS) or without (-NLS) additional nuclear localization sequence. The latter was important, because action of CpB0730 on nuclear targets could not be excluded. Wildtype CpB0730 and gain-of-function mutants (V1 and V2) were cloned into vectors pCS3+ MT and pCS3+ MTNLS. The purified BamH1/Stu1 double-digested PCR products were ligated into Bgl2/Stu1 double-digested and dephosphorylated pCS3+ vectors. Purified inserts and plasmids used for ligation are shown in figure 28. Transformed *E.coli* One Shot Top 10 cells were selected and stored in strain stocks. The cloning procedure is exemplified in figure 7.

5.4.4 Purification of native CpB0730 by IP from lysates of transfected cells

Overexpressed myc₆-CpB0730 or NLS-myc₆-CpB0730 protein was precipitated from lysates of transfected HEK 293 EBNA cells at 48 h post transfection with monoclonal anti c-myc antibody. 10 µL IP aliquots were applied to SDS-PAGE analysis followed by Western blot. Figure 29 shows intense bands of precipitated CpB0730 constructs at molecular weights of approximately 75 and 105 kD. This can be explained by the presence of the c-myc tag and the NLS, which enlarge the protein by 90 amino acids and 14 amino acids respectively. IPs were used fresh for Luminex kinase assays or were stored at -20°C in 30% glycerol for other downstream assays.

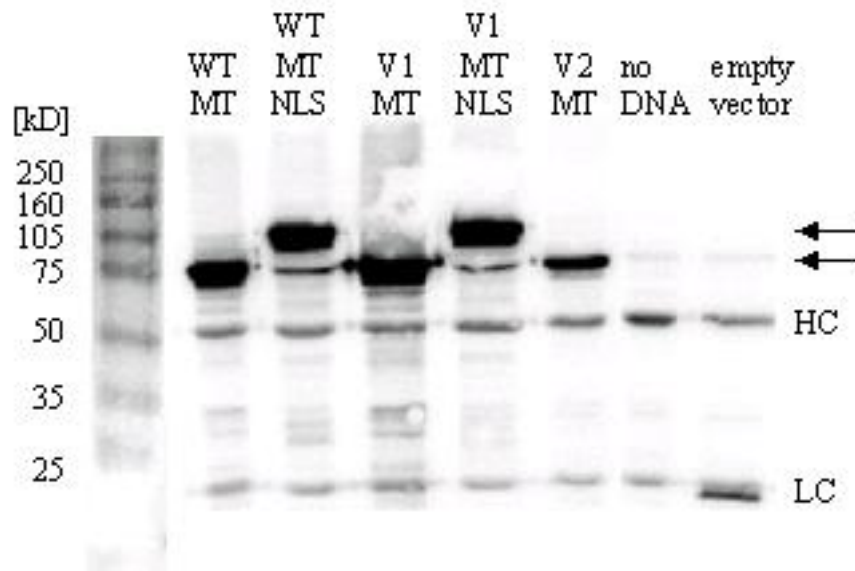


Figure 29: IPs from transfected cell lysates with monoclonal anti c-myc antibody analyzed by 12.5% SDS-PAGE and Western blotting. Anti c-myc antibody was used for detection. Positions of myc₆-CpB0730 and NLS-myc₆-CpB0730 proteins are indicated by arrows. Antibody heavy (HC) and light (LC) chains are also indicated. Lanes are named after the respective construct, which was used for transfection: wt CpB0730 pCS3+ MT (WTMT), wt CpB0730 pCS3+ MTNLS (WTMTNLS), gain-of-function mutant V1 CpB0730 pCS3+ MT (V1MT) and so on. Samples gained from cells transfected without DNA (no DNA) or with empty pCS3+ MT vector only (empty vector) are also shown. M: Full Range Rainbow molecular weight marker.

5.4.5 CpB0730 (Pkn5) is not an active kinase

5.4.5.1 (Auto-)Phosphorylation of CpB0730 in 1D kinase assays

Immunoprecipitated myc₆-CpB0730 or NLS-myc₆-CpB0730 protein was used in kinase assays as described. These kinase assays with radiolabelled ATP were analyzed by SDS-PAGE followed by autoradiography and Western blotting. Figure 30 shows a radioactive kinase assay with Histones as substrate. The SYPRO-Ruby stained SDS-PAGE gel shows that high amounts (approximately 14 ng) of wt and gain-of-function mutant myc₆-CpB0730 were used in each reaction. As also seen on other Western blots, myc₆-CpB0730 was detected as a very narrow double band with the lower one giving the stronger signal.

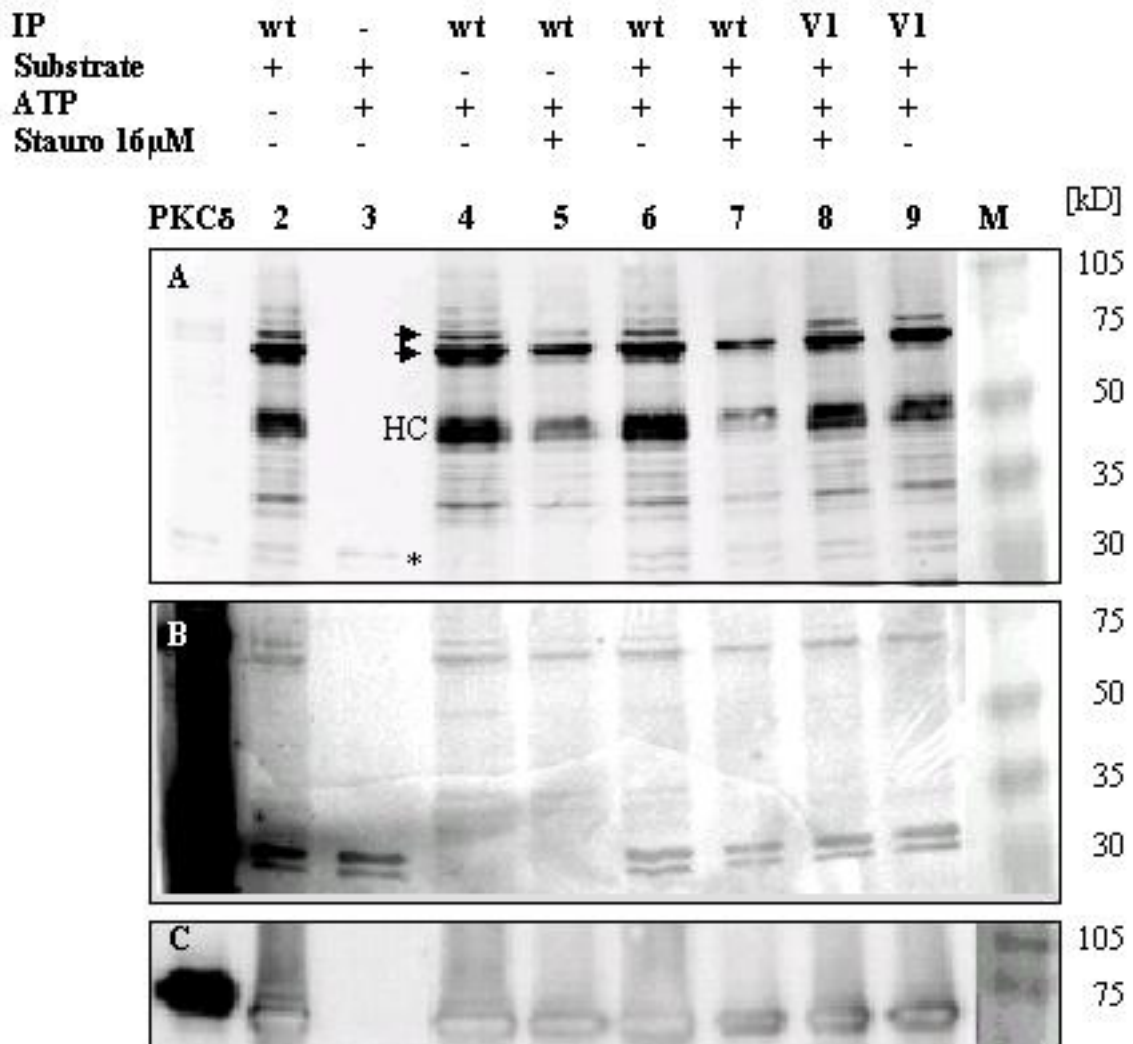


Figure 30: Radioactive kinase assays with Histones as substrate analyzed by SDS-PAGE. Protein was visualized by SYPRO-Ruby staining (**A**). Phosphorylation was detected by autoradiography (**B**) and by Western blotting with anti phospho-Threonine antibody (**C**). Bands corresponding to myc-CpB0730 are indicated by arrowheads, HC marks the antibody heavy chains and histones are indicated by asterisks.

The positive control with PKC δ and histones as substrate demonstrated that the kinase reaction worked very efficiently. The autoradiography shows, that wt and mutant CpB0730 as well as the histones are phosphorylated in every sample. Kinase reactions with gain-of-function mutant V2 displayed exactly the same result (data not shown). The 16 μ M Staurosporine added to every second kinase reaction did not inhibit this

phosphorylation, which can also be observed on the anti phospho-Threonine Western blot (C). However, in lane three histones seem to be phosphorylated without CpB0730 IP or any other kinase present. The same was seen with MBP substrate (data not shown). This means that the here observed radioactive signals probably are due to passive interaction of proteins (CpB0730, histones and MBP) with radioactive ATP and not due to active phosphorylation reactions. It was tried to lower the amount of histone and MBP substrates from 50 to 5 ng/ μ L but this still led to the same results. This meant that detected signals were at background level with the very sensitive autoradiography. As a result an active phosphorylation reaction of CpB0730 could not be detected with this kind of kinase assay. However, as the anti phospho-Threonine Western blot shows, CpB0730 was phosphorylated in every sample at one or several Threonines. This signal could not be explained by passive, unspecific interaction of ATP with CpB0730 but indicated covalent phosphorylation.

To account for possible chlamydial co-activators of CpB0730, IPs with lysates from transfected and infected cells were carried out. However, this approach did not lead to different results. With this strategy an intrinsic CpB0730 (auto-) phosphorylation activity could not be detected.

5.4.5.2 (Auto-)Phosphorylation of CpB0730 in 2D kinase assays

Radioactive kinase assays with CpB0730 but without any substrate were carried out and analyzed by 2D SDS-PAGE analysis at a pH range of 4 to 7. This was done to investigate whether CpB0730 is able to phosphorylate itself. Proteins were visualized by SYPRO Ruby stain and radiolabeled, phosphorylated proteins were detected by autoradiography. Since the gel was dried, reflections of the slightly uneven cellophane foil disturb the visibility (Figure 31). Dried gels have been photographed because the SYPRO Ruby signal was more intense on dry than on wet gels. Nevertheless some of the indicated spots (number 2,3,4, and 6) could not be seen by the naked eye but were identified with the Proteomweaver software spot detection tool (Definiens, Munich, Germany). Only two phosphorylated spots were identified. The spot at pI 7.0 was only phosphorylated very weakly but might correspond to CpB0730. Between pH 5 and 6, at a molecular weight of below 25 kD, a more intensely phosphorylated spot was detected.

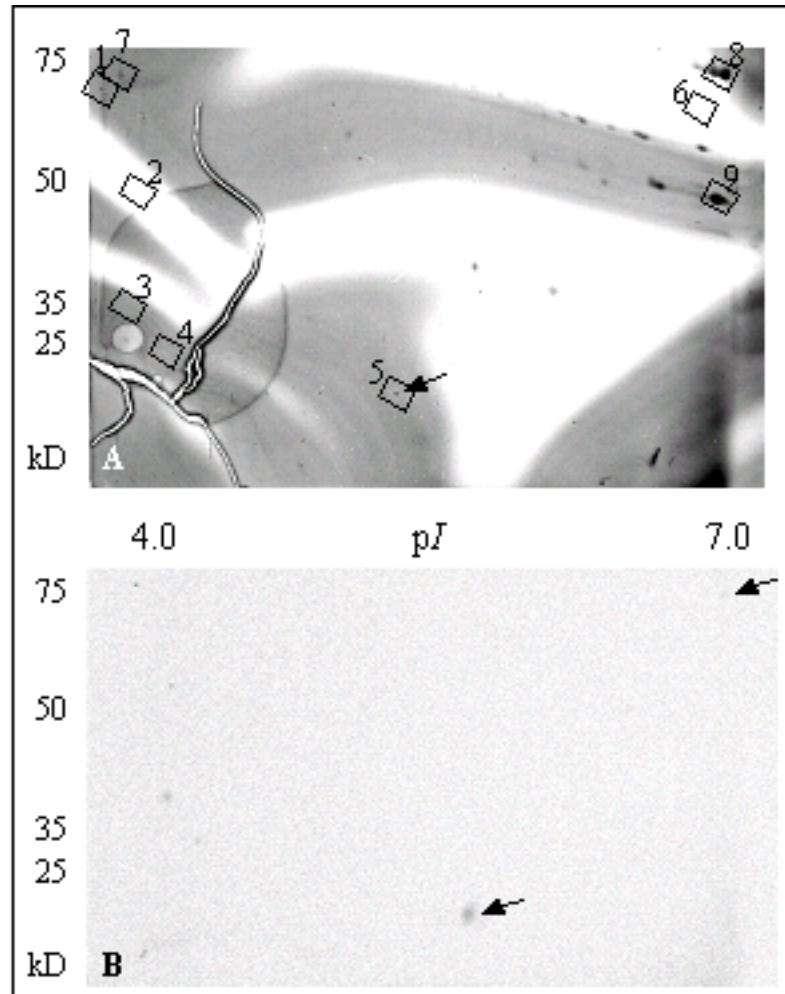


Figure 31: pH4-7 10% 2D SDS-PAGE analysis of radioactive kinase assays with wt CpB0730 protein and without further substrate. **A:** SYPRO Ruby stained, dried gel. Framed spots were cut out and further analyzed by MALDI-TOF analysis. **B:** Autoradiography of the same gel with arrows indicating phosphorylated spots.

These two radioactive spots were cut out of the gel together with other non-radioactive spots for downstream MALDI-TOF analysis. The latter were seen as very faint spots only on the SYPRO Ruby stain. Spot number 8 was identified as bacterial β -galactosidase protein, not as CpB0730 as expected. The presence of this bacterial protein in the gel cannot be explained, since no β -galactosidase gene was present on any

of the used expression vectors. All other spots could not be identified by MALDI-TOF analysis due to too low amounts of protein present in the spots. As a result, the autophosphorylation of wt CpB0730 protein could not be demonstrated by this approach. Therefore another strategy had to be used to look for CpB0730 (auto-) phosphorylation.

5.4.5.3 Kinase activity of CpB0730 in kinase assays with Luminex

With the Luminex system non-radioactive kinase assays were carried out to investigate two questions simultaneously. First, is CpB0730 able to phosphorylate itself? Second, is CpB0730 able to phosphorylate the standard kinase substrates MBP and Histones, as well as two other synthetic peptide substrates?

Sample	Substrates	CpB0730	Histones	MBP	Substrat3	Substrat4
negative control: empty myc-beads		n.d.	135	83	258	251
negative control: empty myc-beads		n.d.	143	68	148,5	209
IP empty vector		376	180,5	238	226,5	250
IP empty vector		735	441	251,5	466	621,5
IP wt CpB0730		261	349	55	101,5	177
IP wt CpB0730		376	262	42,5	106,5	252,5
IP DN CpB0730		1130	329	179	445	405
IP DN CpB0730		n.d.	399	51	153	272

Table 12: Luminex kinase assay results for the phosphorylation of Histones, MBP, synthetic peptide substrates 3 and 4 as well as for the autophosphorylation of CpB0730. Kinase activities were measured in duplicate for each substrate. For each set of data the median is listed. Counts always were above 10, mostly above 30. Counts below 10 are indicated as not determined (n.d.).

Table 12 shows the phosphorylation measured for the indicated substrates as median phosphorylation activities. In this setup the IP from cell lysates overexpressing wt CpB0730 was compared to the IP from lysates of cells transformed with the empty pCS3+ MT vector only. Additionally, the dominant-negative (DN) CpB0730 IP was measured. As additional negative control, kinase assays with empty myc-IP beads were

analyzed. As can be seen in table 12, no kinase activity above background could be observed for CpB0730. The measured activities for the different substrates showed a strong divergence. As activities measured for negative controls and for DN CpB0730 were in the same range as those measured for wt CpB0730 the conclusion is, that no significant kinase activity above background could be determined.

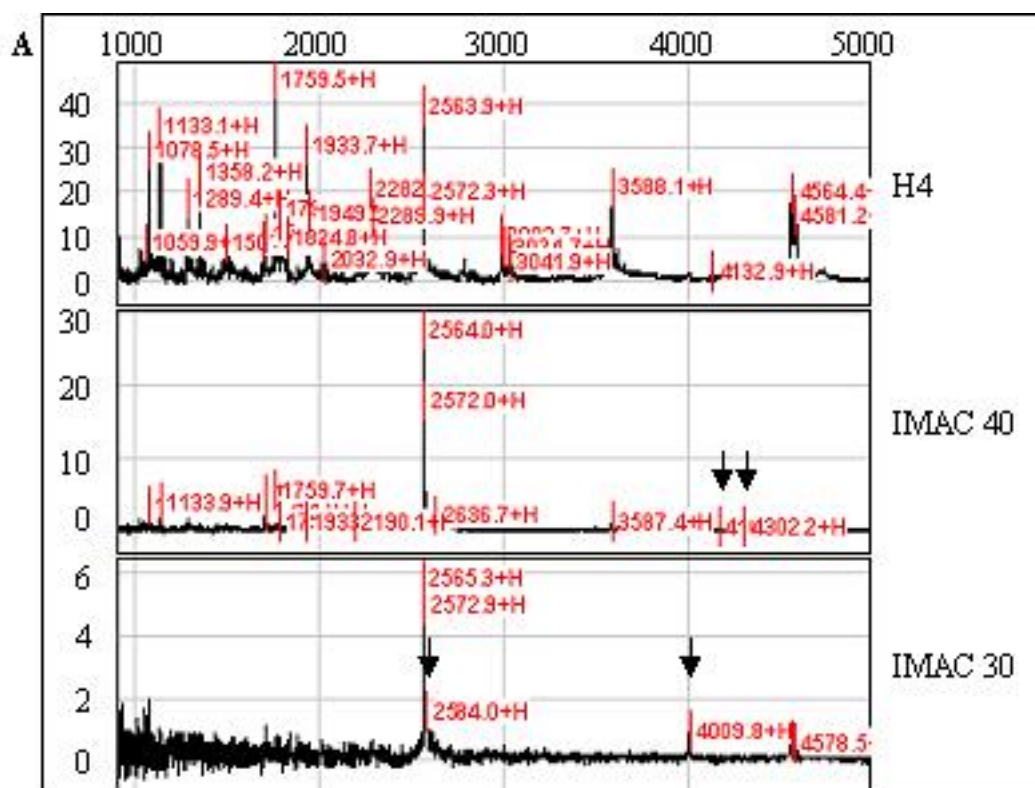
Sample	Substrates	Histones	MBP	Substrat3	Substrat4
negative control: no IP		2	12	35	21,5
negative control: no IP		12	11,5	24	22
negative control: empty IP beads		122	64	91	349
negative control: empty IP beads		101	16	117,5	130
negative control: no primary Ab		77	16,5	51,5	59
negative control: no primary Ab		101	45	129	112
IP wt CpB0730		131,5	293	212	580,5
IP wt CpB0730		207,5	119,5	185	248
IP V1 CpB0730		120	25	78	88
IP V1 CpB0730		83	40	85	82
IP V2 CpB0730		89,5	71,5	69,5	92
IP V2 CpB0730		82	41	88	107,5
IP empty vector		447,5	177	547	506
IP empty vector		1272	330	1003	797
IP DN wt CpB0730		n.d.	24	1946	246
IP DN wt CpB0730		243	169	421	390
IP DN V1 CpB0730		237	219	300	169
IP DN V1 CpB0730		339	243	404	628
positive control: 250 ng PKC theta		3989	4903	4795	4422
positive control: 250 ng PKC theta		3732,5	5961	6049	5603

Table 13 on the previous page: Luminex kinase assay to measure the activity of wt and gain-of-function mutant CpB0730 towards the indicated substrates. Activities were measured for each substrate in duplicate. For each set of data the median is listed. Bead counts always were above 10, mostly above 30. Counts below 10 are indicated as not determined (n.d.).

Table 13 shows a kinase assay setup including the “gain-of-function” mutants V1 CpB0730 and V2 CpB0730. The positive controls with PKC theta proved for all substrates tested that kinase activity could be measured if active kinase was in the kinase assay sample. However, wt as well as V1 and V2 mutant CpB0730 proteins did not display any activity above background. Activities measured with DN CpB0730 constructs (wt and V1) or with IPs from cells transfected with empty vector only were higher than activities measured for wt, V1 and V2 CpB0730 proteins. This indicated that the measured values were background activity. Therefore it must be stated that no kinase activity could be detected for neither CpB0730 construct.

5.4.6 CpB0730 is phosphorylated in the C-terminal putative regulatory domain

The phosphorylation state of wt CpB0730 protein was analyzed with the SELDI technique. Therefore, the band corresponding to wt CpB0730 was cut out from a 10% SDS-PAGE gel of a non-radioactive kinase assay. The protein sample was digested with Trypsin and analyzed with the Ciphergen PBS IIC Biochip Reader on the indicated chip surfaces. On the IMAC 30 and IMAC 40 chips, which have been loaded with Gallium ions in order to enrich phosphorylated peptides, four mass peaks were identified, that fit to the tryptic CpB0730 fragments plus the masses of one or two phosphoryl groups (Figure 32). This is a strong hint that CpB0730 is multiply phosphorylated. Fragment V⁴⁰⁹-C⁴⁴⁴ was detected as double phosphorylated fragment. Putative phosphorylation sites in this fragment would be S⁴¹⁵, S⁴¹⁷, T⁴²⁴, S⁴²⁵ and T⁴³¹. Fragment V⁴³³-R⁴⁶⁷ was identified as tryptic fragment carrying one phosphoryl group with T⁴⁴⁵, S⁴⁵¹, S⁴⁵⁴, T⁴⁵⁷, S⁴⁵⁹ as possible phosphorylation sites. Fragment M²⁵⁸-R²⁹² also was identified as fragment with one phosphorylation. The possible phosphorylation sites in this fragment are S²⁵⁹, T²⁷², Y²⁷⁶, T²⁸¹ and T²⁹⁰.



B MDCRGGIPLPEPQVIGGYHVKKILSKKLRV V HGLHPE TRHSTVIK
 VFSPSPSFTSRVYNFLKEAQSLHQITHPNIVKFHRVYKWKQDCLYIA
 MEYIEGISLREYILAQFISLPQAIDIIFDIAQALEHLHSRNILHKDIKPEN
 ILITPQGGIKLIDFGLADWDTEIQRAHPSVIGTPYYMSPEQRQGESHSP
 ASDIYALGLLAYELILGHLSLGRVFLSLVPERISKILAKALQSPNNRY
 Kinase domain ←
 SSTREFIQDIHRYRMSGDMQEDLRIKDHTV AL YEQLQTQRFWLAPE
 → „Regulatory“ domain
 TLRFPDFISGVLYHQGYPLYPHAYDTLLEGDVFNLWLGYSPISNATIA
 LSVVKSLV CQQDLQRPLLDREVCEINECLIRMKIPIDEMGISILCLEISK
 ENKELSWVACGKTVFWIKRQGRV VQDFESF SPGLGKITSLQIRETKV
 AWEIGDEAVVCTLELEESVA SLKTLSLAELQDRRQKAIFCPIESIHGG
 IQSRQHGSNSPSTLISLKRIR

Figure 32 on the previous page: SELDI analysis of CpB0730 phosphorylation state. **A:** SELDI spectrum from the H4, IMAC 30 and IMAC 40 chip surfaces. **B:** Primary sequence of CpB0730 protein with identified phosphorylated fragments indicated in colour. Double phosphorylated fragments, masses of which could be identified in the SELDI spectrum, are indicated in green. Fragments with one phosphorylation identified in the SELDI spectrum are indicated in red. Green letters with red underline indicate the regional overlap of green and red sequences, since two-colour display of letters was not possible. The proposed domain structure of CpB0730 with kinase and regulatory domain is also indicated. The arrows mark the identified peaks of the respective peptide fragments in the spectrum. Possible phosphorylation sites are written in bold letters.

One phosphorylated fragment (E⁶⁶-K⁸⁶) was identified, which starts with the conserved glutamate E66 from Hanks domain III. This mono-phosphorylated fragment was the only phosphorylated fragment identified from the CpB0730 “kinase domain”. Its putative phosphorylation sites are S⁶⁹, T⁷⁴ and Y⁸⁴. None of these three sites has been described to be conserved or to participate in kinase activity or regulation of other kinases. Furthermore, none of these putative phosphorylation sites are located within one of the conserved Hanks domains. Interestingly all but one of the identified phosphorylated fragments are located within the putative regulatory, non-kinase domain.

5.5 Evaluation of the potential role of CpB0730 as virulence factor

5.5.1 Interaction partners of CpB0730

5.5.1.1 CpB0730 alters the phosphorylation of host cell proteins

In order to elucidate the influence wt and functional mutant V1 and V2 myc₆-CpB0730 might have on the phosphorylation state of host cell proteins, kinase assays were carried out with HEP-2 lysate as substrate. CpB0730 protein was detected on Western blots with anti c-myc antibody.

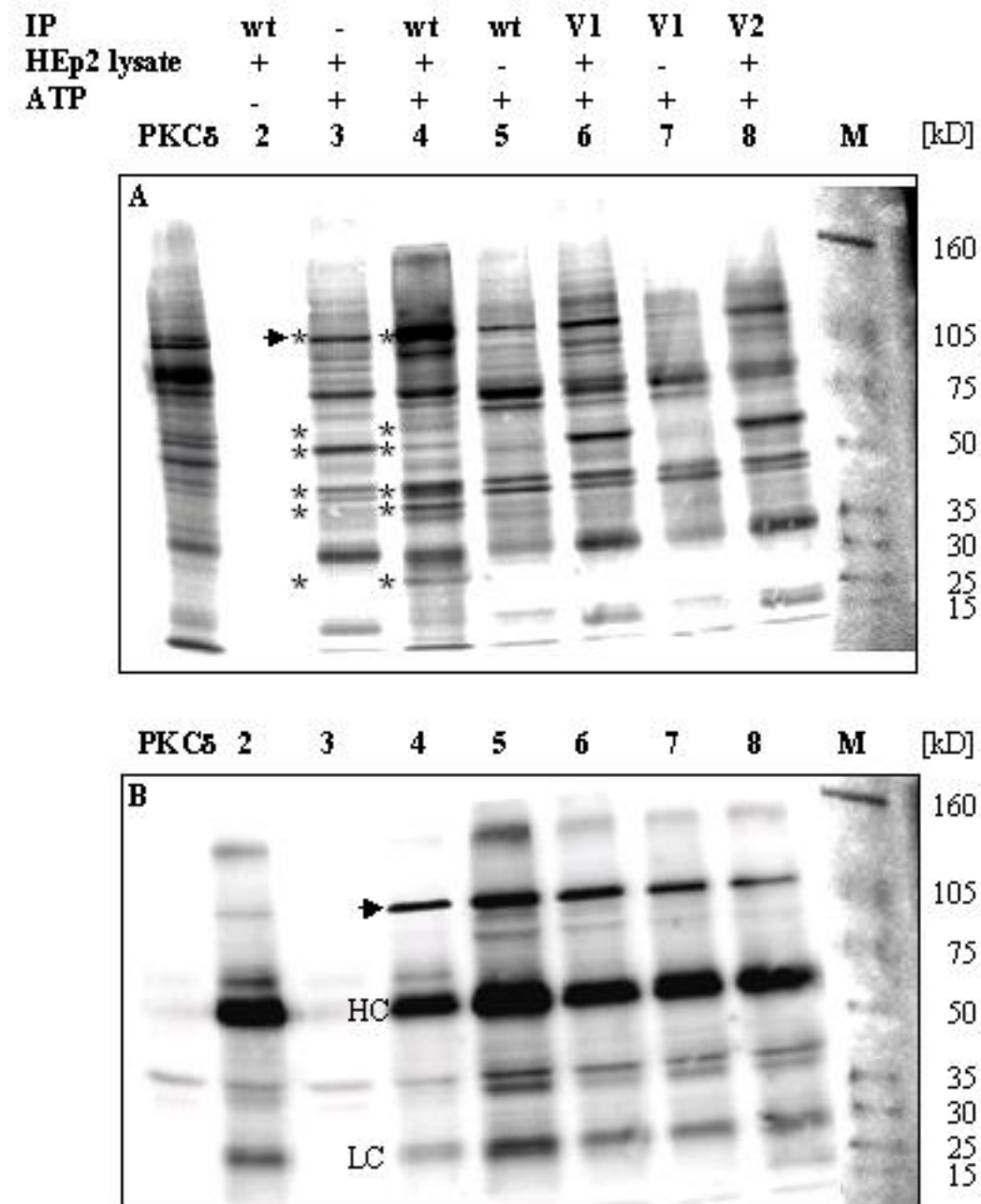


Figure 33: Radioactive kinase assays with HEp-2 lysate as substrate analyzed by SDS-PAGE, Western blotting and autoradiography. **A:** Phosphorylation was detected by autoradiography. **B:** Protein was visualized by Western blot with anti c-myc antibody. Bands corresponding to NLS-myc₆-CpB0730 are indicated by arrowheads, HC marks the antibody heavy chains and LC the light chains. Differences in the phosphorylation pattern, which are possibly due to CpB0730 IP are indicated by asterisks.

Differential phosphorylation of host cell proteins was detected by autoradiography. Figure 33 shows, that wt CpB0730 protein leads indeed to differential phosphorylation

of host cell proteins as indicated by asterisks. Some proteins with molecular weights of approximately 110 kD, 65 kD and 27 kD were stronger phosphorylated when wt CpB0730 was present (lane 4 versus lane 3). A fraction of the 110 kD protein band might be autophosphorylated wt CpB0730 overlaid with a protein already phosphorylated in HEp-2 lysate alone. Another protein(s) was stronger phosphorylated if wt CpB0730 was absent (60 kD). Although approximately identical amounts of CpB0730 proteins were present in kinase assays, the intensities of some differentially phosphorylated bands differed between wt, V1 and V1 CpB0730 proteins. The 10 kD band was stronger phosphorylated with wt (lane 4) than with V1 or V2 mutants (lanes 6 and 8). The latter two lanes displayed intensities at 110 kD comparable to HEp-2 lysate alone (lane 3). This could mean that wt CpB0730 becomes autophosphorylated whereas the putative functional mutants do not. Furthermore, phosphorylation of the 60 kD protein was only attenuated if wt but not if V1 or V2 CpB0730 protein was present. The latter two samples (lane 6 and 8) were in this context identical to HEp-2 lysate alone (lane 3). The other putative activities of wt CpB0730 protein could also not be seen with V1 and V2 mutant proteins. Therefore it seems as if the introduced mutations rendered CpB0730 into non-functional instead of functional mutants, as was originally attempted. It was now of interest what host cell proteins were the substrates for wt CpB0730 protein leading to either stronger or less phosphorylation of these proteins. Since 1D SDS-PAGE did not separate the numerous proteins with high enough resolution, 2D SDS-PAGE analysis had to be performed in order to identify potential interacting proteins of CpB0730.

5.5.1.2 Identification of potential interaction partners and/or substrate proteins of CpB0730

Radioactive kinase assays were carried out in order to identify potential interaction partners or substrates of CpB0730. HEp-2 lysate was incubated in kinase assays either together with CpB0730 or alone as a control. Samples were separated by 2D SDS-PAGE with a pH range from 4 to 7 and a 10% SDS-PAGE gel. Autoradiographies of the dried gels displayed differences in the phosphorylation pattern (Figure 34).

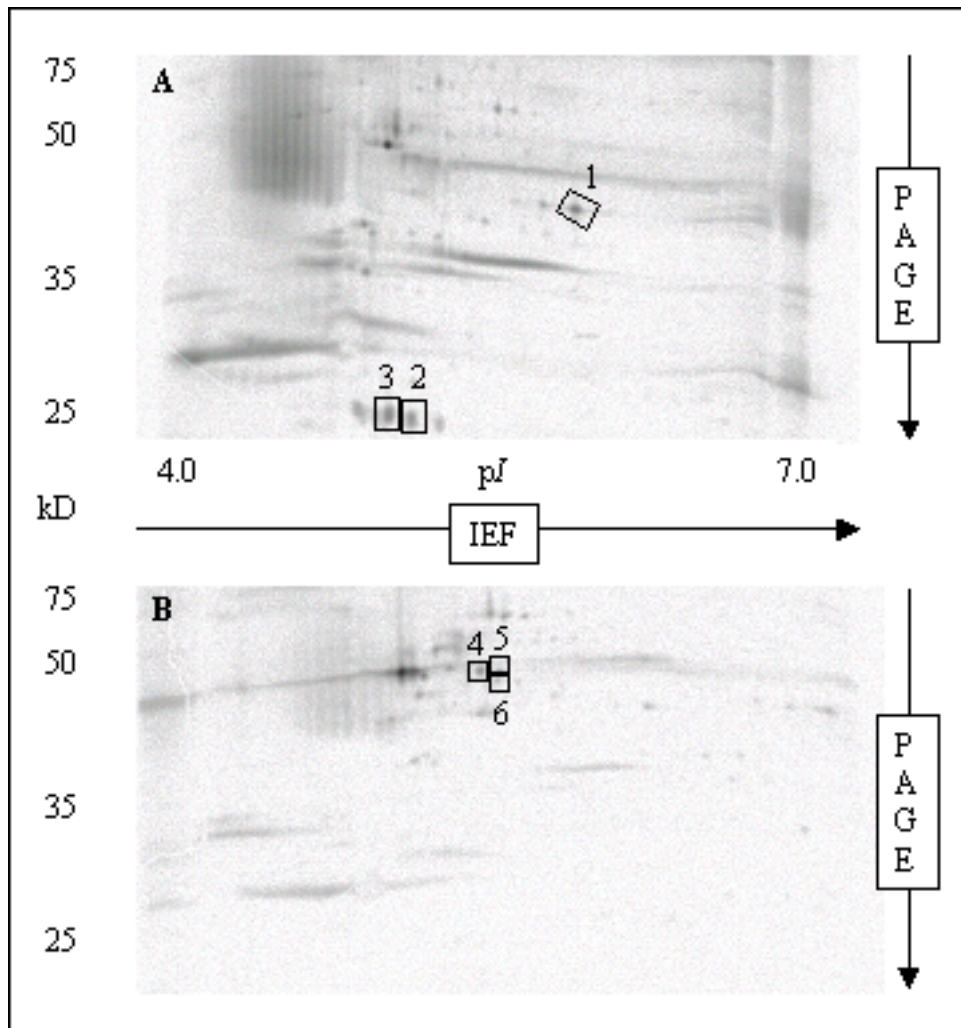
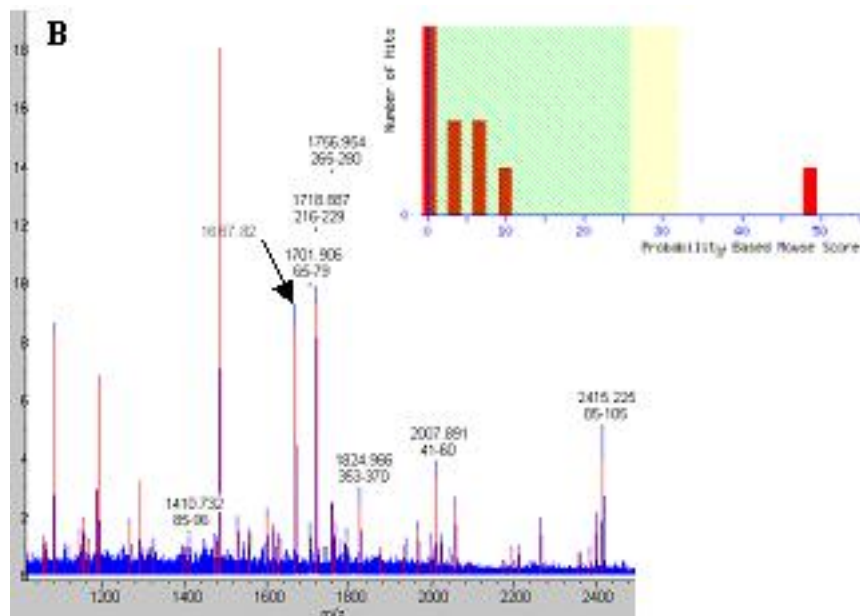
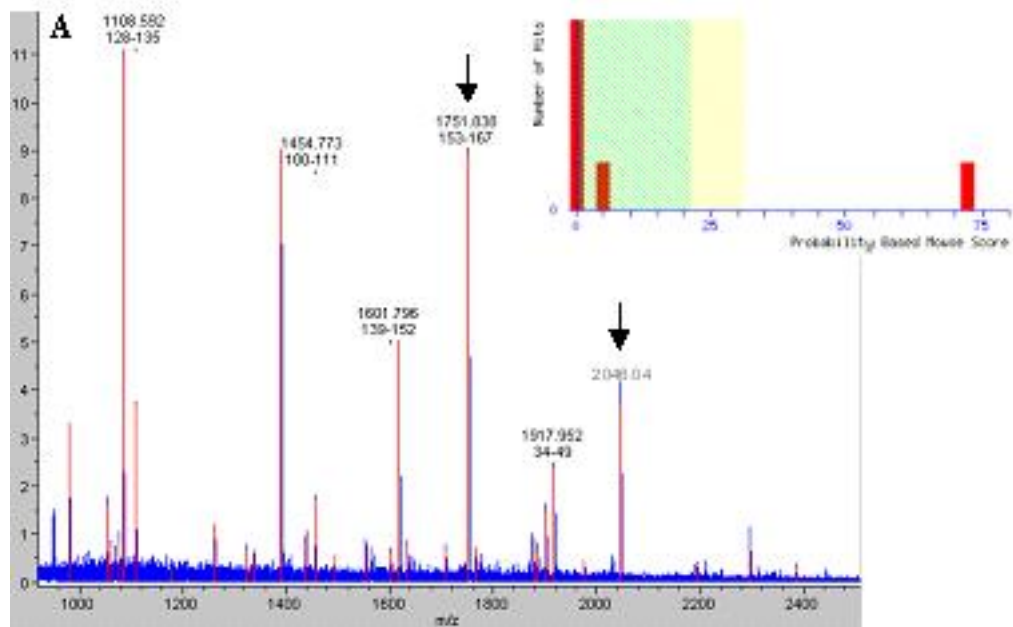


Figure 34: Radioactive kinase assays to identify potential interaction partners of CpB0730 protein. Autoradiographies of 10% 2D SDS-PAGE analyses in a pH range of 4-7 are shown. **A:** Kinase reaction with Hep-2 lysate together with wt CpB0730 IP. **B:** Kinase reaction with Hep-2 lysate alone. Dried radioactive gels were placed on detection screens for 72 h. Indicated, differentially phosphorylated spots were cut out and analyzed by mass spectrometry.

Gel A shows protein spots phosphorylated in the kinase assay with HEP-2 lysate plus wt CpB0730 IP. All spots in Gel B must be phosphorylation products of kinases in the HEP-2 lysate. Theoretically wt CpB0730 protein should be responsible for all observed differences between the two gels. Therefore, differentially phosphorylated spots were cut out, trypsin digested and analyzed by MALDI-TOF. Some mass peaks were additionally analyzed by MS/MS. From six spots analyzed three revealed interesting results (Figure 35). Spot number three, phosphorylated only if CpB0730 protein was

present, was identified as Rho GDP-dissociation inhibitor alpha (gi4757768). Spot number four contained a protein disulfide isomerase-related protein (gi1710248). Spot number five was identified as the alpha regulatory chain of cAMP-dependent protein kinase type I (PKA, gi125193). Phosphorylation of spots four and five was only detectable if wt CpB0730 protein was absent.



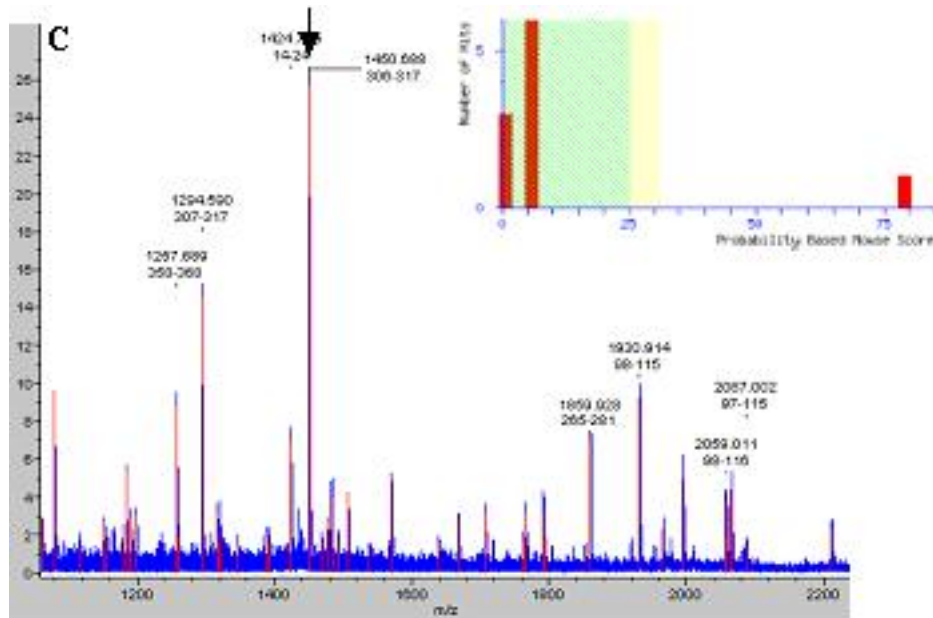


Figure 35: MALDI-TOF mass spectra of tryptically digested, differentially phosphorylated 2D SDS-PAGE spots from kinase assays. Combined Mascot searches were performed using the mass peak lists together with the MS/MS spectra of the peaks indicated with arrows. The Mascot search graph is shown in the upper right of each MALDI spectrum to indicate the identification probability. **A** shows the graphs for the identified Rho GDP dissociation inhibitor alpha. In **B** the data for protein disulfide isomerase-related protein and in **C** data for cAMP-dependent protein kinase type I-alpha regulatory chain are shown.

5.5.2 Influence of CpB0730 on host cell apoptosis

5.5.2.1 Chlamydial infection protects host cells from apoptosis

Apoptosis was measured as the amount of cleaved, active Caspase-7 in cell lysates from Staurosporin treated (0.6 μ M), Staurosporin treated and infected (24 hpi), and untreated HEp-2 cells. Figure 36 shows the grade of apoptosis measured in these cells by means of Caspase-7 detection. HEp-2 cells treated for 4 h with Staurosporine showed a clear activation of Caspase-7 as compared to untreated HEp-2 cells. If HEp-2 cells were infected with *C. pneumoniae* TW183 and treated with Staurosporine for 4 h at 24 hpi, the measured level of active Caspase-7 was almost identical to the level in untreated HEp-2 cells. This means that infected HEp-2 cells were protected from apoptosis at 24 hpi as described in the literature (2, 38, 74, 93, 230, 232, 239, 303, 309).

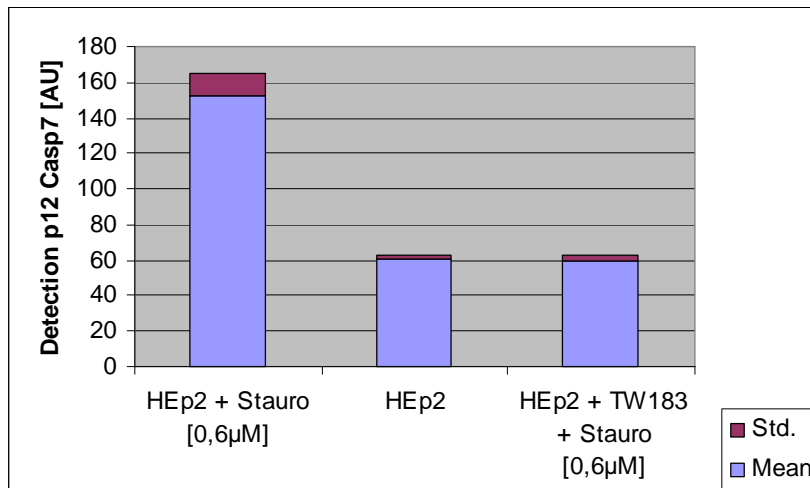


Figure 36: Apoptosis of HEp-2 cell cultures was measured by Luminex Caspase-7 Immunoassay. HEp-2 cells untreated or treated for 4 h with 0.6 µM Staurosporine were lysed as described. Infected HEp-2 cells were treated in the same way at 24 hpi. Cleaved, active Caspase-7 was measured in the lysates. The means of the amount of active caspase-7 are given in arbitrary units (AU) and standard deviations are displayed in red.

However, an involvement of CpB0730 in this anti-apoptotic effect could not be demonstrated in transfected cells overexpressing chlamydial CpB0730. This attempt failed, because the enrichment of transfected cells was unsuccessful (data not shown) and because transfection efficiencies were too low.

5.5.2.2 Antiapoptotic effect of chlamydial infection is independent from the NFκB pathway

Since it has been reported that NFκB activation is involved in the protection of Chlamydia infected host cells from apoptosis (293), the activation status of NFκB along the infection cycle was investigated. This was done with the Cytoplasm-to-nucleus translocation BioApplication in the Cellomics Array Scan. Therefore infected as well as uninfected HEp-2 cells were fixed and immunolabeled for NFκB at different time points along the chlamydial infection cycle as described. The relative localization of NFκB in cytoplasm and nucleus was evaluated with the Cellomics software and is presented in figure 37. Although some alterations in the NFκB distribution could be detected, no

significant differences between infected and uninfected cells could be observed. NF κ B therefore seems not to contribute to the observed anti-apoptotic effect.

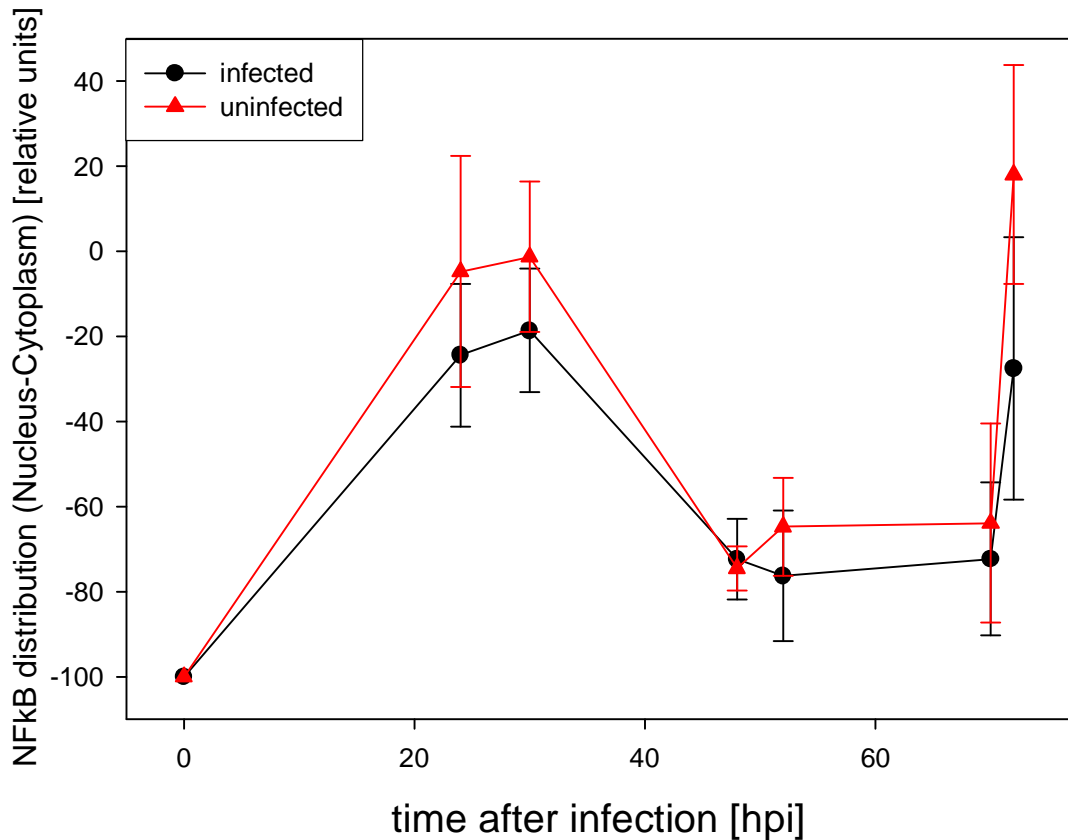


Figure 37: The relative distribution of NF κ B in cytoplasm versus nucleus was measured in infected and uninfected HEp-2 cells along the infection cycle with the Cellomics Array Scan. Data from infected cells are seen in black, uninfected cells are shown in red. The localization of NF κ B in the cell is plotted as amount in the nucleus minus the amount in the cytoplasm (arbitrary units) against the hours post-infection.

5.5.3 CpB0730 interacts with PIASx host cell protein

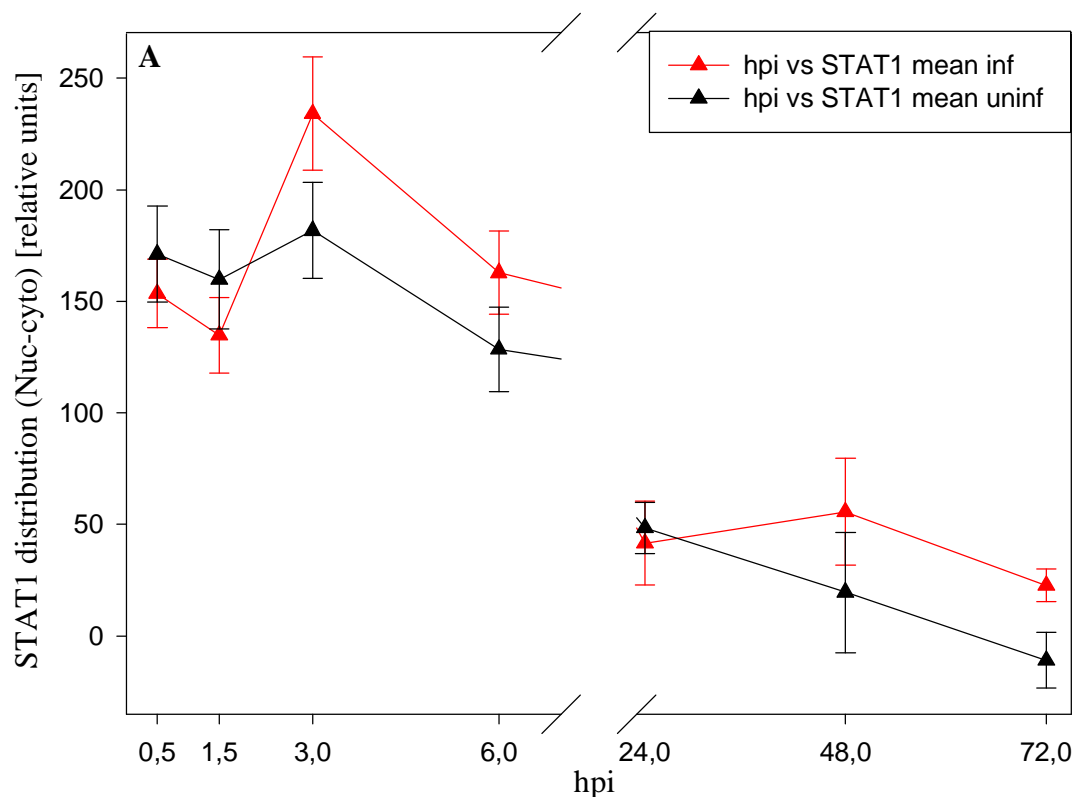
5.5.3.1 PIASx as potential interaction partner of CpB0730

In the context of an external collaboration with GPC Biotech, a potential interaction between chlamydial CpB0730 protein and the PIASx protein was suggested. PIASx was

identified in a series of yeast two-hybrid screens of a human brain library with different baits of CpB0730. Since this finding was interesting for our investigations, we followed this possible interaction during the Ph.D. thesis project presented here.

5.5.3.2 Interference of chlamydial infection with STAT transcription factor translocation

Since PIAS proteins are involved in signal transduction of STAT transcription factors, it was investigated, whether chlamydial infection has an influence on the distribution of STAT transcription factors within infected host cells. Therefore infected and uninfected cells were fixed and immunolabeled for STAT1, STAT2 and STAT3 respectively. This was done at different time points of the infection cycle. The analysis was carried out on the Cellomics Array Scan with the Cytoplasm to nucleus translocation BioApplication.



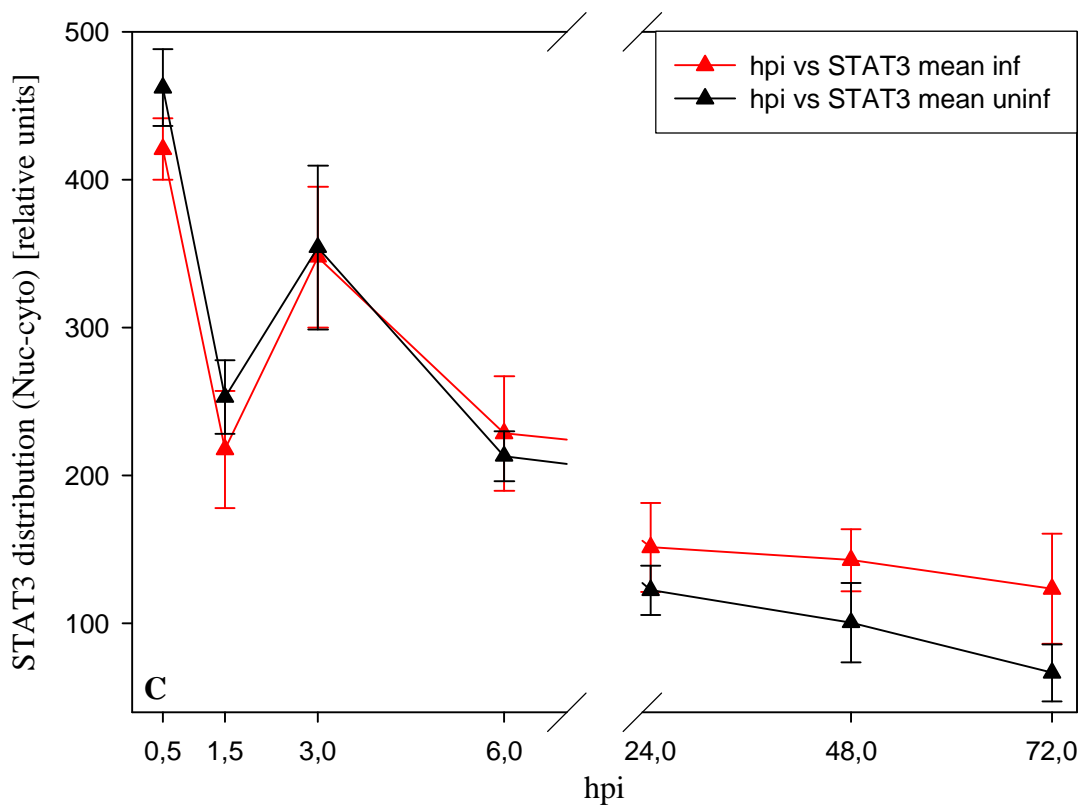
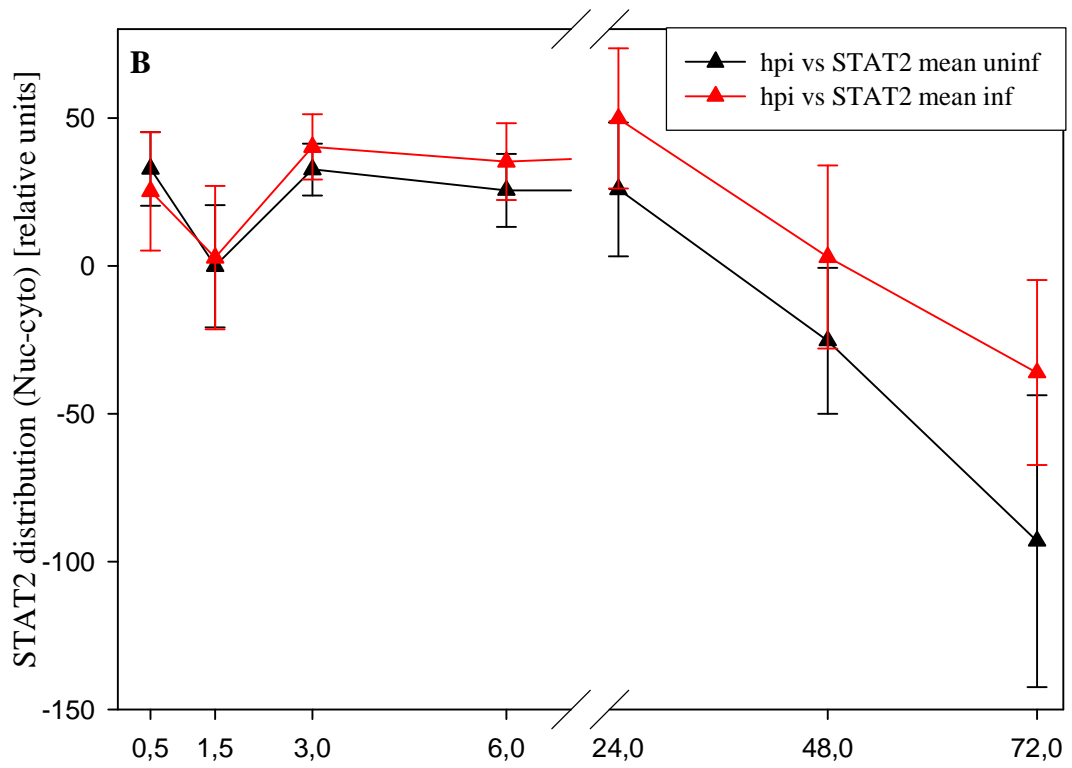


Figure 38 on the previous pages: The relative distribution of STAT1 (A), STAT2 (B) and STAT3 (C) in cytoplasm versus nucleus was measured in infected and uninfected HEp-2 cells along the infection cycle with the Cellomics Array Scan. Data from infected cells are seen in red, uninfected cells are shown in black. The localization of STAT proteins in the cell is plotted as amount in the nucleus minus the amount in the cytoplasm (arbitrary units) against the hours post-infection (hpi).

Figure 38 shows the relative distribution of the STAT transcription factors along the infection cycle. As for NF κ B the relative distribution of STAT1, STAT2 and STAT3 in cytoplasm versus nucleus changes over time along the infection cycle. However, as for NF κ B, there seems to be no different regulation of STAT localization in infected cells compared to uninfected cells. Chlamydial infection seems to have no influence on STAT localization.

5.5.3.3 Posttranslational modification of CpB0730 with SUMO1 by PIASx in transfected cells

As described (145, 216, 258) PIAS proteins can have dual functions. They not only act as inhibitors of STAT proteins inhibiting their translocation into the nucleus, but also act as SUMO-ligases. This enzymatic activity enables PIAS proteins to covalently modify specific substrate proteins with SUMO proteins similar to ubiquitin modification. Therefore it was tested, if CpB0730, putatively interacting with PIASx, might be SUMOylated. This was done by immunoprecipitation of myc₆-CpB0730 with anti c-myc antibody and with anti SUMO antibodies. Figure 39 shows that myc₆-CpB0730 could be precipitated from lysates of HEK 293 EBNA cells transfected with Wild pCS3+ MT with monoclonal anti c-myc as well as with monoclonal anti SUMO1 antibody. This indicated that the chlamydial protein CpB0730 became SUMOylated with SUMO1. The same experiment was carried out with antibody specific for SUMO2/3, but no CpB0730 signal was detected (data not shown). This indicated further, that CpB0730 was specifically SUMOylated with SUMO1. To test whether this SUMOylation was carried out by PIASx protein, IP was performed with PIASx antibody from lysates of transfected cells. Lysates from cells transfected with Wild pCS3+ MT, Wild pCS3+ MTNLS or with empty vector only were used.

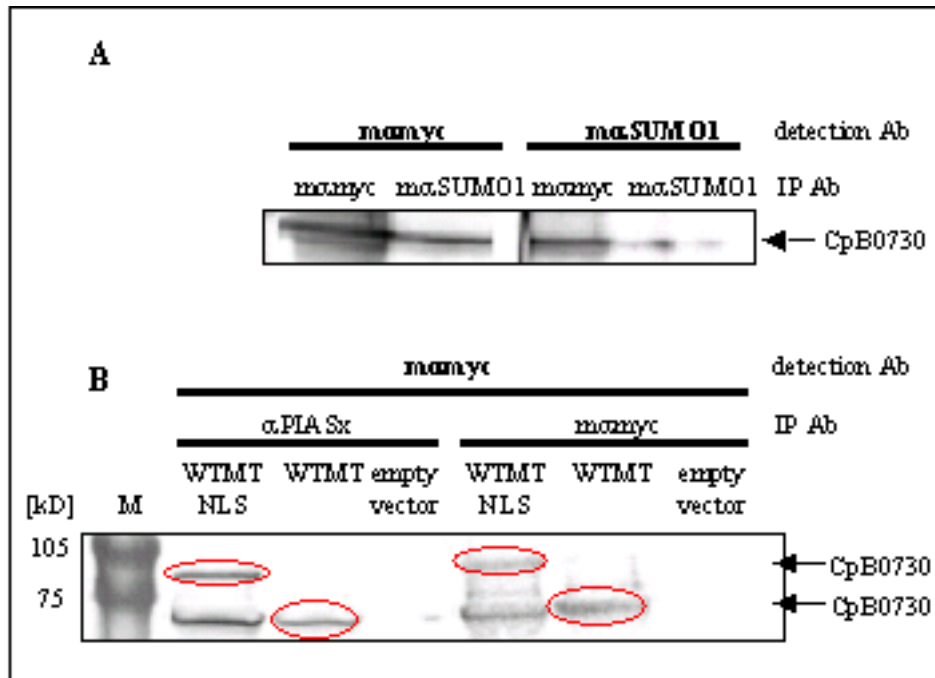


Figure 39: Co-IPs were carried out with antibodies directed against c-myc, SUMO1 and PIASx and lysates from transfected cells. Precipitated protein was analyzed by 10% SDS-PAGE followed by Western blot analysis **A:** Co-IPs from cells transfected with Wild pCS3+ MT. Antibodies used for IP and for detection are indicated. CpB0730 bands are shown. **B:** Co-IPs from cells transfected with plasmids Wild pCS3+ MTNLS, Wild pCS3+ MT and pCS3+ MT (empty vector). Antibodies used for IP and for detection are indicated. Bands of CpB0730 protein with and without NLS are indicated by arrows. M: Full Range prestained molecular weight marker.

Figure 39 shows that myc₆-CpB0730 and NLS-myc₆-CpB0730 co-precipitated with PIASx. Co-IP with lysate from cells transfected with empty vector did not show any CpB0730 band indicating that the detected signals were specific. Hence it was proven that chlamydial CpB0730 became SUMOylated with SUMO1 and interacted with host cell PIASx.

5.5.3.4 Interaction of CpB0730 with PIASx during chlamydial infection

Since the interaction between CpB0730 and PIASx as well as the SUMOylation of CpB0730 were shown in the artificial system of transfected cells, it was investigated whether this interaction also occurs during chlamydial infection. Co-IPs were performed with anti PIASx as well as with anti CpB0730 H15Snew08 antibody and lysates from infected and uninfected cells at 20 hpi. This time point was selected since secretion of CpB0730 into the inclusion membrane and therefore contact to the host cell cytoplasm

occurred at 20 hpi. Figure 40 shows that CpB0730 could be precipitated with both antibodies from infected cells but not from uninfected cells. This proves that the PIASx CpB0730 interaction also occurs during chlamydial infection.

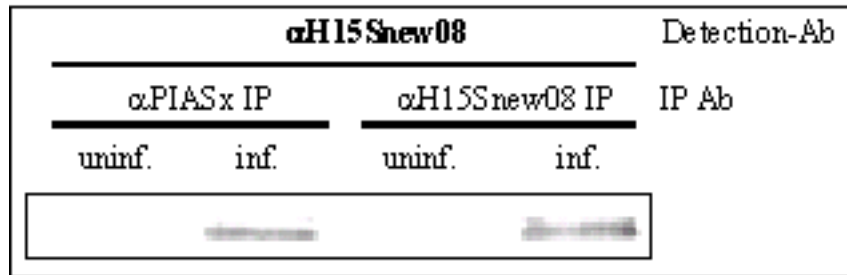


Figure 40: Co-IPs were carried out with antibodies directed against CpB0730 and PIASx and with lysates from infected and uninfected cells. Precipitated protein was separated by 10% SDS-PAGE followed by Western blot analysis. Antibodies used for IP and for detection are indicated. CpB0730 bands are shown.

5.5.3.5 PIASx colocalizes with chlamydial inclusions during infection

It was investigated, whether the demonstrated interaction between CpB0730 and PIASx could also be visualized by ICC with PIASx antibody. Infected and uninfected HEp-2 cells were fixed with formaldehyde at 20, 48 and 72 hpi. Subsequently, fixed cell samples were attributed to immunostaining with anti PIASx primary and FITC-labeled secondary anti rabbit antibody as described. Hoechst 33342 was used to stain chlamydial as well as host cell DNA. At 48 and 72 but not at 20 hpi (the 20 hpi data are not shown) PIASx could be localized at the inclusion-cytosol interface. Figure 42 shows the Nomarsky differential interference contrast and ICC picture overlays from infected cells at 48 hpi. PIASx seems not to be equally distributed at the inclusion membrane outer surface but rather to be localized in foci. Localization of PIASx within the host cell nucleus of infected and uninfected cells was detected at a very low level, which cannot be seen in these overlays because it disappears in the background.

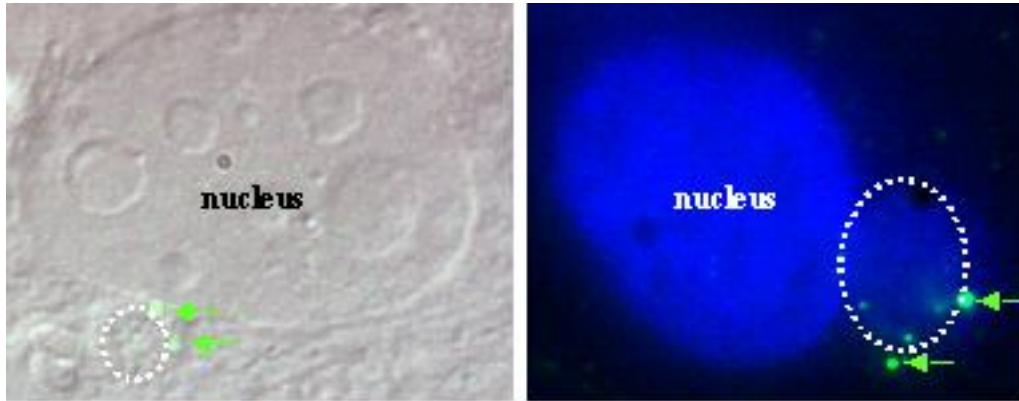


Figure 41: ICC with PIASx antibody in infected HEp-2 cells at 48 hpi. Left: Nomarsky differential interference contrast picture overlaid with anti PIASx ICC visualized by FITC-labeled anti rabbit secondary antibody. Right: ICC with anti PIASx antibody together with FITC-labeled anti rabbit secondary antibody and additional Hoechst staining of chlamydial as well as host cell DNA. White circle line indicates the localization of the chlamydial inclusion. Green arrows show spots of detected PIASx.

5.6 FK506 as cpMip-specific lead structure for the development of anti-chlamydial antimicrobials

5.6.1 Inhibition of *C. pneumoniae* infection with FK506

The immunosuppressive macrolide drug FK506 has been reported to inhibit Mip-like proteins from *Legionella* and *C. trachomatis* on an enzymatic level *in vitro* (80, 159, 185). Furthermore the inhibition of *C. trachomatis* infection in cell culture has been demonstrated (185). Since in this work for the first time secretion of cpMip protein into the inclusion membrane could be shown, the effect of FK506 on infection of cell cultures with *C. pneumoniae* was also investigated. The addition of FK506 at a concentration of 24 μ M following centrifugation of Chlamydia onto the host cell layer

resulted in the impairment of chlamydial infection initiation and/or development of the inclusions (Figure 41).

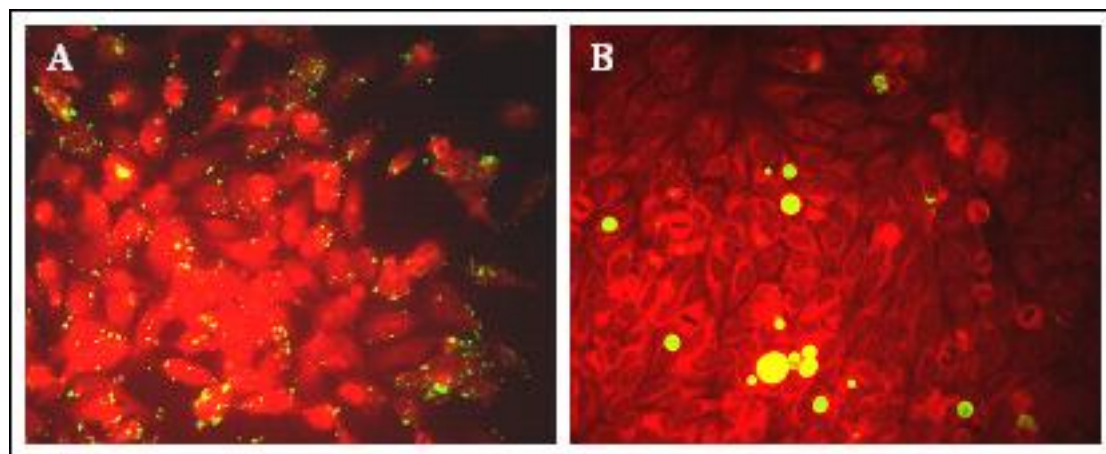


Figure 42: FK506 inhibits *C. pneumoniae* infection. HEp-2 cells were infected with *C. pneumoniae* strain TW183. FK506 was added at 0 hpi, and the cells were incubated until fixation with cold methanol at 72 hpi. Samples were immunocytochemically stained with the Pathfinder kit (Bio-Rad) showing chlamydial inclusions in green and host cell cytoplasm in red colour. **A:** FK506 exhibits a strong chlamydiostatic effect and leads to small, hardly developed inclusions in infected cells treated with 24 μ M FK506. **B:** In contrast, in untreated infected cells infection progresses normally.

The inclusions, seen in figure 41 at 72 hpi, remained smaller and the infection was weaker than without the addition of the macrolide. The appearance of the tiny inclusions reminded of persistent infections (22). Uninfected cells were neither affected by the addition of FK506 nor by addition of DMSO alone (data not shown). Therefore a cpMip specific inhibition of *C. pneumoniae* infection by FK506 is proposed.

5.6.2 Medical and chemical analysis of the macrolide FK506

FK-506 is a macrolide that contains 14 chiral centers. Functionally FK-506 can be divided into three regions: the FKBP-binding region (FKBP: FK506 binding protein), the calcineurin-binding region, and the so-called northwest region (Figure 6). The northwest region of FK-506 does not interact with FKBP-12 or calcineurin and might be suitable for chemical modifications. It has been suggested that the immunosuppressive activity of FK-506 and its toxicity are linked. Calcineurin, the protein whose function FK-506 inhibits, plays a role in the regulation of a wide variety of transcription factors.

Through inhibiting calcineurin's efficacy, FK-506 not only suppresses the immune system but causes the side effects that are linked to FK-506's use. The N-acyl piperidine carbonate substructure encircled in figure 6 is neither part of the FKBP-binding region, nor of the calcineurin-binding region. As displayed in figure 43, this FK506 substructure strongly resembles a prolyl amino acid residue with peptide bonds, which might explain the affinity to peptidyl-prolyl cis-trans isomerases. This region might serve as lead structure for drug development of specific anti-chlamydial antimicrobials.

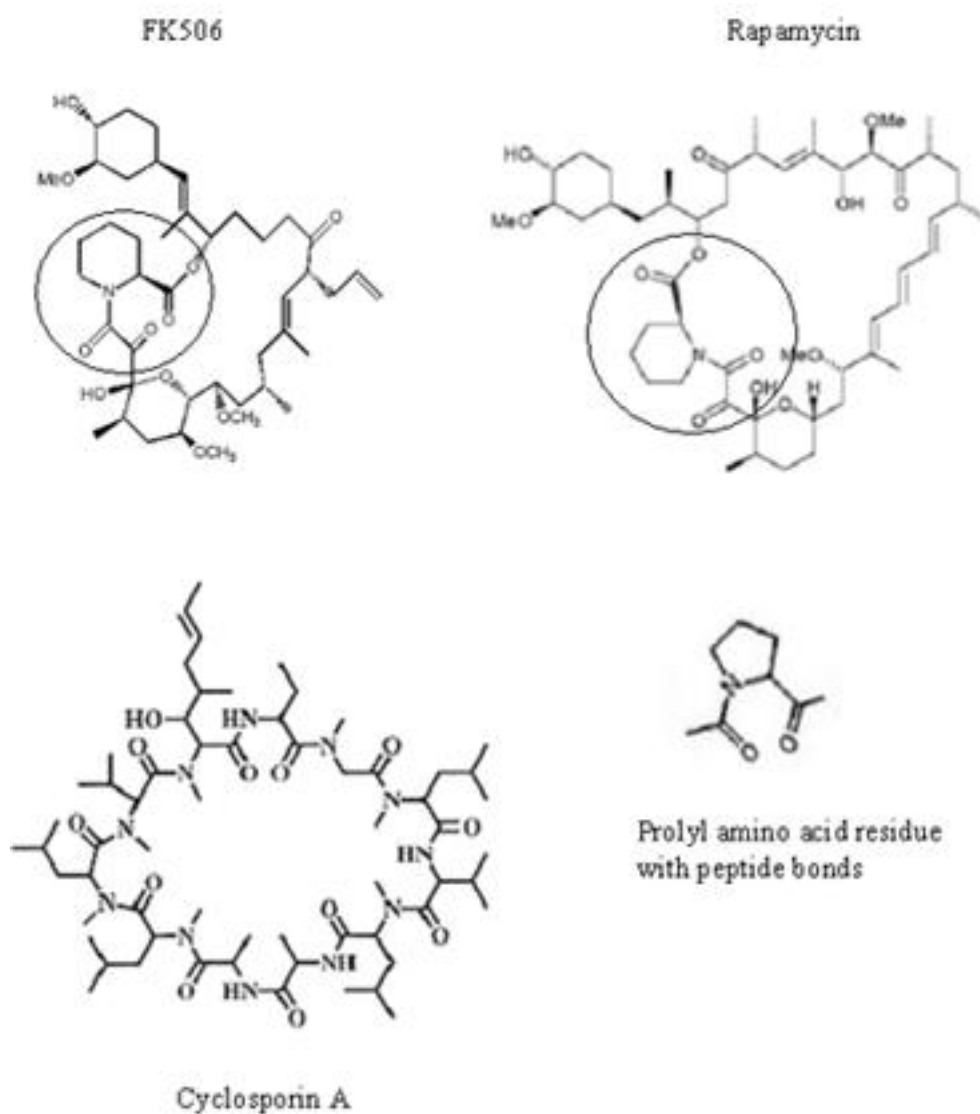


Figure 43: The molecular structures of FK506, Rapamycin and Cyclosporin A are shown together with the structure of a proline residue in a peptide bond. The potentially Mip-inhibiting substructure, which is proposed as new lead structure for antichlamydial antimicrobial development is encircled.

6 DISCUSSION

6.1 Identification of secreted chlamydial Cops

Eight ORFs encoding putatively secreted proteins of the *C. pneumoniae* type III-secretion machinery were selected from the TW183 genome sequence due to the presence of eukaryotic domains or other interesting homologies and properties. Expression, secretion and putative functions of these candidate effectors during chlamydial infection should be investigated. All candidate proteins were expressed during the TW183 infection cycle. Secretion of six proteins into the inclusion membrane could be demonstrated at different times of the infection cycle, which implies that the timing of secretion is regulated in Chlamydia. It was not proven that secretion of these effectors really occurred by means of a type III secretion system (T3SS) and not by another type of secretion system. However, except for cpMip, the genetic loci strongly suggest a type III-dependent mechanism.

6.2 Timing of expression and secretion is independently regulated

Expression and secretion of candidate proteins were not always linked in time. For example, maximal candidate expression was detected for CpB0739 and CpB0856 towards the end of the infection cycle, while secretion occurred only around 20 hpi. The fact that expression of CpB0739 protein increased (towards 72 hpi, Figure 20) long after secretion occurred (20 hpi, Figure 23) supports the idea that expression and secretion are not directly linked in time. These findings lead to the hypothesis that CpB0739 and CpB0856 proteins have more than one function during the chlamydial infection cycle. The fact that 20 hpi was the only time point during which CpB0739 and CpB0856 were detectable within the inclusion membrane hints towards a narrow time window for the secretion of those effector proteins and a rather short half-life. One might speculate, that the presence of CpB0739 within the inclusion membrane for a limited period of time only has an impact on host cell signal transduction pathways. I conclude that effector

protein expression and secretion are regulated independently from one another, as it is the case in yersinia (44, 133, 171).

6.3 Regulation of effector secretion

The investigated chlamydial effector proteins are secreted at different time points of the infection cycle. This might be connected to different roles the putative virulence factors play in the progression of the infection. Temporal regulation of effector secretion is known from other type III-secreting pathogens (44, 112, 133, 171, 182, 194). Interestingly, timing of candidate secretion seems not to be regulated on an inter-inclusional level. Sometimes secretion-active and secretion-inactive inclusions were seen in direct neighbourhood within one single host cell (Figure 22, cpMip). This indicated that there is no quorum sensing-like synchronicity for inclusions within one host cell concerning the type III-secretion of effector proteins. As a consequence, induction of secretion cannot depend on one intracellular parameter or second messenger only. Otherwise all inclusions within one host cell would have to be affected in a similar way. The biological sense thereof remains elusive, since concerted secretion of an effector protein by all inclusions within a host cell occurs advantageous.

A conserved secretion signal in the N-termini of the secreted effector proteins, as was reported for other pathogens (105, 182, 202, 234, 255), was not found. Similarly, no conserved nucleotide sequence stretch could be detected, which might have argued for an mRNA-intrinsic secretion signal (7, 8, 9, 241). Chaperones have been described to be necessary to assist secretion of type III effectors without secretion signal sequence (182). Based on the here presented findings it was concluded that potential signal sequences are either very variable, or that assistance of type III chaperones might be essential in the regulation of effector secretion in Chlamydia.

6.4 The putative role of coiled-coils in type III-secretion

Coiled-coil motifs have been described in T3SS proteins but the implication of this observation is unclear (44, 60, 134, 139). CpB0733, CpB0736 and CpB0739, three chlamydial proteins from the same gene cluster, also have coiled-coil domains predicted

with high probability. Since secretion of CpB0736 and CpB0739 was observed whereas CpB0733 secretion could not be shown, the presence of a coiled-coil domain alone is not indicative for secreted effectors. Since the other reportedly secreted proteins in this work do not display coiled-coil motives, such domains cannot be essential for secretion of type III effectors. However, such an accumulation of coiled-coil domains is unusual and therefore a biological function of these domains is probable. Coiled-coil domains are known for mediating protein-protein interactions. These three candidate proteins could interact with one another during or prior to the secretion process. The secreted coiled-coil proteins possibly interact with host cell proteins via coiled-coil formation. Thereby host cell proteins could be attracted to the inclusion surface. Such a mechanism has been described for the chlamydial CADD protein, which attracts proapoptotic Fas protein to the inclusion surface (271). Participation of coiled-coil structures has not been discussed in this context however.

6.5 CopN (CpB0334) is secreted in late-cycle

The result of Fields and Hackstadt (77), that CopN is secreted into the inclusion membrane, was corroborated for CopN (CpB0334, cpCopN) of *C. pneumoniae* in this work. However, secretion occurred later in the infection cycle (72 hpi, *C. pneumoniae* late-cycle) compared to *C. trachomatis* CopN (20 hpi, *C. trachomatis* mid-cycle). Since we detected constant but increasing expression of cpCopN towards the end of the *C. pneumoniae* infection cycle (Figure 20), and since CopN is thought to regulate and start type III secretion (77), it was surprising that cpCopN was secreted so late. However, Lugert *et al.* also detected cpCopN at 55 hpi within the inclusion membrane (184). This late secretion could point to a special role for cpCopN, since it behaves different from its counterparts in other pathogens. Since Chlamydia, unlike Salmonella (133), only possess one type III secretion system there is a need to regulate secretion of different effectors at different times by another regulatory system. Chlamydia somehow must sense environmental signals of the host cell cytosol and correspondingly coordinate type III secretion within the inclusions. This must be the case, because we observed different groups of secreted proteins being secreted at different times of the infection cycle. One load of type III effectors could be secreted upon contact of Chlamydia with the host

cell, promoting entry into the host cell. A second group of effectors is then secreted around 20 hpi, upon sensing of some intracellular signal by the type III secretion system. A third round of secretion (at 48 to 72 hpi) would then be unleashed with the CopN regulator. This would again occur upon some intracellular or even intra-inclusional signal (*e.g.* quorum sensing within a single inclusion). This is only a model and the inducing signals are totally unknown. It is not very probable that CopN within the chlamydial T3SS has adopted a completely different role as compared to the conserved T3SS of other bacterial pathogens.

6.6 Secretion of CpB0733 and CpB0837 could not be detected

Secretion of CpB0733 and CpB0837 could not be detected at any time point of the infection cycle. Secretion of small amounts of these proteins might be undetectable lacking the tools for genetic manipulation of Chlamydia. The latter would allow the construction of reporter fusion proteins, routinely used for sensitive detection of type III-secreted effector proteins (276). The similarity of CpB0733 to human ubiquitin carboxyl-terminal hydrolase turned this protein into an interesting candidate. Such a hydrolase, able to remove ubiquitin tags, might be useful for intracellular pathogens like Chlamydia, which hide from immunorecognition by the host's immune system. Perrin *et al.* reported, that intracellular bacteria in the cytosol were labelled by ubiquitin for destruction by the proteasome, which in this case translocated to the bacterial surface (233). One could imagine such a scenario also for chlamydial inclusions. In this context, the ability to remove ubiquitin from the inclusion surface would be a great advantage. However, since secretion of CpB0733 could not be shown, this protein probably has a different, intrabacterial function. CpB0837 (YopD) protein was proposed to be a T3SS secreted translocator protein (275). Therefore, it was surprising that secretion of CpB0837 could not be detected, although Lugert *et al.* reported secretion of the neighbouring YopB homologue (CpB0838) as well as of the YopD duplicate CpB1058 at 55 hpi (184). Furthermore, timing of CpB0837 and CpB0838 expression differed, which might imply independent roles of these proteins. Interestingly, the CpB0837, CpB0838 and CpB0840 triple gene cluster exists in two copies in the *C. pneumoniae* genome with CpB1057, CpB1058 and CpB1060 as duplicates (275). Chlamydia are

evolutionary not closely related to other human pathogens, which would explain different roles for common homologues. Another interesting trait was the fact that localization of CpB0837, as assessed by ICC, was not uniform inside the inclusions. CpB0837 was detected in subpopulations of Chlamydia within the inclusions only, probably in EBs. This would explain the dotted pattern that was observed by confocal microscopy (Figure 24). Therefore it was assumed that CpB0837 must play a completely different role compared to its yersinial counterpart YopD.

6.7 CpB0736

Secretion of CpB0736 occurred at 20 hpi, as reported. The presence of a coiled-coil sequence has been discussed above. A growth factor or cytokine receptor signature motif was found in this protein, but since this effector is secreted into the inclusion membrane and not into or outside of the host cell, a function of this motif remains elusive. Binding of this chlamydial protein to any growth factor receptor- or cytokine receptor-like protein within the host cell cytoplasm or within the inclusion membrane is improbable. The presence of a leucine-rich repeat is interesting, because this repeat motif was also found in type III-secreted effectors of Salmonella, Shigella and Yersinia (113, 201, 269, 283). The function of this motif within the effector proteins is unclear but might have to do with protein-protein interaction.

6.8 Potential interaction of multidomain effector protein CpB0739 with host cell signaling

CpB0739 is a secreted chlamydial type III effector with very interesting traits and a big potential for further investigations. The presence of an FHA domain reveals a possible interaction of CpB0739 with host cell phosphoproteins as well as phosphorylation of CpB0739 itself. The potency to interact with phosphoproteins opens the door to the host cell's signaling network. It would be very interesting to identify the host cell interaction partners.

The presence of an extended DXD motif could imply some functional homology with clostridial and chlamydial cytotoxins (23, 33). The morphologies of host cells upon

heavy *C. pneumoniae* infection resemble the reported cell morphologies of cells infected with *C. trachomatis* and *Clostridium sordellii* (23, 33). Secretion of a cytotoxin-like effector as early as 20 hpi is surprising, since the reported antiapoptotic activities induced by chlamydial infection are induced in order to keep the host cell alive until the infection cycle is completed. However, the reported rounding morphology of host cells is visible only upon heavy chlamydial infection of the cells. This morphology could also occur upon metabolic crisis due to bacterial overload, which leads to necrosis and apoptosis of the host cell. Therefore a strong cytotoxic activity of this secreted effector is not probable.

Secretion of this multi-domain effector protein during early mid-cycle might also influence host cell cAMP-levels. The adenylate cyclase homology would fit into the picture of many other bacterial toxins and effector proteins with adenylate cyclase activity (98, 304). Elevation of the intracellular cAMP level usually leads to changes in cell morphology due to reorganization of the host cell cytoskeleton. Many signaling kinases are activated and regulated by cAMP. Another effect of adenylate cyclase toxins is tissue remodelling. Cells then loose contact to each other within a tissue, due to cytoskeletal alterations (53). Maybe Chlamydia use such an effect in order to reach epithelium-underlying tissue for infection of deeper cell layers.

An adenylate cyclase signaling pathway is involved in the cAMP-dependent regulation of the expression of T3SS genes in *P. aeruginosa* (300). Such a regulatory mechanism could also exist in Chlamydia, since I previously hypothesized that several regulatory and sensory mechanisms must exist, which regulate secretion of the different groups of type III effectors in Chlamydia. Maybe cAMP is one such signal. In this case secretion of an effector in midcycle might pave the road and start the signal for the secretion of the next group of effector proteins, being secreted more than 20 hours later. However, it is improbable that cAMP levels elicited by an adenylate cyclase, even with low activity, would rise so slowly that a certain threshold would be reached many hours later.

Cytotoxicity due to the DXD-like domain as well as due to cAMP levels would occur too early at 20 hpi and microscopic observations do not reveal such extreme toxic and remodeling processes at that time. Another possibility, also discussed by Cooper and others is, that these signaling events not necessarily have to occur within the whole

cytoplasm. Cooper mentioned that elevated cAMP might only occur within certain cytosolic compartments and not affect the whole cell (53). This is an intriguing possibility. Having in mind that the host cell cytoplasm is not an aqueous ideal solution, it is possible that second messenger concentrations and thereby induced effects only affect local compartments, as, for example, the surrounding of the chlamydial inclusions. In this context it would make sense to hinder the respective effector proteins from floating free through the cytosol, but to bind and control them within membranes instead. This is a strategy followed by all living cells to compartmentalize reactive, toxic and apoptotic molecules. This would explain, why most secreted chlamydial effector proteins reported to date are secreted into or stay associated with the inclusion membrane.

6.9 CpB0856

This candidate was chosen due to its genetic locus amidst other T3SS genes. No homology or eukaryotic domain was found, which would have helped to investigate the putative function of this secreted effector protein. It must have some important function, however, since the whole T3SS gene cluster is reportedly conserved in Chlamydia and chlamydial ancestors since 700 million years (128).

6.10 CpB0730 (Pkn5)

6.10.1 Influence of Pkn5 secretion on host cell signal transduction

Verma *et al.* reported CT673, the *C. trachomatis* homologue of Pkn5, to be an inactive kinase (290). CT673 and Pkn5 share 41% amino acid sequence identity and both possess all Hanks domains, which are characteristic for STKs (111), except Hanks domain I. However, the absence of Hanks domain I is no absolute prerequisite for a kinase to be active (266). Importantly, the conserved arginine in Hanks domain XI is absent in CT673 but present in its *C. pneumoniae* counterpart Pkn5 (R²⁵⁷). Therefore, it was assumed that *C. pneumoniae* Pkn5 could be active although *C. trachomatis* CT673 was not. This showed not to be the case. *C. pneumoniae* Pkn5 is an inactive, kinase-like,

type III-secreted effector protein. However, also an inactive kinase could have an inhibitory effect on interacting host cell kinases, for example as a dominant-negative kinase by protein-protein interaction. Thereby phosphorylation of host cell proteins by host cell kinases or by other chlamydial kinases (290) could be inhibited. Through protein-protein interactions, host cell kinases also could be targeted to specific substrates, which would lead to the phosphorylation of otherwise unphosphorylated proteins. Thus, a secreted but inactive chlamydial kinase can both promote and inhibit the phosphorylation of host cell proteins, despite the lack of intrinsic kinase activity.

6.10.2 Putative targets of virulence factor CpB0730 (Pkn5) in the host cell

Although a measurable kinase activity of Pkn5 protein could not be detected, influences of this chlamydial effector protein on the phosphoproteome of the host cell were observed. Additionally, Pkn5 was phosphorylated in the C-terminal putative regulatory domain by host cell or chlamydial kinases. Pkn5 was found to inhibit the phosphorylation of two host cell proteins, namely a protein disulfide isomerase-related protein and the regulatory subunit alpha chain of type I protein kinase A (PKA). Furthermore, Rho GDP-dissociation inhibitor alpha protein was phosphorylated only in the presence of Pkn5. The same results were obtained for gain-of-function mutants with restored Hanks domain I. This further argues for an impact of Pkn5 on host cell protein phosphorylation via protein-protein interactions rather than via Pkn5 intrinsic kinase activity. The following paragraphs analyze the possible implications of the observed differential phosphorylations evoked by chlamydial Pkn5 effector protein.

6.10.3 Secreted Pkn5 interferes with host cell protein disulfide isomerase

Phosphorylation of members of the human protein disulfide isomerase (PDI) family has been reported (57, 64, 196, 254, 305), although the consequences thereof are not yet clear. Regulation of isomerase activity by phosphorylation is very probable. Inhibition of phosphorylation of the identified protein disulfide isomerase (PDI) by Pkn5 might block or activate changes in disulfide bonds within or in between host cell proteins. This can influence activity and folding of target proteins. Disulfide bonds in chlamydial proteins have been reported to be important for early steps in the infection process

(242). Host cell PDI has been implicated in inhibition of NF κ B activation and in the induction of cytokines upon LPS stimulation of host cells (124). It was assumed that PDI acts downstream of IL-10 in anti-inflammatory signaling. Since Thioredoxin also had an effect, the observed effects are probably redox-mediated (124). Therefore, the interference of a chlamydial virulence factor with host cell PDI activity makes biological sense. Although no NF κ B activation was detected upon infection, influencing the expression of anti-inflammatory cytokines would be beneficial for chlamydial survival within the host cell. *C. pneumoniae* was reported to induce IL-10 expression of the host cell, which contributes to host cell anti-apoptosis (93).

6.10.4 Secreted Pkn5 possibly leads to activation of PKA

Phosphorylation of the alpha regulatory chain of protein kinase A (PKA) occurred only in the absence of Pkn5. Observation of PKA phosphorylation was surprising at first glance, since PKA is known to be regulated by cAMP binding. However, additional regulation by phosphorylation of the alpha regulatory subunit has also been reported (161, 203). PKA is involved in multiple signaling pathways for example the regulation of expression of inducible nitric oxide (NO) synthase, IL-10 and CD14. NO was reported to play an important role in inflammatory lung damage (147). However, this effect seems to be mediated via NF κ B and an activation of NF κ B in infected cells could not be detected. CD14 is crucial for the induction of an inflammatory response in macrophages upon binding to bacterial LPS (181, 189). IL-10 is an important anti-inflammatory cytokine, which is reportedly upregulated in Salmonella-infected cells by PKA activation with a type III-secreted effector (287). This increase in IL-10 expression leads to better intramacrophage survival of Salmonella. As mentioned above, *C. pneumoniae* infection also induces IL-10 production in order to inhibit host cell apoptosis (93). The peak of IL-10 secretion was reported to occur between 24 and 48 hpi, which would perfectly fit to a secretion of Pkn5 around 20 hpi (63, 93). Therefore, interference with PKA signaling by means of a type III-secreted chlamydial effector makes sense.

6.10.5 Secreted Pkn5 possibly influences the host cell cytoskeleton

RhoGDI (Rho-family-specific GDP-dissociation inhibitor) forms a complex with Rho proteins in the cytosol of mammalian cells. RhoGDI thereby stabilizes the GDP-bound, inactive state of Rho GTPases (28). RhoGDI not only regulates guanine nucleotide binding to Rho proteins, but was also discussed as a molecular shuttle to carry Rho proteins from an inactive cytosolic pool to the membrane for activation (94). The stability of the Rho-RhoGDI protein complex reportedly is modulated by phosphorylation (30). Host cell Rho GDP-dissociation inhibitor alpha was phosphorylated only if Pkn5 was present in kinase reactions. As reported in the introduction, Rho, Rac and Cdc42 proteins are the targets of several bacterial toxins. Pathogenic bacteria thereby modulate the host cell cytoskeleton in their favor. Changes in the cytoskeleton also occur in train of chlamydial infection and an involvement of unknown type III effectors has been proposed (23, 36). A recent publication reported the importance of Rac GTPase for the infection of non-phagocytic cells with *C. trachomatis* (37).

An influence of chlamydial infection on the host cell signal transduction network already between five and 20 minutes post-infection has been reported (52, 164). Pkn5 secretion and therefore contact to the host cell cytoplasm not before 20 hpi seems to be too late to have an influence on these early signaling events. 20 hpi would also be too late for Pkn5 to be involved in chlamydial entry into the host cell. However, modulation of the host cell cytoskeleton could be important for the intracellular transport of inclusions to the perinuclear area. Since Pkn5 could be detected as early as 0.5 hpi in Western blots, a contribution of this putative virulence factor to host cell entry as well as an interference with other early signaling pathways cannot be excluded. At such early time points, observation of secretion was impossible due to the small size of Chlamydia and inclusions. Since a local administration of a Rho modulating protein would be sufficient to influence local cytoskeleton remodelling, secretion of Pkn5 at the sites of chlamydial attachment to the host cell is possible. This might explain the observed local polymerization of actin at the site of chlamydial attachment (36). In summary, I propose a model, where chlamydial type III-secreted effector Pkn5 induces phosphorylation of RhoGDI by a host cell kinase, which then leads to changes in the actin cytoskeleton.

Rho GTPase is not only involved in cytoskeleton remodelling, but also in the formation of superoxide during the oxidative burst in macrophages (154). Inhibition of the oxidative burst by inhibiting Rho with a secreted effector protein like Pkn5 would help Chlamydia to escape destruction by macrophages. This is necessary if one hypothesizes monocytes/macrophages to act as shuttle vectors mediating the transfer of live Chlamydia from the lung to the vasculature (188). This model was proposed to explain the presence of Chlamydia in atherosclerotic plaques.

Rho GTPases were recently reported to be involved in caspase-1 activation and apoptosis (259). This was reported in the context of immune-evasion mechanisms of Yersinia with help of the type III-secreted effectors YopE and YopT. Modulation of Rho activity by altering the stability of a Rho-RhoGDI complex might also influence host cell apoptosis during chlamydial infection.

As reported above, influences of Pkn5 on both protein disulfide isomerase and PKA could lead to antiapoptotic activity involving IL-10 signaling. Although interference with Rho GTPases is classically more discussed in context of cytoskeletal remodelling some interaction with apoptotic signaling was also reported. Therefore I conclude that the chlamydial type III effector Pkn5 possibly enables successful establishment of chlamydial infection in the intracellular niche by interference with the host cell cytoskeleton. Additionally, I propose that apoptosis of the host cell is inhibited by activating the IL-10 response via PKA and PDI.

6.10.6 Implications of the SUMOylation of Pkn5 with SUMO1

In this work it was shown that chlamydial protein CpB0730 (Pkn5) becomes SUMOylated specifically with SUMO1 by PIASx in an artificial system of transfected cells as well as during natural infection. Therefore, this posttranslational modification must play a role in chlamydial infection. Different effects of SUMOylation have been reported ((198), see introduction). Since Pkn5 upon its secretion at 20hpi is located within or attached to the inclusion membrane, SUMOylation might lead to its translocation to some other cellular compartment or protein complex. This has not been observed, however. Pkn5 was either identified within the chlamydial inclusions or secreted into the inclusion membrane. However, a translocation of SUMOylated Pkn5

to another cellular compartment cannot be excluded completely, since ICC only has a limited sensitivity. If the amount of translocated SUMO1-Pkn5 was too small, the identification by ICC might be impossible.

Another effect of SUMOylation of Pkn5 might be protein stability. However, the stability of SUMOylated and unSUMOylated Pkn5 protein was not compared. If stability was affected, I would assume that SUMOylation stabilizes rather than destabilizes chlamydial Pkn5 in order to prolong any beneficial effect this protein might have on chlamydial infection. SUMOylation of Pkn5 might also influence its activity. However, as it is reported here, no kinase activity attributable to Pkn5 could be found. Since nevertheless influences of Pkn5 on host cell protein phosphorylation could be identified, these phosphorylations must be carried out by other chlamydial or host cell kinases. Phosphorylations of host cell proteins could be activated or inhibited by interaction of active kinases with the putatively dominant negative kinase Pkn5. Therefore I propose a model where SUMOylation of Pkn5 by host cell PIASx changes its interactive surface enabling protein-protein interaction with other host cell proteins. These interactions must be beneficial for chlamydial infection in early mid-phase, the time frame during which Pkn5 is secreted. Type III effector proteins from other plant and mammalian pathogens have been described to interfere with the host cell's SUMOylation and the connected signal transduction network (131, 219). SUMOylation of an adenoviral oncoprotein, followed by its translocation into the nucleus, also has been reported (70). However, to date, SUMOylation of a secreted bacterial effector protein has not yet been reported.

6.11 SUMOylation of other secreted effector proteins

Other chlamydial effector proteins besides Pkn5 also display SUMOylation core sequences and therefore might be SUMOylated by inclusion-associated PIASx. CopN, CpB0733 and CpB0739 possess such SUMOylation sequences and might be modified. SUMOylation of CpB0733 is questionable however, since CpB0733 secretion could not be demonstrated and therefore contact to the host cell is not given. SUMOylation of CpB0739 might influence its putative adenylate cyclase and/or FHA-mediated phosphoprotein interaction activities.

6.12 CpMip (CpB0687)

6.12.1 CpMip probably is secreted by the chlamydial T3SS

The chlamydial Mip-homologue CpB0687 (cpMip) is the only candidate with a genetic locus outside of the four described T3SS gene clusters, which might imply a non-T3SS secretion. However, an effector, secreted in a type III-dependent manner, not necessarily has to be located within a T3SS cluster (69, 103, 112). Signal peptidase II leader sequences and detection of cleaved and uncleaved forms on Western blots have been reported for Mip proteins (159, 185, 246). Analysis of lpMip, ctMip and cpMip primary sequences with the Lipop 1.0 signal peptide prediction server from the Center for Biological Sequence Analysis of the Technical University of Denmark (www.cbs.dtu.dk/services/LipoP) resulted in the prediction of signal peptides for all Mip protein sequences. Signal peptidase II usually cleave off the signal peptide of proteins secreted by the Sec system (224). No double band could be observed on Western blots during this work indicating no cleavage of cpMip. Together with the observed secretion into the inclusion membrane, both indicates an alternative secretion of cpMip – e.g. by a T3SS.

6.12.2 Secretion and potential role of cpMip

Other studies reported cpMip and GPIC-Mip (Mip protein from GPIC, a *C. psittaci* strain causing guinea pig inclusion conjunctivitis) to be surface exposed immunodominant proteins found on EBs and RBs (210, 246). It was demonstrated in this work that the Mip-homologue CpB0687 (cpMip) is a secreted effector protein of *C. pneumoniae*, which gives cpMip access to the host cell cytoplasm. The ICC pictures for cpMip resemble those of Fields *et al.* for IncC, which is also an inclusion membrane protein (78). These findings allow the conclusion that the substrate protein(s) of cpMip might either be other chlamydial proteins/virulence factors in the inclusion membrane or host cell proteins whose conformation could be altered by the cpMip intrinsic peptidyl prolyl cis-trans isomerase (PPIase) activity. Human FKBP fulfil basic cellular processes such as protein folding and trafficking. In analogy to human FKBP, secreted chlamydial Mip protein could have various effects on host cell protein folding, signaling

and targeting. FK506 binding proteins with PPIase activity have also been implicated in the protection of proteins from denaturation as well as in chaperone-like activities (138, 222). Therefore, one might hypothesize that cpMip within the inclusion membrane protects other secreted chlamydial effectors from misfolding or damage. Although the Mip substrate proteins have not yet been identified, Mip function seems to be essential for the progress of chlamydial infection. This can be concluded from the finding that inhibition of the PPIase activity of cpMip with FK506 leads to arrest or at least inhibition of the infection.

CpMip protein was expressed throughout the infection cycle, but secretion only occurred between 20 and 72 hpi. Any proposed function of cpMip in chlamydial infection must occur within this time frame. One could imagine this putative virulence factor to play a role in the inhibition of endosome-lysosome fusion, which saves Chlamydia from destruction by host cell lysosomes. However, inhibition of this fusion takes place even before 20 hpi (107). Therefore participation of cpMip in this process can be excluded. Since infections, inhibited by FK506, displayed many tiny instead of one or two bigger inclusions, it is possible that cpMip contributes to the fusion of inclusions. This fusion process might be important in order to better coordinate the infection progress between Chlamydia. As it was reported in this work, a coordinated secretion between different inclusions within one host cell seems not to occur (Figure 22). Therefore the fusion of inclusions might be an essential step in chlamydial infection, which could be blocked by FK506 inhibition of cpMip activity. Furthermore, an involvement of cpMip activity in antiapoptosis or other processes, beneficial for chlamydial infection, is thinkable.

The findings that macrolides like FK506 interfere with chlamydial infectivity and the inhibition of Mip PPIase activity by these drugs (106, 183, 185, 246), turn this protein into one of the prime candidates for a virulence factor of *C. pneumoniae*.

6.12.3 Inhibition of cpMip might induce persistence

An inhibiting effect of FK506 on *C. pneumoniae* infection could be demonstrated in this work, which corroborated the results of Lundemose *et al.* for *C. trachomatis* (185). Inclusions remained small and stopped to grow and develop (Figure 41). One possible

explanation, the involvement of chlamydial Mip in vesicle fusion of small inclusions at the beginning of the infection cycle, was excluded above. The abnormal morphology of chlamydial inclusions after FK506 treatment resembled that of inclusions during persistent infections (228). Persistence can be induced by various methods (4, 20, 21, 228, 277). Persistence is induced experimentally by depriving chlamydia-infected cells of tryptophan or iron or simply by inhibiting chlamydial growth by addition of antibiotics, which are not able to eradicate Chlamydia. Chlamydial inclusions stay small and the ultrastructural morphology of Chlamydia within the inclusions as well as their metabolism changes. It would therefore be possible to clarify the possibility of FK506-induced persistence by taking electron micrographs of FK506-treated chlamydial infections. Another way to pursue this question would be to test Chlamydia from FK506-treated infected cell cultures for their ability to reinfect cells. This reinfection potency is restricted or absent in persistent infections. CpMip must be involved in some essential process, inhibition of which disables chlamydial infection to progress normally. The observation that inhibition of cpMip peptidyl prolyl cis-trans isomerase (PPIase) activity by FK506 possibly induces persistence earns further investigation but so far cannot be explained mechanistically.

6.13 FK506 as potential lead structure for the development of anti-chlamydial antimicrobials

The T3SS as conserved, common and virulence-associated structure of many human pathogens is an attractive target for the development of antimicrobials. Indeed, such attempts have been made and discussed already (149, 150). Resulting antibiotics in this case most probably would be broad-spectrum antibiotics able to hit all bacteria, which express and use a T3SS. Since these are mainly pathogens, this might nevertheless be an interesting worthwhile approach. However, since a putative virulence factor with enzymatic activity was identified, which seems to be essential for chlamydial infection, the development of Chlamydia-specific antimicrobials starting from FK506 as lead molecule is proposed.

The capacity of FK506-derived SMOLS greatly depends on the possibility to break down the big biomolecule into smaller substructures with independent activities. Any FK506-derived antichlamydial antibiotic must be devoid of immunosuppressive quality and of cytotoxic side effects. This seems feasible if one has a look on the molecular regions of this huge molecule (Figure 6). I compared the structures of FK506 and Rapamycin and assessed the common encircled substructure as possibly potent substructure, which could be used as lead for further drug development. The identified N-acyl piperidine carbonate substructure should be devoid of the unwanted immunosuppressive and cytotoxic effects, since it is structurally separated from the Calcineurin-binding and from the FKBP-12 binding region (Figure 6). FK506 and Rapamycin are strongly related molecules and bind to FKBP's whereas Cyclosporin A binds Cyclophilins. Both complexes, however, have immunosuppressive effects. The lack of this substructure in Cyclosporin A might be a hint that this substructure is not involved in immunosuppression (Figure 43).

Inhibition of PPIases and immunosuppressive activity were described to be separate effects of FK506, Rapamycin and Cyclosporin A (25, 163, 268). Additionally it has been reported that neither FKBP nor FK506 nor Rapamycin alone act immunosuppressive, but only the drug-protein complexes (163, 180). Therefore, the synthesis of an antichlamydial non-immunosuppressive molecule with FK506 piperidine ring origin seems to be feasible.

This FK506 substructure strongly resembles a prolyl amino acid residue with peptide bonds, which might explain the affinity to PPIases (Figure 43). The circular conformation of FK506 and Rapamycin molecules is remarkable, and might result in low steric flexibility of the prolyl mimicking part (Figure 43). It would have to be assessed whether the prolyl-substructure itself is active as cpMip inhibitor or if the surrounding molecule with its more rigid conformation is essential for the inhibitory activity. The identified ring structure probably is sufficiently stable and not difficult to synthesize. Therefore enzymatic tests with the purified enzyme as well as inhibition experiments with infected cell cultures could be performed without big efforts in chemical synthesis. With the described PPIase assay (16, 87, 142, 185), an adaptable assay system would already be at hand in order to test for candidate substances.

As mentioned in the introduction, the current clinical problem is a lack of antibiotics, which are specific and potent enough to eradicate chlamydial infections in acute but most importantly in persistent state. If persistence and not inhibition of chlamydial infection progress would be the explanation for the observed small inclusions (which can be tested as described above), I would not propose to use this FK506 substructure as lead for antichlamydial antimicrobial development.

The concentrations of FK506 used in transplantation medicine were reported to be in a range, which would only result in 10% inhibition of PPIase activity (163, 268). Therefore, a new FK506-derived antichlamydial compound should have increased affinity and activity towards the chlamydial PPIase compared to FK506. Care has to be taken when designing new compounds for cpMip inhibition. The goal is to maximally inhibit the chlamydial but not the human FKBP, which might have similar molecular structures and binding pockets. Binding of a new substance to human FKBP might be one source of side effects.

In summary, the FK506-derived N-acyl piperidine carbonate is a promising lead candidate, which could easily be synthesized, tested and chemically modified. Its homology to prolyl residues indicates, that this could indeed be the active structure within the huge FK506 molecule, responsible for the inhibition of cpMip PPIase activity. Therefore, it would be a worthwhile attempt to begin antichlamydial drug development by starting from this structure.

7 CONCLUSION

The aim of this study was to identify effector proteins that are secreted by the T3SS of *C. pneumoniae* and to study their effects on the host cell. The knowledge gained from these host-pathogen interaction studies should be used to extrapolate potential sites of interference for the development of Chlamydia-specific antimicrobials.

Several secreted chlamydial effector proteins have been identified. The characteristics of these effectors strongly suggest a type III-dependent mechanism of these putative virulence factors. Secretion was shown to occur in steps with different groups of effectors being secreted at different times of the infection cycle. A model, how this stepwise secretion could be regulated and coordinated within the host cell between inclusions as well as within the inclusions was proposed. The observation of independent regulation of secretion in inclusions within one host cell leads to the conclusion, that each inclusion individually creates its own microenvironment. This can be explained by secretion of effectors into the inclusion membrane, which does not allow for diffusion of effectors through the cytosol, but rather keeps the effectors and the thereby evoked responses in close proximity of the inclusion.

The potential to interfere with host cell signaling has been evaluated for two of the effectors, Pkn5 (CpB0730) and cpMip (CpB0687), in closer detail. The overall picture of hypothesized and observed activities of the identified effectors fits into the strategy of an intracellular pathogen like *C. pneumoniae* to survive within a host cell in a secure niche. The identified effectors probably lead to the upregulation of IL-10 expression, which protects host cells from apoptosis. A contribution of NF κ B or of STAT1, STAT2 or STAT3 was excluded. A second coordinated activity of the identified effectors seems to be the remodelling of the host cell cytoskeleton by modulating Rho protein activity. Both Pkn5 and CpB0739 effector proteins have the potency to contribute to this remodelling. Pkn5 probably influences Rho activity by phosphorylating RhoGDI protein and CpB0739 has an adenylate cyclase motif, which might influence cAMP levels and thereby also host cell cytoskeletal remodelling. This could explain the

mechanism of the observed localization of chlamydial inclusions in close proximity of the host cell nucleus. Possibly the involved effectors also contribute to the entering process of Chlamydia into the host cell. These effects on the host cell upon chlamydial infection have been reported but attribution of these processes to certain secreted chlamydial type III effectors has not yet been possible, since the participating effectors were unknown.

8 OUTLOOK

Now it is possible to make the link between chlamydial effectors and observed effects in the host cell. Almost every identified secreted effector protein has great potential for ongoing research activities to further study the activities on host cell signaling in detail. Chlamydia still cannot be genetically modified. Therefore investigating the secretion and the activities of the here presented effector proteins in other type III secreting pathogens, which are accessible with molecular genetic methods, would be interesting. The increased knowledge of pathogen-host interaction together with the proposed SMOL lead structure could now be used to develop specific chlamydial antibiotics. Development of such antibiotics with the potency to eradicate also persistent chlamydial infections is of great clinical importance. This work might be one step into this direction.

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