Chlamyphila pneumoniae HflX belongs to an uncharacterized family of conserved GTPases and associates with the Escherichia coli 50S large ribosomal subunit

Adam Polkinghorne,1 Urs Ziegler,2 Yanela González-Hernández,3 Andreas Pospischil,1 Peter Timms4 and Lloyd Vaughan1

1 Institute of Veterinary Pathology, University of Zurich, Winterthurerstrasse 268, Zurich 8057, Switzerland
2 Institute of Anatomy and Center for Microscopy and Image Analysis, University of Zurich, Winterthurerstrasse 190, Zurich 8057, Switzerland
3 Institute of Organic Chemistry, ETH Hönggerberg, Zurich 8093, Switzerland
4 Institute of Health and Biomedical Innovation and School of Life Sciences, Faculty of Science, Queensland University of Technology, Brisbane, Australia

Predicted members of the HflX subfamily of phosphate-binding-loop guanosine triphosphatases (GTPases) are widely distributed in the bacterial kingdom but remain virtually uncharacterized. In an attempt to understand mechanisms used for regulation of growth and development in the chlamydiae, obligate intracellular and developmentally complex bacteria, we have begun investigations into chlamydial GTPases; we report here what appears to be the first analysis of a HflX family GTPase using a predicted homologue from Chlamyphila pneumoniae. In agreement with phylogenetic predictions for members of this GTPase family, purified recombinant Cp. pneumoniae HflX was specific for guanine nucleotides and exhibited a slow intrinsic GTPase activity when incubated with \([^{32}\text{P} ]\text{GTP}\). Using HflX-specific monoclonal antibodies, HflX could be detected by Western blotting and high-resolution confocal microscopy throughout the vegetative growth cycle of Cp. pneumoniae and, at early time points, appeared to partly localize to the membrane. Ectopic expression of Cp. pneumoniae HflX in Escherichia coli revealed co-sedimentation of HflX with the E. coli 50S large ribosomal subunit. The results of this work open up some intriguing possibilities for the role of GTPases belonging to this previously uncharacterized family of bacterial GTPases. Ribosome association is a feature shared by other important conserved GTPase families and more detailed investigations will be required to delineate the role of HflX in bacterial ribosome function.

INTRODUCTION

Members of the phosphate-binding-loop (p-loop) guanosine triphosphatase (GTPase) superfamily are widely conserved and serve crucial roles in signal transduction in all living organisms. The signalling functions of these GTPases are mediated by conformational changes to the GTPase in response to GTP binding, followed by hydrolysis and release of bound GDP, which resets the protein (Bourne et al., 1991). These biochemical properties are conferred to members of the GTPase superfamily by the presence of a characteristic and structurally invariant G-domain. This domain contains conserved sequence motifs (G1, G3, G4) which are responsible for binding and hydrolysing GTP. The more variable effector interaction motif (G2) changes its conformation based on the nucleotide bound state of the GTPase (Bourne et al., 1991; Sprang, 1997).

In bacteria, at least eight GTPase subfamilies (HflX, YihA, YchF, Obg, TrmE, EngA, YjeQ, Era) have been identified (Brown, 2005). A variety of biological processes such as tRNA modification, ribosome biogenesis, DNA replication, chromosome partitioning and stress response regulation have been linked to GTPase function in bacteria (Brown, 2005; Caldon & March, 2003). Amongst these, a common theme emerging is the ability of bacterial-encoded p-loop

**Abbreviations**: CLSM, confocal laser scanning microscope/microscopy; EB, elementary body; GST, glutathione S-transferase; HRP, horseradish peroxidase; p-loop, phosphate-binding loop; PI, post-infection; RB, reticulate body.
GTPases to interact directly with the ribosome (Caldon et al., 2001).

Members of the HflX GTPase family are the last to remain uncharacterized in bacteria. Phylogenetic analysis of HflX family members has revealed a wide distribution and conservation, not only amongst nearly all bacterial species, but also in eukarya and archaea (Leipe et al., 2002). Deletion of the hflX gene in Escherichia coli is not lethal (Gerdes et al., 2003) which, in part, would explain why HflX family members are yet to be examined in detail. A number of transcriptional and genetic studies have hypothesized that HflX may be involved in regulation of proteolysis, based on its presence in an operon with other proteolysis regulatory genes in E. coli (Noble et al., 1993) and its co-regulation with proteolysis genes (Engels et al., 2005) in Corynebacterium glutamicum, although no direct experimental evidence exists.

Our interest in members of this GTPase family developed from observations that the genes encoding hflX and other predicted GTPases in Chlamydophila pneumoniae were differentially transcribed under stress conditions (Belland et al., 2006). Chlamydiae are obligate intracellular bacteria and pathogens of humans and a variety of animal species. Under stress conditions, intracellular vegetative chlamydial forms known as reticulate bodies (RBs) convert to morphologically distinct 'persistent' forms, which are inhibited in their ability to replicate by binary fission and to differentiate into infectious extracellular chlamydial forms known as elementary bodies (EBs) (Koehler et al., 1997; Kutlin et al., 2001). Understanding the molecular mechanisms used to detect stress signals and to trigger these morphological changes is of significant interest to this field since persistent chlamydial infections have been associated with the generation or exacerbation of a range of chronic sequelae including trachoma (Gambhir et al., 2007), tubal scarring (Debattista et al., 2003), arthritis (Silveira et al., 1993), coronary artery disease (Campbell & Kuo, 2004) and late-onset Alzheimer's disease (Gérard et al., 2006). Unfortunately, the fundamental molecular mechanisms utilized to regulate chlamydial growth and development are still poorly understood due to the resistance of these organisms to genetic manipulation and the lack of a host-free growth system.

Here we describe what appears to be the first functional analysis of a member of the HflX family of conserved bacterial GTPases using a HflX gene homologue from C. pneumoniae. Despite the inherent difficulties of working with this obligate intracellular bacterium, we demonstrate that the hflX gene locus encodes a functional GTPase with a slow GTPase hydrolysis ability and specificity for guanine nucleotides. Furthermore, we provide evidence to suggest that HflX GTPases associate with the bacterial ribosome, a feature which is consistent with the function of other important evolutionarily conserved GTPases and may prompt further interest in members of this previously uncharacterized family of signal transduction proteins.

**METHODS**

**Bacterial strains and plasmids.** Escherichia coli strains DH5α and BL21(DE3) were used for molecular cloning and protein expression and purification, respectively (Hanahan, 1983; Studier et al., 1990). E. coli K-12 wild-type strain MG1655 (Blattner et al., 1997) was used for ribosome co-fractionation experiments. Chlamydia pneumoniae strain CWL029 was used for in vitro infection of Hep-2 cell monolayers and HflX protein expression measurements (Grayston et al., 1986).

**E. coli growth conditions.** Unless otherwise stated, E. coli strains transformed with expression constructs were grown in a waterbath shaker in Luria–Bertani broth (LB; 10 g tryptone, 5 g yeast extract, 10 g NaCl l⁻¹) at 37 °C with constant shaking. When necessary, ampicillin was used at a concentration of 75 μg ml⁻¹. Cell growth in liquid medium was assessed by measuring OD₆₀₀.

**Infection and propagation of C. pneumoniae strain CWL029.** Hep-2 cells (ATCC CCL-23) were maintained in minimal essential medium (MEM; Invitrogen), including Earle's salts and 25 mM HEPES, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% (v/v) non-essential amino acids. Prior to infection with C. pneumoniae, Hep-2 cells in 75 cm² cell culture flasks were transferred to MEM without supplements. C. pneumoniae at an estimated m.o.i. of 2–5 were centrifuged onto the Hep-2 cell layers at 1000 g for 1 h at room temperature. Non-adhering C. pneumoniae were removed by rinsing once with MEM and then MEM plus supplements was added and the infected cell layers were incubated at 37 °C in humidified cell culture chambers with 5% CO₂. Under these conditions, C. pneumoniae CWL029 progresses through its full developmental cycle in approximately 72 h, at which point inclusions rupture and EBs are released into the culture medium. For microscopy, Hep-2 cells were cultivated on sterile glass coverslips in 24-well plates.

**Cloning and expression of C. pneumoniae HflX.** Primers used for PCR amplification of HflX and modification are summarized in Table 1. Cloning and expression of recombinant chlamydial HflX for in vitro enzyme assays and antigen production was performed using

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**Table 1. Oligonucleotides used for PCR amplification and modification of C. pneumoniae HflX**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)*</th>
</tr>
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<tbody>
<tr>
<td>HflX-F</td>
<td>TTTTCTCGAGTGGACACCTATAAGGATAGGCACCTGCTGAAGATTTAAC</td>
</tr>
<tr>
<td>HflX-R</td>
<td>CCCGGTACCCTTACCCGCCGAAAGACTCTC</td>
</tr>
<tr>
<td>HflXN-R</td>
<td>CCCGGTACCCTTACCCGCCGAAAGACTCTC</td>
</tr>
<tr>
<td>HflX-F</td>
<td>TTTTCTCGAGATTCCATTATACACAGT</td>
</tr>
<tr>
<td>N349Y-B</td>
<td>CGATCTCACCTTTAGATAGCAGA</td>
</tr>
<tr>
<td>T263A-C</td>
<td>GCCATGGACGACTCTAGTAGC</td>
</tr>
<tr>
<td>S243N-B</td>
<td>ATTAGTAAAGGGTGGTTCCT</td>
</tr>
<tr>
<td>S243N-C</td>
<td>GGAAGAACAGCCTTAATTAAT</td>
</tr>
</tbody>
</table>

*Modified nucleotides are in bold and the altered codons are underlined.
an N-terminal glutathione S-transferase fusion (GST) expression vector system (pGEX; Amersham Biosciences). Initially, pGEX4T2 expression vector was modified following digestion with BamHI and NotI and ligation with insert (5′-GATCCCGGAGAATTCGTGATGGTACCGC-3′) to create a new vector pGEX4T2A with Xhol/Kpn restriction sites in the multiple cloning site. The C. pneumoniae hflX ORF was amplified from C. pneumoniae CWL029 DNA using primers HflX-F and HflX-R to generate a PCR product with 5′-Xhol and 3′-Kpn restriction sites, respectively. Following Xhol and Kpn double digestion, the HflX ORF was ligated using T4 DNA ligase into pGEX4T2A to generate the GST expression vector pAP1, which was double digestion, the HflX ORF was ligated using T4 DNA ligase into pGEX4T2A to generate the GST expression vector pAP1, which was transformed into E. coli BL21(DE3). Transformed cells were grown at 30 °C in 500 ml LB with 75 μg ampicillin ml⁻¹. At an OD₆₀₀ of 0.6, GST fusion protein expression was induced by the addition of 1 mM IPTG for 3 h. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris/HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF and 10 μg pepstatin A ml⁻¹). Cells were lysed by sonication on ice and cell debris and unbroken cells were removed by centrifugation at 15,000 g for 30 min. Supernatant was incubated for 1 h at 4 °C with washed glutathione-Sepharose 4B resin (Amersham Biosciences) with gentle mixing. The beads were then centrifuged at 500 g for 5 min, washed 10 times with washing buffer (50 mM Tris/HCl pH 7.5, 1% Triton X-100) and stored in GST-purify buffer. Recombinant protein yield was determined by Bradford assay using a BSA standard curve as a control. Purified protein samples were resolved on 10% SDS-PAGE gels and detected following staining with Coomassie blue or via Western blotting (after transfer to nitrocellulose) with a mouse anti-GST antibody (0.1 μg ml⁻¹) coupled to horseradish peroxidase (HRP) (Amersham Biosciences).

For E. coli ribosome fractionation experiments, the HflX reading frame was excised by Xhol and Kpn1 double digestion from pAP1 and ligated to a similarly digested and modified N-terminal His-tag expression vector pQE80LAP to make pAP2. pQE80LAP was created by digestion of the pQE80L expression vector (Qiagen) with SacI and PstI and ligation with insert (5′-GTCGACAAATTCTACGTATGG TACC-3′) to form a new vector with SacI and Kpn1 restriction sites in the multiple cloning site allowing direct subcloning from pGEX4T2A expression constructs (SacI digestion products share the same restriction ends as Xhol). All pQE80LAP constructs were transformed into E. coli BL21 and MG1655 for confirmation of protein overexpression. Protein expression was induced by addition of 1 mM IPTG and was detected by Western blotting with a 0.5 μg ml⁻¹ anti-His monoclonal antibody (Invitrogen) and secondary detection with a goat anti-mouse antibody coupled to HRP (Geno Technology).

Modification and mutagenesis of C. pneumoniae HflX. For more detailed analysis of HflX co-sedimentation with the E. coli ribosome, the two domains of C. pneumoniae HflX were separately cloned into pQE80LAP. pAP3, representing the N-terminal domain of HflX (residues 1–228), was prepared by PCR amplification of pAP1 using primers HflX-F and HflX-N and double digestion with Xhol/Kpn1. The resulting insert was cloned into SalI/Kpn1-digested pQE80LAP. The C-terminal GTase domain (residues 229–472) was prepared by PCR amplification of pAP1 with primers HflX-F and HflX-R and Xhol and Kpn1 digestion, followed by ligation into pQE80LAP to make construct pAP4.

Several mutations (S243N, T263A, N349Y) were also introduced into recombinant C. pneumoniae HflX, for analysis and use as controls in in vitro assays, by PCR. To generate site-specific mutations, HflX-F and HflX-R primers were used in combination with the respective reverse (e.g. S243N-B) or forward (e.g. S243N-C) primers in individual reactions to generate two overlapping fragments with modified HflX sequences. These fragments were then combined by annealing the two fragments and amplified in a PCR using the HflX-F and HflX-R primers. Both insert and pGEX4T2A vectors were then treated by Xhol/Kpn1 double digestion and ligated together using T4 DNA ligase. The correct substitution of nucleotides to generate the respective mutants was confirmed by DNA sequencing of the purified constructs.

Monoclonal antibody production. The generation of monoclonal antibodies directed against HflX was performed largely as described by Ferber et al. (1999). In brief, NMRI mice were immunized with Freund’s complete adjuvant for priming, and Freund’s incomplete adjuvant for the subsequent three to five boosts; 20 μg purified GST-HflX antigen was applied for each inoculation and boosting. Harvested splenocytes were fused with P3X63-Ag8.653 myeloma cells using polyethylene glycol (PE 1500, Roche Diagnostics) and the resulting hybridomas were raised in Iscove’s modified Dulbecco’s medium supplemented with 2% interleukin-6-enriched supernatant, 5% fetal bovine serum and gentamicin. Interleukin-6-enriched medium was obtained from the cell line X63161, kindly provided by Ton Rolink from the Institute of Developmental and Molecular Immunology, Basel, Switzerland. Supernatants from clones were selected by ELISA screening using an alkaline-phosphatase-labelled secondary antibody. The microtitre plates were coated with 0.3 μg per well (100 μl) of the antigen used for immunization. The supernatants of the immune-reactive clones were further tested for their suitability both in Western blots and by immunofluorescence staining of E. coli BL21 cells overexpressing C. pneumoniae HflX and of Hep-2 cell monolayers infected with C. pneumoniae CWL029. A number of clones were obtained using this approach and two of these, HflXcCW-7 and HflXcCW-21, were selected for HflX protein detection. Monoclonal antibodies were purified from conditioned hybridoma culture medium by affinity purification over Gamma-bind Sepharose (Amersham Biosciences) according to the manufacturer’s instructions.

Microscopy. Inoculated Hep-2 cells grown on glass coverslips were fixed with ice-cold 4% paraformaldehyde in PBS for 4 min at 4 °C. Following a brief rinse with cold PBS, they were treated with −20 °C methanol for 2 min followed by two wash steps with PBS and transfer to blocking buffer (3% BSA in PBS) for 1 h at room temperature. After incubation in 0.1% Triton X-100 in PBS for 2 min, two additional wash steps in blocking buffer were performed. HflX was detected by incubation with mouse monoclonal antibodies (1–5 μg ml⁻¹) in blocking buffer for 1 h at room temperature followed by five washing steps in blocking buffer, then labelling with goat anti-mouse Alexa-594 antibodies (Invitrogen) in blocking buffer at room temperature for 1 h. After a further five wash steps in blocking buffer, the coverslips were embedded in Fluoromount G (Interchim) and mounted on microscope slides. Chlamydiae were detected for co-localization purposes using a rabbit polyclonal anti-chlamydia antibody, diluted 1:200 in blocking buffer, which primarily recognizes chlamydial LPS and major outer-membrane protein antigens (Cygnus Technologies). The secondary antibody used was goat anti-rabbit Alexa-488 (Invitrogen).

Microscopy was performed with a confocal laser scanning microscope (CLSM; Leica TCS SP5, Leica Microsystems). Alexa-488 and Alexa-594 were excited with the 488 nm and 594 nm laser lines respectively, with emission signals collected between 493–570 nm and 605–670 nm. 3D image stacks were collected sequentially (to prevent green–red channel cross-talk) according to Nyquist criteria and prepared for publication using Imaris (Bitplane, Zurich, Switzerland). To obtain maximal possible resolution, 3D image stacks were deconvolved using Huygens (SVI, Netherlands).

In vitro GTase assays. GTase activity was determined by release of free inorganic phosphate from [γ⁻³²P]GTP as described by Hwang & Inouye (2001). Briefly, reactions were carried out in a 50 μl mixture...
of 50 mM Tris/HCl (pH 7.5) containing 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 2.5 μM GTP, 20 nM [γ-32P]GTP (185 TBq mmol⁻¹; Amersham Biosciences) and 50 pmol purified recombinant proteins at 37 °C. Reactions were terminated at different times by addition of 200 μl 6 % active charcoal. The mixture was incubated for 2 min at room temperature and 70 μl supernatant, obtained by centrifugation, was used for measuring the free inorganic phosphate by Cerenkov counting. Reactions were performed in triplicate.

UV cross-linking experiments. The ability of HflX mutants to bind GTP was analysed using cross-linking experiments, adapted from a previously described protocol (Lin et al., 1999). Purified GST fusion proteins (50 pmol), attached to glutathione-Sepharose, were incubated on ice for 15 min with 1 pmol [γ-32P]GTP (111 TBq mmol⁻¹; Amersham Biosciences) in 50 μl binding buffer [50 mM Tris/HCl (pH 8.0) containing 5 mM ATP, 100 mM KCl, 10 mM MgCl₂, 2 mM DTT, glycerol (10 % w/v)]. Bound [γ-32P]GTP was then cross-linked by UV treatment (254 nm, 1 J cm⁻²). Protein–nucleotide complexes were quantified following repeated washing of the beads with 450 μl binding buffer and measurement of radioactivity using Cerenkov counting. Reactions were performed in triplicate.

Preparation of E. coli cell lysates for ribosome co-fractionation experiments. Preparation and fractionation of E. coli ribosomes were performed effectively as described by Jiang et al. (2006). Briefly, overnight cultures of E. coli MG1655 containing constructs pQEAP2–AP4 were diluted into 100 ml LB and grown to an OD₆₀₀ of 0.1 before 250 μM IPTG was added to induce protein expression. Chloramphenicol was added to a final concentration of 200 μg ml⁻¹ 30 s before harvesting of cells. Cells were harvested at an OD₆₀₀ between 0.5 and 0.8 by centrifugation at 10,000 g 4 °C. The lysate was cleared by centrifugation at 30,000 g 4 °C and an SS-34 rotor (Sorvall). Cell pellets were resuspended in 1 ml lysis buffer (10 mM Tris/HCl pH 7.5, 10 mM MgCl₂, 100 μg chloramphenicol ml⁻¹), mixed with an equal volume of glass beads (300 μm; Sigma) and vortexed for 5 min at 4 °C. The lysate was cleared by centrifugation at 30,000 g 4 °C in an SS-34 rotor (Sorvall). The supernatant was collected and quantified by UV absorbance at 260 nm.

Ribosome fractionation. Solutions of 30 % and 5 % sucrose were prepared in buffer 1 (10 mM Tris/HCl pH 7.5, 10 mM MgCl₂, 100 mM NH₄Cl) and used to form 5–30 % sucrose gradients with a conserved region (residues 128–164) containing numerous variations (Leipe et al., 2002). The predicted G2 domain (Fig. 1) shows strong conservation of features characteristic for members of the GTPase superclass, including: (i) the Walker A motif (GxxxxGKST; G1), required for positioning of the bound nucleotide; (ii) the Walker B motif (DxxG; G3), necessary for stabilization of the γ-phosphate; and (iii) the NxxD motif (G4), which provides guanine specificity (Fig. 1; Bourse et al., 1991; Leipe et al., 2002). The predicted G2 domain (Fig. 1) appears also to be strongly conserved within HflX subfamily members and includes a distinct phenylalanine residue (residue 261 in Cp. pneumoniae HflX) which is shared by members of the Obg subfamily of p-loop GTPases (Leipe et al., 2002).

In vitro assay of recombinant Cp. pneumoniae HflX activity

The ability to bind GTP and hydrolyse this nucleotide to GDP is central to the function of GTPases as molecular switches. GTPase assays were performed by incubating the purified GTPase with [γ-32P]GTP. The initial GTPase hydrolysis reaction rate for GST-HflX over the first 5 min was low (2.1 ± 0.4 pmol min⁻¹). Virtually no 32Pi was released when GST-HflX was replaced with equimolar amounts of GST-only control, even over longer time periods (Fig. 2a). To determine the nucleotide specificity of Cp. pneumoniae HflX, competition experiments were performed.
Fig. 1. Alignment of C. pneumoniae HflX with other predicted HflX subfamily GTPase orthologues. Identical residues and those sharing similar physicochemical properties across all (black) or most (grey) predicted proteins are indicated by shading. Gaps are indicated by dashes. G1, G2, G3, and G4 motifs are indicated above the alignment in blue. Purple arrows indicate residues selected for mutagenesis of C. pneumoniae HflX. Red asterisks denote glycine residues of the C. pneumoniae HflX glycine-rich segment. The N-terminal and C-terminal domains selected for cloning are indicated with red and green arrows, respectively.

A ribosome-associated GTPase, HflX, in Chlamydia

carried out with other nucleotides (Fig. 2b). In the presence of 1.25 mM unlabelled GTP and GDP (500-fold excess), the rate of \[^{32}\text{P}\]GTP hydrolysis was markedly reduced. GMP, ATP, ADP, AMP, UTP and CTP at the same concentration were not inhibitory. These data support the prediction of chlamydial HflX as a GTPase and demonstrate the guanine nucleotide specificity of this enzyme.
In vitro analysis of \textit{Cp. pneumoniae} HflX mutant proteins

To confirm that the activity of recombinant \textit{Cp. pneumoniae} HflX is not associated with contamination of other co-purifying \textit{E. coli} proteins, we constructed a series of mutants targeting the conserved G motifs responsible for GTP binding and hydrolysis. Amino acids selected for this analysis (Fig. 1) are analogous to well-described mutations used to study other eukaryotic and prokaryotic GTPases and include: (i) Walker A mutants such as S243N, comparable to the S17 residue in Ras-like GTPases, which is critical for GTP-binding activity (John et al., 1993); (ii) T263A, involving modification of a G2 motif threonine residue, important for GTP binding and hydrolysis (Pai et al., 1990); and (iii) N349Y, a G4 motif mutant that modifies an essential asparagine residue (Clanton et al., 1986). Assay of the GTPase hydrolysis of these mutants failed to detect any release of inorganic phosphate, compared to the wild-type \textit{Cp. pneumoniae} HflX control (Fig. 2a), confirming that the GTPase activity observed in our earlier assays was from our purified recombinant protein.

To confirm whether these phenotypes were due to a loss of GTPase activity or were associated with an inability to bind GTP, a UV-cross-linking approach was used to examine the HflX GTPase mutants. GST-tagged HflX and mutants, bound to glutathione-Sepharose beads, were incubated with [$\alpha$-32P]GTP and exposed to UV irradiation, before washing with GTP-binding buffer and detection of radioactivity associated with the protein-bound bead samples (Fig. 2c). Relative to wild-type HflX, all the mutants bound [$\alpha$-32P]GTP weakly, as indicated by a 10-, 4- and 15-fold reduction in the radioactivity associated with the S243N, T263A and N349Y mutants, respectively.

HflX protein expression in \textit{Cp. pneumoniae}

To determine if \textit{Cp. pneumoniae} hflX encodes a protein of the expected size, a sample of predominantly \textit{Cp. pneumoniae} RBs from a 48 h post-infection (PI) culture was harvested by centrifugation over a renografin density gradient and subjected to SDS-PAGE for screening by immunoblotting with monoclonal antibody HflX Cpn-7. This analysis revealed a clear single band of $M_r$ 52,000, which is the predicted size of the full-length encoded HflX polypeptide (data not shown).

More detailed HflX expression analysis was performed on \textit{Cp. pneumoniae} CWL029-infected Hep-2 cell monolayers at 24, 48 and 72 h PI by CLSM (Fig. 3). HflX Cpn-7 was used for detection of \textit{Cp. pneumoniae} HflX and a commercial polyclonal anti-chlamydia antibody, recognizing the chlamydial LPS and membrane proteins, was used for co-localization purposes. Using these reagents, HflX staining could be detected through all time points of chlamydial growth investigated, although the spatial distribution appeared to change with time. Higher-magnification 3D image stacks collected at 24 h PI revealed that HflX could be specifically localized to small chlamydial inclusions containing two to eight RBs. Interestingly, when compared with signal observed with the polyclonal anti-\textit{Chlamydia} membrane antibodies, HflX staining appeared to partly co-localize to membrane structures, suggesting that at this time point, HflX may be partly membrane associated. At later time points, this association was less clear, although a part of the signal still showed a membrane association.

\textbf{Fig. 2.} Biochemical analysis of \textit{Cp. pneumoniae} HflX and mutants. (a) GTP hydrolysis activity of HflX. Reaction rates for HflX (■), a GST control (▲) and the three HflX mutants, S243N (△), T263A (□) and N349Y (○), are shown. (b) Relative GTPase activity of HflX in the presence of competitors. Reactions for HflX were performed for 30 min in the presence of unlabelled nucleotides (500-fold excess). The activity with [$\alpha$-32P]GTP alone was taken as 100%. (c) Relative GTP-binding activity of HflX and mutants. Proteins bound to glutathione-Sepharose beads were incubated in the presence of [$\alpha$-32P]GTP for 5 min on ice before washing and detection of radioactivity by Cerenkov counting. The activity of wild-type HflX with [$\alpha$-32P]GTP was taken as 100%. All reactions were performed in triplicate and the standard deviation from the mean is indicated.
Recombinant *Cp. pneumoniae* HflX co-fractionates with the *E. coli* 50S ribosomal subunit

The majority of other universally conserved bacterial GTPase families are capable of associating with the ribosome (Caldon & March, 2003). To establish whether members of the HflX GTPase subfamily may associate with the ribosome, we examined the localization of *Cp. pneumoniae* HflX in a heterologous *E. coli* expression model system by sucrose density gradient ultracentrifugation of ribosomes and

Fig. 3. Spatio-temporal expression of HflX protein during chlamydial development. HflX expression in *Cp. pneumoniae*-infected Hep-2 cells at 24, 48 and 72 h PI was detected using mouse monoclonal HflX<sub>Cpn</sub>-7 antibody, followed by anti-mouse Alexa-594 antibodies (red). Chlamydial membranes were labelled with rabbit anti-chlamydia antibody (Cygnus), which preferentially recognizes chlamydial LPS and membrane MOMP protein, followed by secondary goat anti-rabbit Alexa-488 antibodies (green). 3D image stacks were collected with a Leica SP5 CLSM according to Nyquist criteria and processed by deconvolution (see Methods). The panels on the left show the overlaps (yellow) of HflX expression (red) and chlamydial membranes (green). Orthogonal images are provided for the 24 h time point, with cross-hairs showing the locations of the orthogonal planes selected. Chlamydial RBs are approximately 1 μm in diameter and EBs 300 nm.
associated proteins from *E. coli* MG1655 overexpressing His-HflX from *Cp. pneumoniae* and subsequent detection of the protein in fractions of the gradient using the HflX<sub>7</sub> antibody. This system was used for this analysis to avoid difficulties associated with isolating sufficient pure bacterial ribosome fractions from infected cell culture material, a procedure which we were unable to replicate from the one existing protocol describing this technique (Tamura, 1967). Measurement of A<sub>254</sub> in each fraction collected from the 5–30 % gradient revealed good separation of the free 50S and 30S ribosome subunits from the 70S monosome (Fig. 4a). The ribosomal profile of *E. coli* overexpressing *Cp. pneumoniae* HflX was unchanged from that of control cells containing the empty vector (data not shown). The majority of cellular proteins, as judged by A<sub>380</sub>, remained at the top of the gradient (i.e. free proteins), with the remainder distributed between peaks corresponding to the 70S monosome and 50S and 30S subunits (data not shown). Immunoblotting with anti-HflX monoclonal antibody revealed that His-HflX localized specifically to the 50S ribosomal subunit or was found in the non-ribosomal fractions of the gradient (Fig. 4a).

In an attempt to identify the regions of HflX responsible for this ribosome association, preliminary experiments were performed using truncated forms of HflX. Two truncations of HflX were prepared for this analysis and involved removal of either the carboxyl-terminal GTPase (HflX-N) or the amino-terminal HflX domain (HflX-C). Test experiments confirmed that the truncated HflX forms were expressed equally as well as full-length HflX when overexpressed in MG1655 cells (data not shown). Immunoblotting of cell lysates overexpressing HflX-C, following ultracentrifugation over sucrose gradients, using HflX<sub>7</sub> revealed the loss of any specific association with the 50S subunit (Fig. 4b). Similar experiments performed with HflX-N also showed a loss of specificity, following immunoblotting with HflX<sub>Cpn-21</sub> (Fig. 4b). These data indicate that intact full-length HflX is required for specific association with the 50S ribosome subunit and, furthermore, this association does not appear to be a result of a specific motif within the amino- or carboxyl-half of the GTPase alone.

**DISCUSSION**

In this paper, we provide the first characterization of a member of the widely conserved HflX subfamily of GTPases from any species. This analysis, using a homologue conserved in the genome of *Cp. pneumoniae*, revealed that HflX is, indeed, a GTPase with nucleotide specificity for guanine nucleotides and a slow GTPase activity. Other conserved bacterial GTPase families have been shown to regulate ribosome activity and biogenesis. Consistent with such a role in a process central to the biology of the organisms, deletion mutants of these GTPases have also proven to be lethal (Brown, 2005). This stands in contrast to the non-lethal HflX knockout mutant (Gerdes *et al.*, 2003), raising the question of the function of HflX and whether, indeed, HflX associates at all with the ribosome. We have now clarified this point, showing in an ectopic expression system that there is a specific association between recombinant *Cp. pneumoniae* HflX and the 50S large ribosomal subunit from *E. coli*. This observation can be reasonably extrapolated to wild-type HflX function since previous experiments with recombinant His-tagged GTPases revealed similar co-fractionation characteristics to native protein experiments (Wout *et al.*, 2004). This approach was necessary as we were not able to isolate sufficient quantities of the fragile *Cp. pneumonia* RBs, intact and free of contaminating cellular ribosomes, to perform these experiments with native material.

So what might be the role of HflX in ribosomal function? Members of the related and conserved Obg family of GTPases are essential in eukaryotes and bacteria (Kobayashi *et al.*, 2001; Park *et al.*, 2001) and mutations lead to defects in the synthesis of ribosomal subunits (Datta *et al.*, 2004; Lin *et al.*, 2004; Jiang *et al.*, 2006). These data, combined with detailed biochemical investigations into Obg GTPases (Buglino *et al.*, 2002), suggest that these proteins are primarily responsible for promoting ribosome biogenesis in a nutritionally rich environment (Sato *et al.*, 2003). Measurement of A<sub>254</sub> in each fraction collected from the 5–30 % gradient revealed good separation of the free 50S and 30S ribosome subunits from the 70S monosome (Fig. 4a).
In comparison to Obg family GTPases, HflX gene knockout is not lethal in *E. coli* (Gerdes et al., 2003) and members of this family are not as widely distributed (Leipe et al., 2002). Furthermore, *hflX* displays a different transcriptional response to other ‘ribosome biogenesis’ GTPase-encoding genes, in response to stress (Polkinghorne et al., 2006). These points of evidence are not consistent with a role for this GTPase in ribosome biogenesis and indicate an alternative role for HflX in ribosome function. More details of this function will emerge with further analysis of the native HflX interaction with ribosomes and evaluation of other factors affecting the putative ribosome association (i.e. presence and concentrations of guanine nucleotides, salts and other cofactors).

As already mentioned, biochemical analysis revealed that *Cp. pneumoniae* HflX has a very slow GTPase activity. A slow GTPase activity is also characteristic for members of the Obg family of GTPases, although the rates measured in this study were also slower than those reported for purified recombinant proteins from the Obg family (Welsh et al., 1994; Buglino et al., 2002; Wout et al., 2004). A low GTPase hydrolysis rate would suggest that HflX can be predominantly found in a GTP-bound configuration. This observation will have important ramifications for our understanding of HflX in vivo function and, along with analysis of the GTP and GDP exchange rates for this GTPase, will help to assess the role of HflX family members as molecular switches that other conserved bacterial GTPases are proposed to fill (Caldon et al., 2001).

Visualization of HflX expression through the developmental cycle of *Cp. pneumoniae* provided unexpected evidence for a partial HflX co-localization to the RB membrane. This was seen most clearly in inclusions early in the chlamydial developmental cycle containing only a few RBs, a situation which also favors the optical resolution of the signal, relative to later stages with multiple RBs and EBs. HflX contains no sequences that would indicate a direct membrane association, which raises the question of a possible association with membrane proteins or structures. HflX has also been shown to be membrane-associated in *Corynebacterium glutamicum* (Engels et al., 2005). It is unclear at this stage what the functional significance of a membrane association might be. Early reports suggested that HflX might be involved with regulation of proteolysis. This presumption was based on its presence in an *E. coli* operon with the genes *hflK* and *hflC* (Noble et al., 1993), which encode modulators of the membrane-bound protease FtsH (Kihara et al., 1997; Shotland et al., 1997). *hflK* and *hflC* are both absent from the chlamydial genome (Stephens et al., 1998), so if a partial membrane association of chlamydial HflX can be substantiated biochemically, the question remains as to the nature of the target protein(s).

In this study, we provide an important first step in the characterization of a member of the previously undescribed HflX family of GTPases and present the first GTPase to be characterized from a chlamydia. Further understanding of the role of this GTPase in growth and development would require detailed analysis of the biochemical properties of HflX and further examination of the relationship between these proteins and the ribosome, amongst other putative functions. Such studies will undoubtedly reveal insights into the role of members of this family, not only in this unique intracellular parasite, but across all bacterial species where this GTPase is conserved.

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