Synthesis in *Escherichia coli* of two smaller enzymically active analogues of *Coxiella burnetii* macrophage infectivity potentiator (CbMip) protein utilizing a single open reading frame from the *cbmip* gene

Yin-yuan MO^{*1,2}, J. SESHU^{*1,3}, Dong WANG^{†4} and Louis P. MALLAVIA^{*5}

^{*}Department of Microbiology, Washington State University, Pullman, WA 99164-4233, U.S.A., and [†]Department of Biophysics and Biochemistry, Washington State University, Pullman, WA 99164-4233, U.S.A.

FK506-binding proteins (FKBPs) have been identified in a variety of eukaryotic and prokaryotic organisms. Macrophage infectivity potentiator (CbMip, 23.5 kDa) protein of the obligate intracellular bacterium, *Coxiella burnetii*, was shown previously to belong to the family of FKBP proteins based on sequence homology and peptidyl-prolyl *cis/trans* isomerase (PPIase) activity. Further characterization of the *cbmip* gene has identified two additional proteins with molecular masses of 15.5 and 15.0 kDa that are synthesized, in addition to the 23.5 kDa CbMip, when expressed in *Escherichia coli*. Amino acid sequencing at the N-terminus combined with transcription and translation fusion expression revealed that the two proteins were synthesized from the same open reading frame of the *cbmip* gene, but starting at different internal translation start codons, probably by translational reinitiation. When the internal methionines serving as start sites were replaced with lysine by site-directed mutagenesis, the synthesis of 15.5 and 15.0 kDa proteins was abolished even though the synthesis of 23.5 kDa CbMip was intact. This confirmed that the 15.5 and 15.0 kDa proteins are indeed

generated by translational reinitiation and are not degradation products of the 23.5 kDa protein. Like other FKBP proteins, both 15.5 and 15.0 kDa proteins exhibit PPIase activity. Because they share significant sequence homology with FKBP proteins and have a similar PPIase activity, 15.5 and 15.0 kDa proteins are designated as *C. burnetii* FKBP (Cb-FKBP) analogues I and II, respectively. *TnphoA* mutagenesis demonstrated that whereas the large protein (CbMip) is secreted, Cb-FKBP analogues I and II are cytoplasmic, indicating that structural variations could allow for different subcellular compartmentalization of similar proteins. Western-blot analysis of lysates of purified *C. burnetii* using a CbMip-specific monoclonal antibody revealed the presence of a protein migrating at ≈ 15 kDa, indicating the presence of smaller Cb-FKBP analogue(s) in *C. burnetii*, although at much lower levels compared with 23.5 kDa CbMip. This unique gene organization seen with *cbmip* may provide the organism with a mechanism of efficient use of its limited genetic information to synthesize proteins that are structurally different yet functionally similar.

INTRODUCTION

FK506-binding proteins (FKBPs) and cyclophilins are two protein superfamilies designated as immunophilins because of their ability to bind immunosuppressive drugs [1]. Cyclophilins bind to the cyclic undecapeptide cyclosporin A, whereas FKBP proteins form complexes with the macrolide FK506. These immunophilins have been characterized recently in great detail because of their potential function in the T-cell signal-transduction pathway [2–4]. All FKBP proteins and cyclophilins have been shown to exhibit peptidyl-prolyl *cis/trans* isomerase (PPIase) activity, which catalyses conformational interconversion of proline-containing peptides and proteins [5]. In addition, FKBP proteins and cyclophilins assist in the folding and maturation of proteins, as well as in the translocation of mitochondrial precursor proteins [6,7]. In T cells, immunophilin-immunosuppressant complexes interact with the protein phosphatase, calcineurin, and block a critical step in T-cell activation by inhibiting the nuclear transport of the

transcriptional factor, nuclear factor of activated T cells (NFAT) [8].

Many bacterial species, like *Legionella pneumophila* [9], *Chlamydia trachomatis* [10], *Neisseria meningitidis*, *Bartonella quintana*, *Rickettsia* spp. and *Ehrlichia* spp. [11], as well as *Escherichia coli*, produce proteins that share significant sequence homology with FKBP proteins or cyclophilins of higher organisms. Like their eukaryotic counterparts, these bacterial immunophilins exhibit PPIase activity that can be inhibited by immunosuppressants. Based on size and location, two types of immunophilins have been identified among the pathogenic bacteria. One is represented by macrophage infectivity potentiator (Mip)/Mip-like proteins from intracellular bacteria like *Legionella* and *Chlamydia*. These proteins are approximately 24–27 kDa, with the C-termini being homologous with FKBP proteins [9,10]. These proteins have been shown to be on bacterial cell surfaces and are important for infection of macrophages by these pathogenic bacteria [12]. The second type is represented by the immunophilin

Abbreviations used: FKBP, FK506-binding protein; Cb-FKBP, *Coxiella burnetii* FKBP; Mip, macrophage infectivity potentiator; CbMip, *C. burnetii* Mip; PPIase, peptidyl-prolyl *cis/trans* isomerase; ORF, open reading frame; IPTG, isopropyl β -D-thio-galactopyranoside; mAb, monoclonal antibody; RBS, ribosome-binding site; ECL, enhanced chemiluminescence; β -Gal, β -galactosidase; IVTT, *in vitro* transcription and translation.

¹ Contributed equally to the work and hence should be considered as joint first authors.

² Present address: Cancer Center, University of Illinois at Chicago, Chicago, IL 60607, U.S.A.

³ To whom correspondence should be addressed (e-mail jseshu@mail.wsu.edu).

⁴ Present address: Department of Neurobiology, Harvard Medical School, Boston, MA 02115, U.S.A.

⁵ Deceased.

identified in *N. meningitidis*, which is a 12 kDa cytoplasmic protein that is homologous with FKBP and the C-termini of Mip proteins [13,14]. Whereas some bacteria have been shown to synthesize both types/sizes of protein [15]; to date, individual genes have been shown to encode only one type of protein.

The cellular location of Mip bacterial proteins may be critical to the functions of these proteins, which may partially explain why bacteria have multiple genes encoding immunophilins that are localized either to the outer surface, periplasm or cytoplasm. In addition to the membrane-associated FKBP25 Mip protein, *L. pneumophila* has also been shown to have an 18 kDa cyclophilin (L.p.Cyp18) with considerable sequence similarity to cytoplasmic and periplasmic cyclophilins of *E. coli* [15]. This argues for the presence of proteins of similar enzymic functions in different locations and this localization is achieved by synthesis of proteins with structural variations that allow for targeting of proteins to different compartments has been shown to be achieved by different mechanisms: (i) at the genomic level, synthesis of analogues or isoforms from separate genes with structural differences that allow sorting to different compartments [16]; (ii) at the transcriptional level, synthesis of multiple mRNAs with variable 5' ends from a single gene leading to synthesis of products with variable structure/length that facilitate sorting [17,18]; and (iii) at the translational level, synthesis of multiple protein products from a single open reading frame (ORF) by translational initiation at different start sites [19,20]. Molecular analysis of genes encoding proteins that exhibit enzymic properties in each of the subcellular locations may provide clues as to the regulation of synthesis and targeting of these proteins.

Coxiella burnetii, the etiological agent of Q fever [21], produces a 23.5 kDa Mip-like protein encoded by the *C. burnetii* *mip* (*cbmip*) gene [22]. Surprisingly, we consistently observed two additional proteins, with molecular masses of approximately 15.5 and 15.0 kDa, coexisting with the 23.5 kDa protein during expression of the *cbmip* gene in *E. coli*. The purpose of the present study was to determine if the two smaller proteins are degradation products of the 23.5 kDa protein or are proteins synthesized independently of the larger protein. Further, we determined whether the two smaller proteins are the direct translation products of the *cbmip* gene using different translation start codons and if they are functional proteins with respect to PPIase activity. These observations provide unique information as to how a single ORF is able to encode proteins of three different sizes while retaining the enzymic activity in all of them.

EXPERIMENTAL

Construction of plasmids and sequencing

Plasmids pYMA1.1 and pYMA1.6 carry the *cbmip* gene on a 1.7 kb *EcoRI*–*ClaI* fragment in the vector pSK (Ap^R cloning vector in *E. coli* strain XL1-Blue; Stratagene, La Jolla, CA, U.S.A.). pYMA1.3-1 and pYMA1.3-2 are PCR clones (pCRII vector, Ap^R, Km^R, PCR cloning vector in *E. coli* strain INVαF') generated using the TA cloning kit as per manufacturer's protocol (Invitrogen, Carlsbad, CA, U.S.A.), containing a 553 bp DNA fragment of *cbmip*, amplified using primers A1R-1 and A1U-1 (Table 1, primers based on *cbmip* sequence in [22], GenBank accession no. U14170) without the potential promoter and N-terminal regions of *cbmip*. The *cbmip* DNA fragment in pYMA1.1 and pYMA1.3-1 is in the same orientation as the *lac* promoter (Figure 1A), whereas in pYMA1.6 and pYMA1.3-2, it is in the opposite orientation to the *lac* promoter.

To express *cbmip*, pYMA1.14-1, pYMA1.18-1, pYMA1.42-1 [22], pYMA1.43-1 and pYMA1.54-1 were constructed in either

pTrcHis (Invitrogen) or pET3 (Novagen, Inc., Madison, WI, U.S.A.) vectors. To generate pYMA1.14-1 (Figure 1B), a 694 bp DNA (from the first methionine to the translation stop codon) was first amplified by PCR using primer pair A1R-0 and A1U-1 from pYMA1, the original *cbmip* clone isolated from a *C. burnetii* genomic library [22]. The PCR product was separated on a 1.5 % agarose gel, purified by GeneClean Kit II (Bio101 Inc., Vista, CA, U.S.A.) and cloned into the pCRII vector (Invitrogen). Recombinant plasmids were identified by white colonies on ampicillin-containing LB plates supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. An insert of the recombinant plasmid was excised by digestion with *EcoRI*, and ligated into *EcoRI*-digested pTrcB. The resultant plasmid (pYMA1.14-1) carried the *cbmip* gene that was fused in-frame to the leader peptide of the vector, and expression of the gene was controlled by the isopropyl β-D-thio-galactopyranoside (IPTG)-inducible *trc* promoter [23] on pTrcB. The same cloning strategy was used to construct pYMA1.18-1, except the vector pTrcA was used instead of pTrcB so that *C. burnetii* Mip (CbMip) was out-of-frame with the polyhistidine leader peptide. The same DNA fragment also was cloned into pET3-a to generate the translation fusion pYMA1.54-1 in which expression was driven by the T7 promoter [24]. pYMA1.42-1 was generated from a DNA fragment amplified by PCR using primers A1R-1A and A1U-1 and cloned into pTrcB, and pYMA1.43-1 contained a DNA fragment generated using primers A1R-1.1 to A1U-1 and cloned into pTrcA.

Recombinant plasmids were verified by nucleotide sequencing using the Sequenase Version 2.0 DNA sequencing kit (Amersham Life Science, Arlington, IL, U.S.A.). Amino acid sequencing was performed using an Applied Biosystems 475A by the Biochemistry and Bioanalysis Service at Washington State University.

Site-directed mutagenesis of translation initiation sites on *cbmip* gene

Site-directed mutagenesis was performed on the plasmid pYMA1.1 that has the *cbmip* gene on a 1.7 kb *EcoRI*–*ClaI* fragment in the vector pSK (Table 1) using the QuickChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Briefly, oligonucleotide primers (Table 1) complementary to the opposite strands of the plasmid pYMA1.1 were synthesized with single base alterations of T → A resulting in replacing methionines at CbMip residue 1, residue 91 and residue 95 with lysine. Mutagenesis was performed using *Pfu* DNA polymerase with temperature-cycling conditions set according to the manufacturer's protocol. Following temperature cycling, the parental template DNA was digested with *DpnI* to select for a mutation containing synthesized DNA, which was used to transform Epicurian Coli™ XL1-Blue supercompetent cells. Transformants were selected on LB plates supplemented with 100 µg/ml ampicillin. Plasmids derived after mutagenesis at these residues were used to further replace methionine at other start sites to get double and triple mutants. The nucleotide sequence of the mutagenized plasmids was determined by using a double-stranded DNA cycle-sequencing system (Gibco-BRL Life Technologies, Gaithersburg, MD, U.S.A.).

In vitro transcription and translation (IVTT)

Plasmid DNA (2 µg) was mixed with 10 µl of a premix of amino acids minus methionine, 7.5 µl of S30 extract (Promega, Madison, WI, U.S.A.) and 1 µl of [³⁵S]methionine (1200 Ci/mmol; NEN Life Science Products, Boston, MA, U.S.A.) in a final volume of 25 µl. The mixture was incubated for 30 min at 37 °C, precipitated

Table 1 Bacterial strains, plasmids and primers

Strain	Relevant characteristics	Source or reference
<i>E. coli</i>		
XL1-blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qΔM15Tn10</i> (Tet ^r)]	Stratagene
INVαF ⁺	F' <i>endA1 recA1 hsdR17</i> (rk ⁻ ,mk ⁺) <i>supE44 thi-1 gyrA96 relA1</i> 180 <i>lacZΔ(lacZYA-argF)</i> U169	Invitrogen
Top10	F' <i>mcrAD</i> (<i>mrr-hsdRMSmcrBC</i>)180 <i>lacZ</i> ΔM15D <i>lacX74 deoR recA1 araD139 Δ(ara-leu)</i> 7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
CC118	<i>araD</i> 139 <i>Δ(ara-leu)</i> 7697 <i>ΔlacC74 phoAΔ</i> 20 <i>galE</i> <i>thi rps rhoB argE recA1</i>	[29]
<i>C. burnetii</i>		
Nine Mile phase 1	Acute disease isolate plasmid QpH1	[25]
RSA493 clone 7		
Plasmid		
pUC19	Ap ^R cloning vector	GIBCO BRL
pSK	Ap ^R cloning vector	Stratagene
pCRII	Ap ^R Km ^R cloning vector	Invitrogen
pET3-a	Ap ^R expression vector	Stratagene
pTrcB/pTrcC	Ap ^R expression vector	Invitrogen
pYMA1.1	pSK carrying <i>cbmip</i> on a 1.7kb <i>Clal-EcoRI</i> fragment	[22]
pYMA1.6	pUC19 carrying <i>cbmip</i> as in pYMA1.1 but opposite in orientation to <i>lac</i> promoter	[22]
pYMA1.3-1	pCRII Ap ^R Km ^R cloning vector containing a 553bp DNA fragment of <i>cbmip</i> amplified using primers A1R-1 and A1U-1 without the potential promoter and N-terminal regions of <i>cbmip</i>	This study
pYMA1.3-2	pCRII carrying 553 bp <i>cbmip</i> fragment as in pYMA1.1 but opposite in orientation to <i>lac</i> promoter	This study
pYMA1.14-1	pTrcB carrying 694 bp <i>cbmip</i> DNA from first methionine to translational stop residue	This study
pYMA1.18-1	pTrcA carrying same insert as in pYMA1.14-1 so that <i>cbmip</i> was out-of-frame with polyhistidine leader peptide	This study
pYMA1.18-2	pTrcA carrying same insert as in pYMA1.18-1 but in opposite orientation to <i>trc</i> promoter	This study
pYMA1.35-1	pTrcC carrying the insert from pYMA1.3-1 fused in-frame with the leader peptide of the vector, resulting in translational fusion protein	This study
pYMA1.42-1	pTrcB carrying <i>cbmip</i> DNA fragment generated using primers A1R-1A and A1U-1	This study
pYMA1.43-1	pTrcA carrying <i>cbmip</i> DNA fragment generated using primers A1R-1.1 and A1U-1	This study
pYMA1.54-1	<i>cbmip</i> DNA fragment (694 bp) as in pYMA1.14-1 cloned into pET3-a with expression driven by T7 promoter	This study
pJS505	As pYMA1.6 except for base substitution in <i>cbmip</i> corresponding to M1 → K	This study
pJS511	As pYMA1.1 except for base substitution in <i>cbmip</i> corresponding to M91 → K	This study
pJS521	As pYMA1.1 except for base substitution in <i>cbmip</i> corresponding to M95 → K	This study
pJS531	As pYMA1.1 except for base substitutions in <i>cbmip</i> corresponding to M1 → K and M91 → K	This study
pJS541	As pYMA1.1 except for base substitutions in <i>cbmip</i> corresponding to M1 → K and M95 → K	This study
pJS551	As pYMA1.1 except for base substitutions in <i>cbmip</i> corresponding to M91 → K and M95 → K	This study
pJS565	As pYMA1.6 except for base substitutions in <i>cbmip</i> corresponding to M1 → K, M91 → K and M95 → K	This study
Primers	Sequence (mutations are underlined)	
A1R-0	5'-GAAACGATTGATTTTAC-3'	
A1R-1	5'-CTGGAAGAGCTTTCAGA-3'	
A1R-1A	5'-GCAACACAAAATGAAAC-3'	
A1R-1.1	5'-CAATCCCTGCAAAAAAT-3'	
A1U-1	5'-CTGAGGTTTTATTCTT-3'	
MIP-23.5	5'-AATTCAGGAGATCGCTGAAAGAAACGATTG-3'	
MIP-23.5R	5'-CAATCGTTTCITTCAGCGATCTCCTGAATT-3'	
MIP-15.5	5'-CAATCCCTGCAAAAAAGCAACACAAA-3'	
MIP-15.5R	5'-TTTGTGTTGCTTTTTTTGCGAGGATTG-3'	
MIP-15	5'-GCAACACAAAAGAAACAAACAGCTCAACAG-3'	
MIP-15R	5'-CTGTTGAGCTGTTGTTTCTTTTTGTTGTC-3'	

with four volumes of acetone and finally dissolved in 100 μ l of 1 $\times$ Laemmli buffer [25]. The labelled proteins were separated by SDS/PAGE (12 % gels). The IVTT products obtained from using site-directed mutagenized plasmids were separated by SDS/PAGE (20 % gels). The gel was dried and autoradiographed using X-OMAT film (Eastman Kodak Co., Rochester, NY, U.S.A.).

Induction and purification of recombinant proteins

The cloned DNA fragments were expressed in *E. coli* Top10, which was grown in SOC medium (20 g/l tryptone/5 g/l yeast extract/0.5 g/l NaCl/2.5 mM KCl/10 mM MgCl₂/20 mM glucose, pH 7.0) [22] supplemented with 100 μ g/ml ampicillin at 37 °C. Cultures were induced with 1 mM IPTG when the attenuation reached $\approx$ 0.6. Expression of the proteins was analysed by SDS/PAGE. At least 500 ml of each Top10 culture

carrying an appropriate plasmid was used for protein purification.

Purification of the fusion protein was done by using nickel resin (Invitrogen) as per the manufacturer's protocol. Because preliminary experiments showed that the fusion proteins were soluble, non-denaturing buffers were used throughout the purification. In brief, after equilibration with binding buffer (20 mM NaH₂PO₄/500 mM NaCl, pH 7.8), a 5 ml protein sample was loaded on a nickel-resin syringe column and incubated at room temperature for 15 min with gentle shaking. After allowing for the fusion proteins to bind, the solution was removed and another 5 ml protein sample was loaded and processed as above. Non-specifically bound proteins were removed by sequential washes with 20 mM NaH₂PO₄/500 mM NaCl at pH 6.3, 6.0, 5.5 and then 4.0. Finally, the recombinant protein was eluted using pH 3.8 elution buffer (20 mM NaH₂PO₄/500 mM NaCl). The purity of the isolated protein was examined by SDS/PAGE.

Figure 1 Plasmids used for expression of the *cbmip* gene

(A) The plasmids used in IVTT. Direction of the *cbmip* gene is as indicated. (B) The plasmids used to overexpress recombinant proteins. Plasmid pYMA1.14-1 encoded the 23.5 kDa CbMip, whereas pYMA1.43-1 and pYMA1.42-1 encoded 15.5 and 15.0 kDa CbMip respectively. Vectors for each plasmid are indicated on the left.

For purification of non-fusion proteins, gel filtration and ion-exchange chromatography or HPLC were employed. The cell extract of the *E. coli*-harbouring pYMA1.14-1 was fractionated using Bio-Gel P10 (Bio-Rad Labs, Hercules, CA, U.S.A.) filtration to separate the large protein (23.5 kDa) from the small proteins (15.5 and 15.0 kDa). After separation, pooled samples of each major peak corresponding to the 23.5 kDa and a mixture of the 15.5 and 15.0 kDa proteins were further purified using either HPLC or ion-exchange chromatography. HPLC used a dual-pump Rainin system connected to a Waters 991 photodiode array detector with a C-8 reverse phase column (Aquapore RP-300, Brownlee Labs). The loaded proteins were eluted with a 1%/min gradient of 0.1% trifluoroacetic acid/65% acetonitrile at a flow rate of 1 ml/min. The fractions were monitored by absorbance at 214 nm. Due to their similar molecular masses, the two smaller proteins could not be separated and were eluted as a mixture as judged by SDS/PAGE. Because the 23.5 kDa protein tended to aggregate, and HPLC was ineffective in purifying this protein, ion-exchange chromatography was employed. The protein was loaded on to a 1.0 cm × 20 cm CM-Sephadex C25 column that was equilibrated with 10 mM NaH₂PO₄/0.5 M NaCl, pH 7.0, at 4 °C, and eluted with increasing concentrations of NaCl.

Preparation of antibodies and Western blotting

Polyclonal antibodies were prepared by immunizing female BALB/c mice (6–8 weeks old) intraperitoneally with each of the purified proteins (150 µg) emulsified with Freund's incomplete adjuvant (Sigma, St. Louis, MO, U.S.A.). Following the initial injection, 50 µg of each protein with the incomplete adjuvant were administered intraperitoneally on days 14 and 21. Sera were collected on day 28 and stored at –20 °C until used.

Western blotting was conducted as follows: total cell extracts of *E. coli*, *C. burnetii* Nine Mile phase 1 (RSA 493, clone 7) [26] and purified protein samples were separated in SDS/PAGE (15% gels) and transferred to a nitrocellulose membrane. Immunoblotting was carried out according to Sambrook et al. [27]. Signals were detected by horseradish peroxidase-conjugated goat anti-mouse antibodies in the presence of 4-chloro-1-naphthol and hydrogen peroxide. Monoclonal antibodies (mAbs) were prepared according to standard methods [28] and two, 6H10.1B8 and 6G10.1F10, which reacted to the 15.5 and 15.0 kDa CbMip proteins, were characterized (J. Seshu, D. Howe, K. L. McIvor

and L. P. Mallavia, unpublished work). For detection of *C. burnetii* FKBP (Cb-FKBP) analogues in *C. burnetii*, mAb 6H10.1B8 was used and signals were detected using Enhanced Chemiluminescence (ECL; Amersham) according to the manufacturer's protocol with minor modifications. The blots were blocked for 1 h in PBS (pH 7.5) with 9% non-fat milk/0.1% Tween-20. Both the primary antibody (6H10.1B8 ascites, 1:2000 dilution) and secondary antibody (sheep anti-mouse horseradish peroxidase-conjugated; Amersham) were diluted in the above blocking buffer and the blots were incubated for 1 h. The blots were developed with ECL detection reagents and exposed to Reflection™ (NEN Life Science Products) autoradiography film.

Northern hybridization

Total RNA was isolated from *E. coli* strain XL1-Blue carrying pYMA1.1, pYMA1.6 or pSK using TRIzol (Gibco-BRL) as per the manufacturer's protocol, separated in 2.2 M formaldehyde agarose gels (1.5%), and transferred to Nytran membranes (Schleicher & Schuell Inc., Keene, NH, U.S.A.). The probe was the 553 bp insert from pYMA1.3-1, labelled with [α -³²P]dCTP using a random primer labelling kit (Promega) as per the manufacturer's protocol. RNA size markers (RNA Millennium markers, Ambion Inc., TX, U.S.A.) were loaded at 5 µg/lane and were detected on Northern blots by using [α -³²P]dCTP-labelled *Hind*III-cut λ DNA molecular-mass markers (Gibco-BRL). Hybridization and membrane washes were carried out under high stringency as per standard procedures [27].

TnphoA mutagenesis

Plasmids pYMA1.6 and pYMA1.3-2 were mutagenized by λ ::TnphoA according to the method of Manoil and Beckwith [29]. Transfectants (CC118) carrying potential TnphoA insertions on plasmids were plated on kanamycin-containing LB plates supplemented with 5-bromo-4-chloro-3-indolyl-phosphate. Physical mapping was used to identify insertions of interest and further analysed by alkaline phosphatase activity assays. When needed, the positions of TnphoA insertions were determined by nucleotide sequencing.

Enzyme assay

PPIase activity of purified proteins was assayed based on a published method [30,31]. The measurement of the PPIase activity was carried out using the protease-coupled assay as described by Kofron et al. [30]. Briefly, the assay is based on the observation that α -chymotrypsin cleaves the C-terminal amide bond only in the *trans*-Xaa-Pro conformer of the chromogenic substrate succinyl-Ala-Xaa-Pro-Phe-4-nitroanilide. At pH 7.8 this substrate exists in an equilibrium of about 5–20% *cis* and 80–95% *trans* conformer. α -Chymotrypsin cannot cleave off the 4-nitroaniline residue in the *cis* conformer, but in the presence of PPIase (CbMip), which results in the slow isomerization of the *cis* form to the cleavable *trans* substrate, the consumption of the chromogenic substrate at varying concentrations of CbMip would be a measure of its PPIase activity. This was measured according to Harrison and Stein [31]. The assay used succinyl-Ala-Phe-Pro-Phe-*p*-nitroanilide (Bachem Bioscience, Inc., King of Prussia, PA, U.S.A.), which has been shown to be the most favourable substrate for the CbMip protein [9,22]. Enzymic activity was measured in 35 mM Hepes buffer (pH 7.8) containing 0.25 mg/ml α -chymotrypsin at 0 °C. The reaction was started by adding 75 mM substrate and colour changes were monitored at 390 nm.

RESULTS AND DISCUSSION

CbMip, Cb-FKBP analogues I and II are synthesized from the same ORF

The full-length *cbmip* gene has the potential to encode for three proteins of 23.5, 15.5 and 15.0 kDa in size, as observed by either IVTT or overexpression of plasmids carrying this gene. The consistent production of three proteins from a single cloned gene was an intriguing observation, which prompted us to determine the origin of these proteins. To identify if the two additional

proteins were new proteins or degradation products of the larger protein, various constructs of the *cbmip* gene were made for expression. IVTT of both pYMA1.1 and pYMA1.6 resulted consistently in three protein bands corresponding to 23.5, 15.5 and 15.0 kDa, respectively (Figure 2A). These results suggested that *cbmip* carries its own promoter within a 1.7 kb *EcoRI*–*ClaI* fragment. When the upstream region, including the nucleotides encoding the N-terminus (40 amino acids), of CbMip was deleted (pYMA1.3-1), the 23.5 kDa protein band was no longer evident, but the other proteins were still produced (Figure 2A). This

Figure 2 Expression of the *cbmip* gene in *E. coli*

(A) IVTT. Plasmid DNAs used are indicated above each lane and are described in the text and Table 1. Plasmids pYMA1.6 and pYMA1.3-2 were counterparts of pYMA1.1 and pYMA1.3-1, respectively, with inserts in opposite orientation. After IVTT, the proteins were separated on SDS/PAGE (12.5% gels) and subjected to autoradiography. The large and two small proteins are noted by arrows. (B) Overexpression of the recombinant proteins from the transcription and translation fusions. Cultures of *E. coli* Top10 in SOC medium + ampicillin bearing various plasmids with *cbmip* were induced with 1 mM IPTG and the proteins analysed by SDS/PAGE followed by staining with Coomassie Brilliant Blue G-250. pYMA1.14-1 carries the full-length *cbmip* gene fused in-frame with the leader peptide of pTrcB, whereas pYMA1.18-1 has the same *cbmip* gene fused out-of-frame with pTrcA. pYMA1.14-2 had the same construct as pYMA1.14-1, except in the opposite orientation. (C) Western-blot analysis. All three proteins were recognized by polyclonal antibodies to CbMip. Lane 1, total proteins of *E. coli* cell extracts harbouring pYMA1.14-1; lane 2, purified 23.5 kDa protein; lane 3, purified 15.5 and 15.0 kDa proteins. (D) Western-blot analysis of lysates of purified *C. burnetii* Nine Mile (RSA493 clone 7) phase I organisms using a CbMip-specific mAb 6H10.1B8. In addition to the 23.5 kDa CbMip, another band corresponding to protein(s) of 15.0 kDa is observed. Lane 1, *C. burnetii* phase I, 25 µg; lane 2, *C. burnetii* phase I, 50 µg; lane 3, *C. burnetii* phase I, 75 µg; lane 4, *E. coli* pYMA1.54-1. The bands were visualized using the ECL detection system. (A, B, D) Molecular-mass (MW) markers are indicated in kDa.

suggested that production of the 15.5 and 15.0 kDa proteins was independent of the 23.5 kDa protein. A $\approx$ 26 kDa band (Figure 2A) was observed consistently in lanes with the recombinant plasmids but was absent in the lane with the vector used as the template alone. The origin of this protein is not known, but it does not seem to be related to the CbMip ORF, because the protein was not recognized by CbMip-specific antibodies (results not shown). The levels of the 26 kDa protein band appeared to be more abundant in pYMA1.3-2 in comparison with other constructs (Figure 2A), but this could be due to variations in the concentration of proteins loaded in different lanes. The identity of 23.5 kDa CbMip is indicated by an arrow in Figure 2(A) and is distinct from the 26 kDa band found in all the constructs. In addition, both short clones (pYMA1.3-1 and pYMA1.3-2) no longer express the 23.5 kDa CbMip and are not long enough to encode a protein with a molecular mass of 26 kDa.

To further verify that the synthesis of 15.5 and 15.0 kDa proteins are from their own translation initiators with ribosome-binding sites (RBSs), the *cbmip* gene was cloned into expression vectors to generate translation (pYMA1.14-1) and transcription fusion (pYMA1.18-1) products. For translation fusion, the entire CbMip ORF was fused with the leader peptide of the vector (pTrcB) in-frame, so that the translation of the ORF was initiated by the RBS in the vector. As shown by IVTT experiments, three similar protein bands were also observed in Coomassie Brilliant Blue G-250-stained gels for pYMA1.14-1 (Figure 2B). When the same fusion was made in the pET3 system, identical bands were detected as in the pTrcHis system (results not shown), showing that the proteins were not associated with a particular vector. Moreover, when the insert from pYMA1.3-1 (lacking the nucleotides encoding the N-terminus of CbMip) was cloned into pTrcC (pYMA 1.35-1) there was translational fusion resulting in a 20 kDa CbMip protein synthesized from the leader peptide of the vector, as well as the 15.5 and 15.0 kDa CbMip proteins (results not shown).

The transcription fusion was made by cloning the same DNA fragment into pTrcA, instead of pTrcB, so that the CbMip ORF was out of frame with respect to the leader peptide. Hence it was expected that no protein would be synthesized by the transcription fusion if there were no other ORFs within this region. As predicted, no 23.5 kDa protein was observed in pYMA1.18-1. However, the two small proteins were still synthesized (Figure 2B). This strongly suggests that the small proteins are the direct translation products of that DNA fragment.

But how did the transcription fusion still produce the small proteins if they were synthesized from the same DNA fragment? Examination of *cbmip* did not reveal any obvious ORFs in addition to the 23.5 kDa protein. The most reasonable possibility was that the smaller ORFs are present within *cbmip*. There are eight internal methionines in the CbMip sequence, but only two purine-rich regions (or potential RBSs) in front of ATGs with reasonable spacing (11 and 12 bases from the ATGs, respectively). These were detected at nucleotides 313–318 and 329–334 (GAAAAA and AAAAAA) [22]. However, no potential promoter was found within this region, which would be recognized by *E. coli* RNA polymerase. If translation starts with ATG at nucleotide 334 (ORF2), the predicted protein has a relative molecular mass of 15386 Da, whereas if it starts with ATG at nucleotide 346 (ORF3), it will be 14861 Da. Based on data obtained using pYMA1.14-1, it is predicted that the large protein is initiated at the first methionine using the RBS on the vector, whereas the other two proteins must be translated using *C. burnetii* RBSs within the *cbmip* gene. The expression level for the two proteins was about equal to that of the 23.5 kDa CbMip (Figure 2B), indicating that the *C. burnetii* RBSs are about as

efficient as the *E. coli* RBSs, assuming a single species of mRNA is made from this construct.

Western-blotting data also supported that the three proteins shared similar epitopes, suggestive of the same origin. Polyclonal antiserum to CbMip detected all three proteins (Figure 2C) in *E. coli* expressing the *cbmip* gene and a mAb to CbMip detected the presence of all three proteins in purified *C. burnetii* Nine Mile phase I organisms (Figure 2D). Whereas the two smaller proteins were present in much lower quantities compared with the 23.5 kDa CbMip, they were detectable without ambiguity with greater than 25 μ g of purified *C. burnetii* per lane as well as employing the highly sensitive ECL detection system (Figure 2D). There was no reactivity of identical amounts of *C. burnetii* with an isotype-matched irrelevant mAb (MOPC195, IgG2b, results not shown). Hence it is very likely that all three proteins result from the *cbmip* gene.

To further confirm that the synthesis of the 15.5 and 15.0 kDa proteins was due to internal initiation sites and not due to proteolytic degradation of 23.5 kDa CbMip, we performed site-directed mutagenesis on the plasmid pYMA1.1, whereby the methionines at position 91 (pJS511, M91 $\rightarrow$ K) and 95 (pJS521, M95 $\rightarrow$ K) were changed to lysine so that the translational start sites of the 15.5 and 15.0 kDa CbMip proteins, respectively, were abolished. Analysis of these plasmids by IVTT (Figure 3A, lane 3) showed that whereas the 15.5 kDa protein was abolished when pJS511 was used as template, there was synthesis of both the 23.5 kDa as well as the 15.0 kDa CbMip proteins. Similarly, the synthesis of 15.0 kDa CbMip was abolished on using the plasmid pJS521 whereas the synthesis of both the 23.5 and 15.5 kDa CbMip proteins was intact (Figure 3A, lane 4). Moreover, IVTT analysis of pJS551, where methionines at both position 91 and 95 were replaced with lysine, resulted in the loss of both 15.5 and 15.0 kDa proteins while there was synthesis of 23.5 kDa protein (Figure 3A, lane 5). All three proteins were synthesized when pYMA1.1 (Figure 3A, lane 1) was used as the template. There was no synthesis of proteins of similar size when pSK (Figure 3A, lane 2), the vector common to all the plasmids, was used in this analysis, though there was a faint band corresponding to β -lactamase. Since the *cbmip* gene in pYMA1.1 is cloned in-frame with the *lac* promoter [22], it was possible to have CbMip fused to β -galactosidase (β -Gal), and hence a product larger than 23.5 kDa was also synthesized and has been designated as a putative β -Gal–CbMip fusion protein. The experimental reasons for the conclusion that the two smaller CbMip analogues are synthesized due to translational reinitiation at internal start sites are two fold. (i) When nucleotides encoding the N-terminus (40 amino acids) of CbMip were deleted, the 23.5 kDa protein was not evident (Figure 2A, pYMA1.3-1), whereas the 15.5 and 15.0 kDa products were present. (ii) Site-directed mutagenesis of internal start sites replacing the methionines with lysines abolished the synthesis of the two smaller CbMip analogues (Figure 3A). Taken together, these observations preclude the possibility of degradation of 23.5 kDa CbMip as the source of the two smaller CbMip proteins.

Western-blot analysis of *E. coli* expressing various mutagenized plasmids was in agreement with the IVTT data. Anti-CbMip mAb 6H10.1B8 was used as the primary antibody, as it reacts with the C-terminal half of CbMip and hence has the advantage of detecting the presence or absence of different CbMip analogues. The specificities of mAb 6H10.1B8 for all three CbMip proteins are shown by its reactivity with 23.5, 15.5 and 15.0 kDa proteins expressed in *E. coli* carrying the plasmid pYMA1.1 (Figure 3B, lane 1), whereas no such reactivity was seen with pSK without insert (Figure 3B, lane 2). In addition to these proteins, a product larger than 23.5 kDa also reacted with

Figure 3 Site-directed mutagenesis of internal start sites in *cbmip* gene abolishes synthesis of smaller analogues

(A) IVTT of plasmids with site-directed mutagenesis replacing methionines serving as translation start sites for CbMip analogues with lysines. After IVTT, the proteins were separated on SDS/PAGE (20% gels) and subjected to autoradiography. Plasmid DNAs used as templates for IVTT were: lane 1, pYMA1.1; lane 2, pSK; lane 3, pJS511 (M91 $\rightarrow$ K); lane 4, pJS521 (M95 $\rightarrow$ K); and lane 5, pJS551 (M91 $\rightarrow$ K, M95 $\rightarrow$ K). Molecular-mass markers are indicated in kDa. (B) Western-blot analysis of *E. coli* expressing the site-directed mutagenized plasmids using anti-CbMip mAb 6H10.1B8. Lane 1, pYMA1.1; lane 2, pSK; lane 3, pJS521 (M95 $\rightarrow$ K); lane 4, pJS505 (M1 $\rightarrow$ K); lane 5, pJS511 (M91 $\rightarrow$ K); lane 6, pJS565 (M1 $\rightarrow$ K, M91 $\rightarrow$ K, M95 $\rightarrow$ K); lane 7, pJS531 (M1 $\rightarrow$ K, M91 $\rightarrow$ K); lane 8, pJS541 (M1 $\rightarrow$ K, M95 $\rightarrow$ K); and lane 9, pJS551 (M91 $\rightarrow$ K, M95 $\rightarrow$ K). The bands were visualized using the ECL detection system. Molecular-mass markers are indicated in kDa.

Table 2 Determination of the N-terminal sequence of Cb-FKBP analogues I and II

The ratio of analogue I to analogue II was calculated by comparing the amount of non-overlapped amino acids of each protein to the total amount of the amino acids and was determined to be 35:65. The yields for Met, Gln and Ala in cycles 1, 7 and 9 are derived from both analogues I and II.

Cycle number	Analogue I		Analogue II	
	Amino acid	Amount (pmol)	Amino acid	Amount (pmol)
1	Met	—	Met	143
2	Gln	278	Lys	673
3	His	159	Gln	475
4	Lys	322	Thr	333
5	Met	351	Ala	545
6	Lys	338	Gln	419
7	Gln	—	Gln	536
8	Thr	146	Asn	310
9	Ala	—	Ala	654
10	Gln	210	Glu	275
11	Gln	175	Lys	397

6H10.1B8 and was observed on expressing pYMA1.1 (Figure 3B, lane 1) or its mutagenized derivatives. Since the *cbmip* gene in pYMA1.1 was cloned in-frame with the *lac* promoter [22], as mentioned earlier, that it was possible to have CbMip fused to the first 36 amino acids of β -Gal. This putative β -Gal-CbMip fusion protein showed reactivity with 6H10.1B8, similar to the 23.5 kDa CbMip, except that it had an additional 36 amino acids of β -Gal as well as amino acids encoded by nucleotides upstream of the start codon of 23.5 kDa CbMip, accounting for its bigger size. There was reactivity to only the 23.5 and 15.5 kDa proteins with pJS521 (M95 $\rightarrow$ K; Figure 3B, lane 3) whereas there was reactivity to 23.5 and 15.0 kDa proteins with pJS511 (M91 $\rightarrow$ K;

Figure 3B, lane 5). This indicated that replacement of the methionine serving as the start site with lysine resulted in the loss of initiation of translation of either 15.5 kDa (pJS511, M91 $\rightarrow$ K) or 15.0 kDa (pJS521, M95 $\rightarrow$ K) proteins, whereas there was initiation at the methionines for the other two CbMip proteins. Moreover, neither 15.5 nor 15.0 kDa proteins (Figure 3B, lane 9) could be detected when the initiation methionines for both the smaller proteins were replaced by lysine, whereas there was reactivity to 23.5 kDa CbMip, indicating that the two smaller CbMip analogues are not degradation products of the 23.5 kDa CbMip. In addition to these mutants, we also replaced the initiation methionine for the 23.5 kDa CbMip with lysine to confirm that the smaller CbMip proteins are synthesized independently of the 23.5 kDa CbMip. When the *cbmip* gene was cloned into pUC19 (with the *cbmip* gene in the opposite orientation as the *lac* promoter), after mutagenesis at position 1 (M1 $\rightarrow$ K, pJS505) there was no reactivity with the 23.5 kDa protein, though there was reactivity with both 15.5 and 15 kDa proteins (Figure 3B, lane 4). This was because there was no synthesis of 23.5 kDa CbMip either from its own start site or as the β -Gal-CbMip fusion protein. Replacement of two methionines, serving as start sites for 23.5, 15.5 and 15 kDa proteins, respectively, were changed to lysine in conjunction with cloning the *cbmip* gene in opposite orientation as the *lac* promoter there was no synthesis of any CbMip proteins (Figure 3B, lane 6). Taken together these observations confirm that the synthesis of the two smaller CbMip analogues is due to translational reinitiation at internal start sites and hence they are not breakdown products of the 23.5 kDa CbMip.

Figure 4 A putative gene organization with three ORFs within the *cbmip* gene and the deduced amino acid sequence

ORF1 encompasses the entire CbMip protein and its mature product has a calculated molecular mass of 23.5 kDa. ORF2 (Cb-FKBP analogue I) and ORF3 (Cb-FKBP analogue II) start at nucleotides 334 and 346, and produce proteins of 15.5 and 15.0 kDa, respectively. Only the nucleotide sequences with relevant features are shown. Three ATG codons serving as possible translation initiators are italicized and blocked. The cleavage site of the CbMip precursor is indicated by a downward arrow.

These conclusions were further confirmed by sequencing the purified proteins obtained from *E. coli* expressing the relevant plasmids (Table 2). The N-terminus of the 23.5 kDa CbMip protein was determined previously to be ATPLKTE [22], which corresponds to amino acids 22–28 of the mature CbMip. On the other hand, the two smaller proteins were determined to be a mixture of two proteins whose N-terminal sequences are MQHKMKQTAQQ (Cb-FKBP analogue I) and MKQTAQQNAEK (Cb-FKBP analogue II) at a ratio of 35:65 (Table 2).

Based on the data obtained, a possible gene organization for this DNA fragment is shown in Figure 4. In essence, two small ORFs exist within a single ORF, such that all three proteins are synthesized utilizing a single ORF. It is suggested that the two small ORFs be designated subsidiary ORFs. The ability of *E. coli* ribosomes to initiate at different internal start sites on a single ORF is not unique, as a similar mechanism has been shown to operate for the synthesis of the heat-shock protein ClpB [20]. By PCR-aided site-directed mutagenesis it was shown that this protein-activated ATPase (ClpB) consists of two proteins of 93 (ClpB93) and 79 kDa (ClpB79), encoded by the same ORF of the *clpB* gene. The 93 kDa protein is translated from the 5'-end ATG start site and 79 kDa protein from the 149th codon which is an internal GTG start site [20]. ClpB79 is functional in spite of lacking 148 N-terminal amino acids of ClpB, as it retains the consensus sequences for adenine nucleotide binding with high homology to the ClpA family of proteins. ClpB79 was also shown to inhibit the casein-activated ATPase activity of ClpB93 in a dose-dependent manner without any effect on its inherent ATPase activity. One possible reason suggested for this observation was that the smaller protein may control the enzyme activity of the larger protein or its other unknown functions. The expression of multiple proteins from a single ORF has also been observed in other organisms such as *Saccharomyces cerevisiae*, where *MOD5*-encoded tRNA-modifying isoenzymes are synthesized from different start sites on mRNAs having the same 5' end [19]. The smaller isoenzyme is localized only in the

cytoplasm whereas the larger isoenzyme (11 amino acids longer) can partition into the mitochondria [19]. Two different cyclophilins with molecular masses of 18 and 19 kDa, respectively, are synthesized in *E. coli* from two different genes and their structural differences explain one being found in the periplasm and the other in the cytoplasm. The *E. coli* PPIase a, or Rot (pI $\approx$ 9.0), has a 24 amino acid signal peptide and is found in the periplasm, whereas PPIase b (pI $\approx$ 5.0) is located predominantly in the cytoplasm. These data give credence to the observation that gene organization as seen with the *cbmip* gene, which results in proteins of different sizes originating from a single ORF, may be a mechanism for conserving genetic information, yet having the possibility of synthesizing isoforms or analogues that function in different subcellular compartments.

23.5 kDa protein is exported, whereas the other two proteins are cytoplasmic

Amino acid sequence data of the N-terminal region of CbMip suggests that this protein is processed and probably exported. To confirm this as well as to determine the subcellular locale of the two smaller proteins, we used the *phoA* gene-fusion approach that readily identifies genes that encode exported proteins utilizing the property of the bacterial alkaline phosphatase, which in order to be active must be localized extracytoplasmically [32]. Signal sequence mutations that block its export render this enzyme inactive. Export and activity of this enzyme can be restored by fusing restriction fragments containing a truncated *phoA* gene (lacking secretion signals) to portions of genes encoding the signal sequences of heterologous genes. This approach was modified by Manoil and Beckwith [29], who inserted a *phoA* restriction fragment near one end of Tn5 to create a transposon designated Tn_{phoA} that can randomly generate gene fusions to *phoA* upon insertion into a cloned target gene or chromosome. The hybrid proteins expressed by such gene fusions display alkaline phosphatase activity only if the target gene encodes a membrane, periplasmic, outer membrane

Figure 5 *TnphoA* mutagenesis analysis of CbMip and Cb-FKBP analogues

E. coli strain CC118 (*PhoA*⁺) was first transformed with pYMA1.6 or pYMA1.3-1, then transfected with λ ::*TnphoA* [20]. Potential *cbmip*::*TnphoA* fusions were selected on LB agar plates with kanamycin and 5-bromo-4-chloro-3-indolyl-phosphate and identified by physical mapping of the constructs. PhoA activity for each insert was measured as units of alkaline phosphatase. Abbreviations used: C, *Cla*I; H, *Hind*III; and R, *Eco*RI.

or extracellular protein. When a DNA fragment covering the full length of the *cbmip* gene (pYMA1.6) was subjected to *TnphoA* mutagenesis, we found that over 50 % of the kanamycin-resistant blue colonies carried a *phoA* insertion within *cbmip*. Those insertions expressed significant amounts of alkaline phosphatase activity over the background (Figure 5). In contrast, the Cb-FKBP analogues I and II appeared to be cytoplasmic. When pYMA1.3-1 was subjected to *TnphoA* mutagenesis, none of the insertions selected were shown to express PhoA activity (Figure 5), suggesting that the proteins stay in the cytoplasm. The structural features of the 23.5 kDa protein, such as the presence of a potential N-terminal signal peptide [22], different from what has been observed with the 15.5 and 15.0 kDa proteins may account for the differential subcellular localization of these proteins. This ability to target analogues of CbMip to various compartments may be similar to *MOD5*-encoded isoenzymes of *S. cerevisiae* while conserving the genetic information needed to accomplish this. The subcellular distribution of the *MOD5*-encoded isoenzymes suggests that they have different physiological functions and may be akin to the presence of *E. coli* PPIases in the periplasm as well as the cytoplasm [16]. This functional diversification of PPIases, even within a single cell, may be needed for assisting the folding of polypeptides located on either side of the inner membrane, as many proteins are translocated in an unfolded state [33]. The Gram-negative structure of the *C. burnetii* outer membrane and the presence of a single copy of the *cbmip* gene, while not precluding the presence of other functional PPIases, serves to strengthen the need for enzymically active analogues of CbMip that could function within the cytoplasm as well as extracellularly.

A single species of mRNA is produced from the *cbmip* gene

To determine whether the DNA fragment is transcribed as a single species of mRNA, Northern blotting was carried out for total RNA from *E. coli* cells carrying either pYMA1.1 or pYMA1.6. A band corresponding to a predicted size of *cbmip* mRNA (≈ 900 bases) was detected by the *cbmip* probe (Figure 6, lanes 2 and 4). In addition, there seems to be two bands, one slightly larger than 900 bases, detected from pYMA1.1. This is most likely due to a transcript initiating upstream of *lacZ* (extra ≈ 100 bases) in the vector fused with the *cbmip* gene. Since no other major transcript(s) smaller than ≈ 900 bases have been observed in both lanes 2 and 4 (Figure 6), this indicates that a single species of mRNA is produced from the *cbmip* gene. IVTT also suggests that there appears to be a single species of mRNA made because the promoterless construct pYMA1.3-2 did not express the two smaller proteins (Figure 2A).

All three proteins are PPIases

Because the three proteins are translated from the same ORF, all of their amino acid sequences are highly homologous with FKBP. For instance, when compared with FKBP from human [34], mouse [35], *Neurospora crassa* [36] and *N. meningitidis* [13], over 35% amino acid identity was observed using the GAP program [37] (Figure 7).

To examine if the two smaller proteins exhibit PPIase activity like CbMip, enzyme assays were carried out for all three proteins. Because the two small proteins are so close in molecular mass, we were unable to separate them. To overcome this problem,

Figure 6 Northern-blot analysis of total RNA from *E. coli* carrying plasmids with *cbmip*

Total RNA extracted from *E. coli* XL1-Blue with either the *cbmip* gene in the same (pYMA1.1) or opposite (pYMA1.6) orientation to the *lac* promoter. Lane 1, RNA size standards; total RNA from *E. coli* with (lane 2) pYMA1.1, (lane 3) pSK and (lane 4) pYMA1.6. Note a band ($\approx$ 900 bases) corresponding to *cbmip* mRNA detected in both pYMA1.1 and pYMA1.6, as indicated by the arrow. RNA standards (kb) are indicated on the left.

plasmids pYMA1.42-1 and pYMA1.43-1 were constructed, in which the 15.0 and 15.5 kDa proteins were synthesized and individually isolated as fusion proteins with a nickel affinity column. The purified fusion proteins were assayed for PPIase

Figure 8 Comparison of PPIase activity of the recombinant proteins

PPIase activity at various concentrations of CbMip or its analogues was measured colorimetrically using succinyl-Ala-Phe-Pro-Phe-*p*-nitroanilide as the peptide substrate in the presence of α -chymotrypsin. The observed first-order rate constant K_{obs} for isomerization was calculated according to Harrison and Stein [31].

activity. All three of the purified proteins were shown to exhibit similar PPIase activities (Figure 8), demonstrating that the small proteins are also functional. Thus, they are Cb-FKBP analogues based on their enzymic activity and sequence homology.

	1				50
Hum - Fkbp				GVQVET	ISPGDGRTFP
Mou - Fkbp				GVQVET	ISPGDGRTFP
Neu - Fkbp			 MTI	PQLDGLQIEV	QQEGQGTRFT
Nei - Fkbp				..MGSLIIED	LQESFG.KEA
Cb - Fkbp	MQHKMKQTAQ	QNAEKSR AFL	TANKNKP GVK	TLANGLQYKV	LQAGQGQS.P
	I II				
	51	▼	▼	▼ ▼▼▼▼	▼ 100
Hum - Fkbp	KRGQTCVVHY	TG MLEDGKKF	DSSRDRNKPF	KFMLGKQEV I	RGWEEGVAQM
Mou - Fkbp	KRGQTCVVHY	TG MLEDGKKF	DSSRDRNKPF	KFVLGKQEV I	RGWEEGVAQM
Neu - Fkbp	RRGDNDVDVHY	KG VLTSGKKF	DASYDRGEPL	NFTVGQGQVI	KGWDEGLLGM
Nei - Fkbp	VKGKEITVHY	TG WLEDGTKF	DSSLDRRQPL	TITLGVGQVI	KGWDEGFGGM
Cb - Fkbp	TLNDEVTVNY	EGRLINGTVF	DSSYKRGNPA	TFPL..KSVI	KGWQEALTRM
	101	▼	▼	▼	147
Hum - Fkbp	SVGQRAKLTI	SPDYAYGATG	HPGIIPPHAT	LVFDVELLKL	E.....
Mou - Fkbp	SVGQRAKLTI	SPDYAYGATG	HPGIIPP NAT	LIFDVELLKL	E.....
Neu - Fkbp	KIGEKRLTI	APHLAYGNRA	VGGIIPANST	LIFETELVGI	KG VQKGE...
Nei - Fkbp	KEGGKRKLTI	PSEMGYGAHG	AGGVIPPHAT	LIFEVELLKV	YE.....
Cb - Fkbp	KPGAIWEIYV	PPQLAYGEQG	APGVIGPNEA	LIFKVNLI SV	KKK.....

Figure 7 Amino acid sequences of Cb-FKBP analogues I and II aligned with those of human (Hum), mouse (Mou), *N. crassa* (Neu) and *N. meningitides* (Nei)

The identical or conserved residues common to four or five of the five proteins are in bold. The triangles indicate those amino acids involved in FK506 binding [4].

Even though much of the data presented in this study involved expression of the *cbmip* gene in a heterologous system (*E. coli*), it has been possible to detect the presence of the FKBP analogues (15.0 and 15.5 kDa proteins) in *C. burnetii* cell extracts using mAbs specific to CbMip. However, the smaller proteins are detected only when the gels are loaded with a 10-fold higher concentration of *C. burnetii* than that required to detect the 23.5 kDa protein. It is interesting to speculate that some of the possible reasons for lower levels of the 15.5 and 15.0 kDa CbMip analogues could be that: (i) the smaller proteins may be tightly regulated and only synthesized during certain growth stages [38] or in an unique environmental niche of the organism; or that (ii) the levels of CbMip analogues may be extremely low, as this may be one mechanism of controlling isomerization of critical proteins in the cytoplasm that are destined for export. It remains to be seen if these possibilities are true once a well-defined genetic system to manipulate *C. burnetii* is established.

In conclusion, three proteins have been shown to be synthesized from the same ORF starting at different translation initiators (Figure 4). This demonstrates a unique mechanism of translational regulation for the obligate intracellular pathogen, *C. burnetii*. Such a translational regulation might be a mechanism to use more efficiently limited genetic information, particularly for an organism like *C. burnetii*, whose genome is only one-third the size of *E. coli*'s genome [39].

We thank M. E. Konkel and K. L. McIvor for critical reading of the manuscript and S. Schmitt for her technical assistance. The software cited is part of the VADMS Center, a campus-wide computer resource at Washington State University. This work was supported by grant AI20190 from the National Institutes of Health.

REFERENCES

- Hacker, J. and Fischer, G. (1993) Mol. Microbiol. **10**, 445–456
- Schreiber, S. L. (1991) Science **251**, 283–287
- O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J. and O'Neil, E. A. (1992) Nature (London) **357**, 692–694
- Eitzkorn, F. A., Stoley, L. A., Chang, Z. and Walsh, C. T. (1993) Curr. Opin. Struct. Biol. **3**, 929–933
- Fischer, G. and Schmid, F. X. (1990) Biochemistry **29**, 2205–2212
- Baker, E. K., Colley, N. J. and Zuker, C. S. (1994) EMBO J. **13**, 4886–4895
- Rassow, J., Mohrs, K., Koidl, S., Barthelmess, I. B., Pfanner, N. and Tropschug, M. (1995) Mol. Cell. Biol. **15**, 2654–2662
- Clipstone, N. A. and Crabtree, G. R. (1992) Nature (London) **357**, 695–697
- Engleberg, N. C., Carter, C., Weber, D. R., Cianciotto, N. P. and Eisenstein, B. I. (1989) Infect. Immun. **52**, 1263–1270
- Lundemose, A. G., Rouch, D. A., Birkelund, S., Christiansen, G. and Pearce, J. H. (1992) Mol. Microbiol. **6**, 2539–2548
- Cianciotto, N. P., O'Connell, W., Dasch, G. H. and Mallavia, L. P. (1995) Curr. Microbiol. **30**, 149–153
- Cianciotto, N. P. and Fields, B. S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 5188–5191
- Sampson, B. A. and Gotschlich, E. C. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 1164–1168
- McAllister, C. F. and Stephens, D. S. (1993) Mol. Microbiol. **10**, 13–23
- Schmidt, B., Tradler, T., Rahfeld, J.-U., Ludwig, B., Jain, B., Mann, K., Rucknagel, K. P., Janowski, B., Schierhorn, A., Kullertz, G. et al. (1996) Mol. Microbiol. **21**, 1147–1160
- Hyano, T., Takahashi, N., Kato, S., Maki, N. and Suzuki, M. (1991) Biochemistry **30**, 3041–3048
- Natsoulis, G., Hilger, F. and Fink, G. R. (1986) Cell **46**, 235–243
- Oda, T., Funai, T. and Ichiyama, A. (1990) J. Biol. Chem. **265**, 7513–7519
- Gillman, E. C., Slusher, L. B., Martin, N. C. and Hopper, A. K. (1991) Mol. Cell. Biol. **11**, 2382–2390
- Park, S. K., Kim, K., Woo, K. M., Seol, J. H., Tanaka, K., Ichihara, A., Ha, D. B. and Chung, C. H. (1993) J. Biol. Chem. **268**, 20170–20174
- Baca, O. G. and Paretsky, D. (1983) Microbiol. Rev. **47**, 127–149
- Mo, Y., Cianciotto, N. P. and Mallavia, L. P. (1995) Microbiology **141**, 2861–2871
- Amann, E., Brosius, J. and Ptashne, M. (1983) Gene **25**, 167–178
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, W. (1990) Methods Enzymol. **185**, 60–89
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Hendrix, L. R., Samuel, J. E. and Mallavia, L. P. (1991) J. Gen. Microbiol. **137**, 269–276
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor
- Aydintug, M. K., Inzana, T. J., Letonja, T., Davis, W. C. and Corbeil, L. B. (1989) J. Infect. Dis. **160**, 846–857
- Manoil, C. and Beckwith, J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8129–8133
- Kofron, J. L., Kuzmic, P., Kishore, V., Colon-Bonilla, E. and Rich, D. H. (1991) Biochemistry **30**, 6127–6134
- Harrison, R. K. and Stein, R. L. (1990) Biochemistry **29**, 3813–3816
- Maloy, S. R., Stewart, V. J. and Taylor, R. K. (1996) in Genetic Analysis of Pathogenic Bacteria: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor
- Rothman, J. E. (1989) Cell **59**, 591–601
- Standaert, R. F., Galat, A., Verdine, G. L. and Schreiber, S. L. (1990) Nature (London) **346**, 671–674
- Nelson, P. A., Lippke, J. A., Murcko, M. A., Rosborough, S. L. and Peattie, D. A. (1991) Gene **109**, 255–258
- Tropschug, M., Wachter, E., Mayer, S., Schonbrunner, E. R. and Schmid, F. X. (1990) Nature (London) **346**, 674–677
- GCG (1991) Program Manual for GCG Package, Version 7, Madison, WI
- McCaul, T. F. (1991) in Q Fever: The Biology of *Coxiella burnetii* (Williams, J. C. and Thompson, H. A., eds.), pp. 223–258, CRC Press, Boca Raton
- Myers, W. F., Baca, O. G. and Wissemann, Jr., C. L. (1980) J. Bacteriol. **144**, 460–461