Mip protein of *Legionella pneumophila* exhibits peptidyl-prolyl-*cis/trans* isomerase (PPlase) activity

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Summary

Legionella pneumophila **is an intracellular parasite which is able to survive and multiply in human monocytes and alveolar macrophages. The Mip (macrophage infectivity potentiator) protein has been shown to be an essential virulence factor. A search of translated nucleic acid data bases has shown that the Mip protein from strain Wadsworth possesses regions homologous to those found in the FK506-binding proteins (FKBPs) of several different eukaryotic organisms. FKBPs are able to bind to the immunosuppressant macrolide FK506 and possess peptidyl-prolyl** *cis/trans* **isomerase (PPlase) activity. The gene coding for the Mip protein was cloned from the chromosome of** *L. pneumophila* **strain Philadelphia I and sequenced. It was synthesized in** *Escherichia coli* **K-12 and after purification it exhibited PPlase activity catalysing the slow** *cis/trans* **isomerization of prolyl peptide bonds in oligopeptides. Mip is inhibited by FK506 and fully resistant to cyclosporin A, as was also found for the recently characterized FKBP-type PPlases of eukaryotes. However, the** *N*-**terminal extension of Mip and/or the substitutions of the variable amino acids in the** *C*-**terminal FKBP core lead to variations, when compared with eukaryotic FKBPs, in substrate specificity with the oligopeptide substrates of type Suc–Ala–Xaa–Pro–Phe–4-nitroanilide. Nevertheless, the** *Legionella* **Mip factor represents a bacterial gene product which shares some characteristics normally found in eukaryotic proteins. In view of the** activity of PPlases in protein-folding reactions, such prokaryotic FKBP analogues may represent a new class of bacterial pathogenicity factors.

Introduction

Legionella pneumophila is the aetiological agent of Legionnaires' disease, a distinct form of pneumoniae in humans (McDade *et al.*, 1977; Winn, 1988; Horwitz, 1988). The bacterium multiplies intracellularly in human blood monocytes, human alveolar macrophages and tissue-culture cells. *L. pneumophila* is phagocytosed by an unusual coiling phagocytosis mechanism (Horwitz, 1984) and is able to inhibit phagosome–lysosome fusion and phagosome acidification of the phagocytes (Horwitz, 1983; Horwitz and Maxfield, 1984; Horwitz, 1988).

In order to identify the gene products involved in the pathogenesis of *L. pneumophila* (for a review, see Engleberg and Eisenstein, 1991) the determinants coding for two toxins and for three different protein antigens have been cloned and sequenced (Black *et al.*, 1990; Wintermeyer *et al.*, 1992; in preparation; Hoffman *et al.*, 1990; Engleberg *et al.*, 1989; Ludwig *et al.*, 1991). To date, only the Mip (macrophage infectivity potentiator) protein, a polypeptide of 24 kDa, is known to contribute to the virulence of *L. pneumophila* (Cianciotto *et al.*, 1989; 1990b). Using isogenic *Legionella* strains with and without an intact *mip* gene it was shown that Mip potentiates the uptake of *L. pneumophila* by macrophages and/or contributes to early survival processes in the phagocytic cell. The amino acid sequence of the *C*-terminal part of the Mip protein was deduced from the DNA sequence of the *mip* gene from strain Wadsworth (Engleberg *et al.*, 1989) and revealed homology to the sequences of FK506-binding proteins (FKBPs) found in various eukaryotic organisms (Standaert *et al.*, 1990; Maki *et al.*, 1990; Tropschug *et al.*, 1990; Wiederrecht *et al.*, 1991; Jin *et al.*, 1991; Lane *et al.*, 1991). The eukaryotic FKBPs are able to bind to the immunosuppressant macrolide, FK506. FKBPs and cyclophilins form the substance class of the immunophilins (Schreiber, 1991). The latter have the capacity to bind cyclosporin A (Fischer *et al.*, 1989; Price *et al.*, 1991). Immunophilins possess peptidyl–prolyl *cis/trans* isomerase (PPlase) activity and catalyse the slow isomerization of prolyl bonds in oligopeptides and proteins *in*
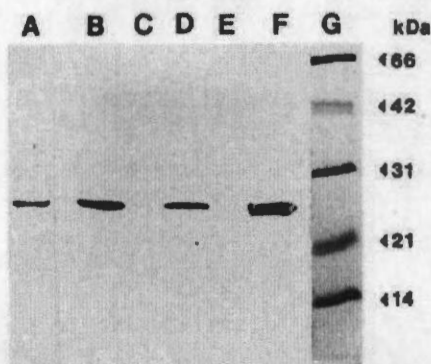
Fig. 1. Western blot analysis of cell extracts and Mip protein using anti-serum prepared against the Mip-positive *E. coli* K-12 clone HB101 (pBLL3039). Lanes: A, cell extract of *L. pneumophila* Philadelphia I; B, cell extract of *E. coli* K-12 (pBLL3039); C, cell extract of *E. coli* K-12 (pLAFR2) (control); D, cell extract of *E. coli* K-12 (pBLL106); E, cell extract of *E. coli* K-12 (pBR322) (control); F, purified Mip protein of *E. coli* K-12 (pBLL106); G, molecular weight markers (BioRad Laboratories).

vitro (Lang *et al.*, 1987; Fischer and Schmid, 1990; Tropschug *et al.*, 1989). Recently, it has been suggested that the timing of the triple-helix formation of procollagen I in chick-embryo tendon fibroblasts is related to PPIase activity *in vivo* (Steinman *et al.*, 1991). In addition, immunophilins and their complexes with FK506 or cyclosporin A seem to play a key role in the signal-transduction pathways of T cells (Schreiber, 1991).

In order to test whether both PPIase activity and FK506 sensitivity are conserved within the *Legionella* Mip protein, we have cloned and sequenced the corresponding

gene from strain *L. pneumophila* Philadelphia I and have purified the protein. It is shown that Mip has PPIase activity which approximates the same level as that found in the eukaryotic FKBPs. The PPIase activity of Mip is strongly inhibited by 100 nM FK506 but is completely resistant to micromolar concentrations of cyclosporin A.

Results

Cloning and expression in Escherichia coli K-12 of the mip gene from L. pneumophila strain Philadelphia I

A genomic library of *L. pneumophila* Philadelphia I (Hacker *et al.*, 1991; Ludwig *et al.*, 1991) was screened by immuno-colony blotting using anti-*Legionella*-specific antiserum. Seventy-six clones reacted strongly and the cell lysates were further analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting. Proteins of 24 kDa were produced by 21 recombinant clones. DNA isolated from each of these clones was found to react in a DNA–DNA dot blot using a 30-mer oligonucleotide probe with a *mip*-specific sequence (Engleberg *et al.*, 1989). A *Bam*HI–*Cla*I DNA fragment of 4.5 kb was subcloned from one of the cosmid clones, HB101(pBLL3039), into the vector pBR322. Western blots with a polyclonal monospecific anti-Mip-antiserum showed that the resulting clone, HB101(pBLL106), produced the Mip protein (Fig. 1).


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    ATGAAGATGAAATTGGTGACTGCAGCTGTTATGGGGCTTGCAATGTCAACAGCAATGGCT
-20  M  K  M  K  L  V  T  A  A  V  M  G  L  A  M  S  T  A  M  A  -1

    GCAACCGATGCCACATCATTAGCTACAGACAAGGATAAGTTGTCTTATAGCATTGGTGCC
+1   A  T  D  A  T  S  L  A  T  D  K  D  K  L  S  Y  S  I  G  A

    GATTTGGGGAAGAATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAATGGCTAAA
21   D  L  G  K  N  F  K  N  Q  G  I  D  V  N  P  E  A  M  A  K

    GGCATGCAAGACGCTATGAGTGGCGCTCAATTGGCTTTAACCGAACAGCAAATGAAAGAC
41   G  M  Q  D  A  M  S  G  A  Q  L  A  L  T  E  Q  Q  M  K  D

    GTTCTTAACAAGTTTCAGAAAGATTTGATGGCTAAGCGTACTGCTGAATTCAATAAGAAA
61   V  L  N  K  F  Q  K  D  L  M  A  K  R  T  A  E  F  N  K  K

    GCGGATGAAAATAAAGTAAAAGGGGAAGCCTTTTTAACTGAAAACAAAAACAAGCCAGGC
81   A  D  E  N  K  V  K  G  E  A  F  L  T  E  N  K  N  K  P  G

    GTTGTTGTATTGCCAAGTGGTTTGCAATACAAAGTAATCAATTCTGGAAATGGTGTTAAA
101  V  V  V  L  P  S  G  L  Q  Y  K  V  I  N  S  G  N  G  V  K

    CCCGGAAAATCGGATACAGTCACTGTCGAATATACTGGTCGTCTGATTGATGGTACCGTT
121  P  G  K  S  D  T  V  T  V  E  Y  T  G  R  L  I  D  G  T  V

    TTTGACAGTACCGAAAAAAACTGGTAAGCCAGCAACTTTCCAGGTTTCACAAGTTATCCCT
141  F  D  S  T  E  K  T  G  K  P  A  T  F  Q  V  S  Q  V  I  P

    GGATGGACAGAAGCTTTGCAATTGATGCCAGCTGGATCAACTTGGGAAATTTATGTTCCC
161  G  W  T  E  A  L  Q  L  M  P  A  G  S  T  W  E  I  Y  V  P

    TCAGGTCTTGCATATGGCCCACGTAGCGTTGGCGGACCTATTGGCCCAAATGAAACTTTA
181  S  G  L  A  Y  G  P  R  S  V  G  G  P  I  G  P  N  E  T  L

    ATATTTAAAATTCACTTAATTTCAGTGAAAAAATCATCTTAA
201  I  F  K  I  H  L  I  S  V  K  K  S  S  -
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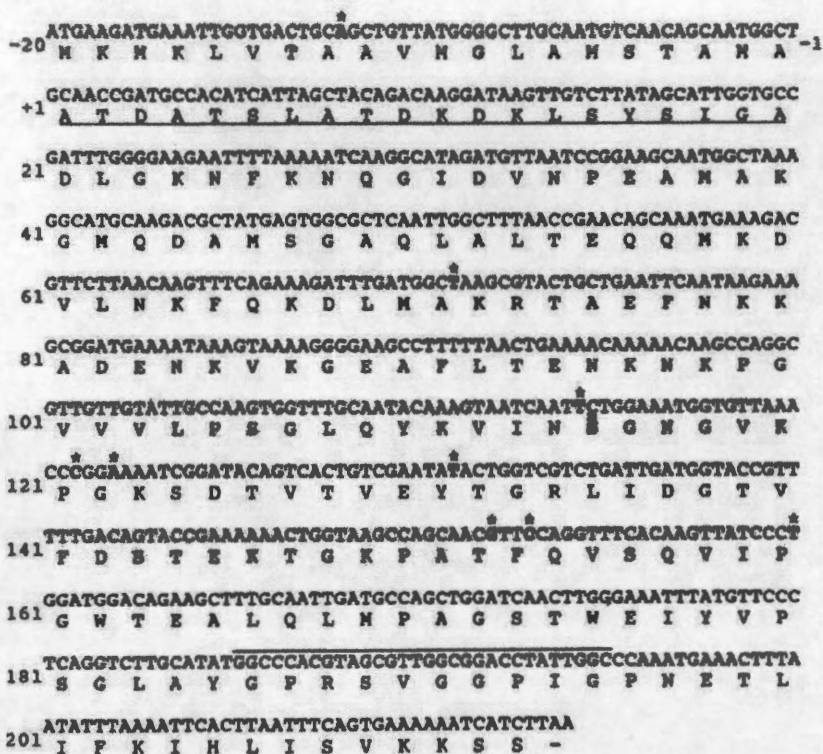
Fig. 2. Nucleotide and protein sequence of the *mip* gene from strain *L. pneumophila* Philadelphia I. The nine nucleotides and the amino acid at position 115 which differ from the published sequence of strain Wadsworth are indicated in bold type and by asterisks. The N-terminal amino acid sequence underlined was determined by Edman degradation. The overlined nucleotides represent the oligonucleotide sequence used for DNA–DNA hybridization. These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries.
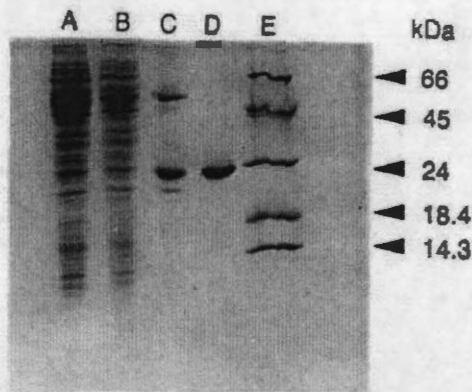
Fig. 3. Purification of recombinant Mip protein. The various fractions were subjected to SDS–PAGE and protein was stained with Coomassie brilliant blue. Lanes: A, crude cell extract of subclone HB101(pBLL106); B, Mip containing fractions after DEAE-sephacel passage; C, Affigel-blue chromatography; D, combined phenyl-sepharose fractions; F, molecular weight markers (Sigma).

DNA sequence of the mip determinant of strain L. pneumophila *Philadelphia I*

The DNA sequence of the *mip* gene from strain Philadelphia I was determined (Fig. 2). This sequence revealed nine nucleotide substitutions relative to the *mip* sequence of strain Wadsworth (Engleberg *et al.*, 1989). One of these substitutions resulted in the replacement of an alanine at amino acid position 115 with serine. The other eight nucleotide substitutions did not lead to alterations of the Mip amino acid sequence.

Isolation of the recombinant Mip protein

As shown in Fig. 3 and lane F of Fig. 1, the Mip protein of approximately 24 kDa produced by clone HB101 (pBLL106) was isolated and obtained in a purified form using a four-step procedure. Table 1 summarizes the steps and yields as well as the enrichment factors thus obtained. No contaminating cyclophilin-like *E. coli* PPIases could be detected in the final Mip preparation.

Table 1. Purification steps leading to homogeneous Mip-PPIase.

Purification step	Total protein (mg)	Total Mip activity (arbitrary units)	Specific activity (arbitrary units per mg protein)	Recovery (%)
DEAE-Sephacel[a]	275	4152	15	100
TSK Affigel Blue	4.7	1020	215	30
Phenyl-Sepharose	0.61	346	568	12

a. Mip activity measured toward Suc–Ala–Pro–Phe-4-nitroanilide; differentiation of Mip enzymatic activity from *E. coli* PPIase is only possible after the DEAE-Sephacel step

N-*terminal protein sequence of Mip*

In order to demonstrate that the isolated 24 kDa polypeptide was indeed Mip, *N*-terminal sequencing of the mature protein was undertaken. As indicated in Fig. 2, the initial amino acids of the mature protein are alanine, threonine, and aspartate. This clearly demonstrates a cleavage of the first 20 amino acids following transport. Each of the 20 *N*-terminal amino acid residues of the mature Mip protein are identical to those predicted from the DNA sequence of *mip* indicated in Fig. 2.
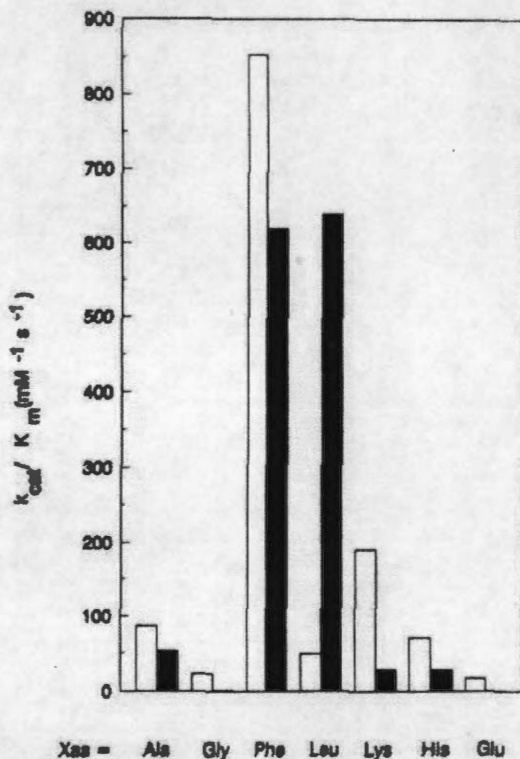

Fig 4. Comparison of the specificity constants k_{cat}/K_m of Mip PPIase (40–293 nM) activity (open columns) and human FKBP (shadowed columns, data from Harrison and Stein, 1990) toward Suc–Ala–Xaa–Pro–Phe-4-nitroanilide (pH 7.8; 0.035 M^{-1} HEPES buffer) at 10.0°C.

Determination of PPIase activity of Mip

The Mip protein was tested for PPIase activity upon Suc–Ala–Xaa–Pro–Phe-4-nitroanilide with the aid of isomer-specific proteolysis using chymotrypsin as the protease. The particular conditions of the assay result in k_{cat}/K_m for the *cis/trans* isomerization of the –Xaa–Pro bond.

We found enzymatic activity which slightly exceeds the highest value of k_{cat}/K_m observed for eukaryotic FKBPs (Fig. 4) using the most favoured substrate, Suc–Ala–Phe–Pro–Phe-4-nitroanilide. Mip additionally shows an elevated activity towards a substrate with a Lys in the P_1 position (using the nomenclatural system proposed by Schechter and Berger (1967) for the reactive peptide
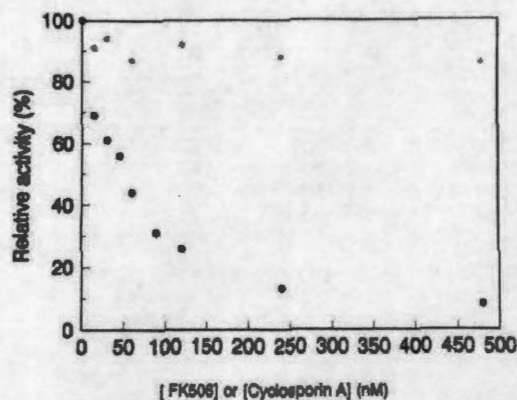
Fig. 5. PPIase activity as a function of the cyclosporin A (∗) and of the FK506 (o) concentration.

bond of oligopeptide substrates). The characteristic 5–10-fold drop in activity for the entire set of substrate peptides that was found in prokaryotic and eukaryotic cyclophilin-like PPIases (G. Fischer, H. Bang, M. A. Marahiel, R. Schönbrunner and F. X. Schmid, submitted for publication) is not observed with FKBPs.

More interestingly, Mip lacks the pronounced Leu specificity for the P_1 position demonstrated for human FKBP (Harrison and Stein, 1990). A 600-fold decrease of k_{cat}/K_m was determined for human FKBP in Xaa=Gly *cis/trans* isomerization. This value, like that obtained using the human FKBP optimal substrate, was significantly reduced in Mip. This suggests that the hydrophobicity of the amino acid side chains cannot satisfactorily explain the influence of the P_1-amino acid on catalysis in Mip. The control strain HB101(pBR322) does not exhibit any PPIase activity with a specificity pattern similar to the Mip enzyme.

Inhibition studies with FK506 and cyclosporin A

As it was shown that all FKBPs possessing PPIase activity could be inhibited with nanomolar amounts of FK506 (Tropschug *et al.*, 1990; Siekierka *et al.*, 1990), we decided to examine the effect of increasing concentrations of FK506 as well as those of cyclosporin A, the inhibitor of cyclophilin-like PPIases, upon the enzymatic activity of Mip (Fig. 5). PPIase activity could be considerably influenced by FK506 although it was not significantly inhibited by cyclosporin A or by the non-immunosuppressive cyclosporin H. The IC_{50} value (50% inhibitory concentration) calculated from the data of Fig. 5 is about 45 nM, indicating that the K_i value may be even smaller.

Discussion

The amino acid sequence of the Mip protein, a virulence

factor of *L. pneumophila* (Cianciotto *et al.*, 1989), the aetiological agent of Legionnaires' disease in humans, shows homology to amino acid sequences of human, *Neurospora* and yeast FK506-binding proteins (Standaert *et al.*, 1990; Maki *et al.*, 1990; Tropschug *et al.*, 1990; Wiederrecht *et al.*, 1991; Jin *et al.* 1991; Lane *et al.*, 1991; see Fig. 6). Cyclophilins and eukaryotic FKBPs represent a family of peptidyl–prolyl *cis/trans* isomerases (PPIases) termed immunophilins which can catalyse slow *cis/trans* interconversion of prolyl imidic peptide bonds in oligopeptides and proteins (Lang *et al.*, 1987; Fischer *et al.*, 1989; Fischer and Schmid, 1990; Tropschug *et al.*, 1989; Price *et al.*, 1991; Schreiber, 1991).

When we tested the recombinant Mip protein cloned from *L. pneumophila* strain Philadelphia I we found PPIase activity comparable in magnitude to other cyclophilin-type prokaryotic PPIases and to eukaryotic FKBPs. The comparison of the P_1 subsite specificity of the recombinant Mip with human FKBP (Fig. 4) results in a slightly different pattern of k_{cat}/K_m. The highest catalytic constant that could be obtained for the peptide with Xaa=Phe ($850\ 000\ M^{-1}\ s^{-1}$) exhibits an 18-fold lower activity when compared with the highest activity yet measured for a PPIase (pig kidney 17.8 kDa cyclophilin acting on Suc–Ala–Ser–Pro–Phe-4-nitroanilide). The clear dependence of k_{cat}/K_m on hydrophobicity of the P_1 amino acid side chain using human FKBP (Harrison and Stein, 1990) is not valid in Mip catalysis as even charged side chains can fulfil the requirements for favourable catalytic interactions.

The inhibitory effect of FK506 on Mip is similar of that observed for human FKBP although the interaction may be slightly weaker. However, the large PPIase concentration of 47 nM used in our inhibition assay does not allow us to determine a K_i range much smaller than this value. As in the case of P_1 subsite specifity, this may result from either the N-terminal extension of Mip or the variable amino acids located in the FKBP analogue C-terminal part (Fig. 6).

As indicated in Fig. 6, unidentified open reading frames (URFs) in the genome of the bacterial pathogens *Neisseria meningitidis* (Perry *et al.*, 1987) and *Pseudomonas aeruginosa* (Kato *et al.*, 1989) resembling FKBPs are located in close proximity to virulence-associated loci, near two silent copies of truncated pilin genes in *N. meningitidis* and a region encoding regulatory proteins involved in the alginate biosynthesis of *P. aeruginosa*. Furthermore, Mip of *L. pneumophila* is closely related to a homologous protein of *Legionella micdadei* (Bangsborg *et al.*, 1991) and to the L2 protein of *Chlamydia trachomatis* (Lundemose *et al.*, 1991). Both are pathogenic organisms that exhibit virulence features similiar to those of *L. pneumophila* (Moulder, 1985; Horwitz, 1988). The possibility that putative gene products of the URFs and of

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L.pneumophila phiII Mip  ..AT------ ---DATSLAT DKDKLSYSIG ADLGKMFKNQ GIDVNPEAMA KGMQDAMSG- AQLALTEQQM KDVLNKFQKD

Human (cy) FKBP          .......... .......... .......... .......... .......... .......... .......... ..........
Human (mem) FKBP         .......... .......... .......... .......... .......... .......... .......... ..........
Bovine FKBP              .......... .......... .......... .......... .......... .......... .......... ..........
S.cerevisiae FKBP        .......... .......... .......... .......... .......... .......... .......... ..........
N.crassa FKBP            .......... .......... .......... .......... .......... .......... .......... ..........
N.meningitidis URF       .......... .......... .......... .......... .......... .......... .......... ..........
C.trachomatis L2protein  .......... .......... .......... .......... .......... ......LVEV IKGNQSEIDG QSAPLTDTE. ------YEKQ
L.micdadei Mip           ..ATATTDAT TSAPGTSLTT DTEKLSYSIG ADLGKMFKKQ GIEISPAAMA KGLQDGMSG- GQLLLTDDQM KDVLMKFQKD
P.aeruginosa URF         .......... ........AP PKDELAYAVG ARLGTRLQQE MPDLELSELL LGLRQAYRG- EALEIPPERI EQLL--LQHE
Consensus sequence       ..AT...... ....*TSL.. ..*.L.Y.*G A.LG..*..* ..**.....* ....*,.... ..*.*..... .**LNK.***


L.pneumophila phiII Mip  LMAKRTABFN KKADBNKVKG BAFLTBNKNK PGVVVLPSGL -QYKVINSGN GVRPGKSD-- TVTVEYTGRL IDGTVFDSTB

Human (cy) FKBP          .......... .......... .......... ......MGV -QVETISPGD GRTFPKRGQ- TCVVHYTGML EDGKKFDSSR
Human (mem) FKBP         .......... .......... .......... ..TGABGKRK LQIGVKKRVD HCPIKSRKGD VLHMHYTGKL EDGTEFDSSL
Bovine FKBP             .......... .......... .......... ......MGV -QVETISPGD GRTFPKRGQ- TCVVHYTGML EDGKKFDSSR
S.cerevisiae FKBP       .......... .......... .......... ..MSEVIEGN VKIDRISPGD GATFPKTGD- LVTIHYTGTL ENGQKFDSSV
N.crassa FKBP           .......... .......... .......... .MTIPQLDGL -QIEVQQEGQ GTRETRRGD- NVDVHYKGVL TSGKKFDASY
N.meningitidis URF       .......... .......... .......... ......MGS LIIEDLQESF GKRAVKGK-- EITVHYTGWL EDGTKFDSSL
C.trachomatis L2protein MAEVQKASFE AKCSENLASA BEFLKBNKEK AGVIELEPNK LHDRVVKEGT GRVLSGKP-- TALLHYTGSF IDGKVFDSSB
L.micdadei Mip          LMDKRSABFN KKABBNKSKG BAFLNBMKSK EGVVSLPSGL -QYNILERGD GAKPTKDD-- VVTVBYTGKL XDGQVFDSTB
P.aeruginosa URF        NATTETPRIT PABAR----- --FLANBKAR F---GVRBLT GGVLVSELRR GQGNGIGAAT QVHVRXRGLL ADGQVFDQSB
Consensus sequence       .........*. .....N...* B.FL.**K.* .......... .......... .......... .....Y.G.. ..G..FD.*.


L.pneumophila phiII Mip  KTGKPATFQV S--QVIPGWT BALQLMPAGS TWHIYVPSGL AYGPRSVGGP IGPMETLIFK IHLISVKKSS ..........

Human (cy) FKBP          DRNKPFKFML GKQEVIRGWE BGVAQMSVGQ RAKLTISPDY AYGATGHPGI IPPHATLVFD VELLKLE... ..........
Human (mem) FKBP         PQNQPFVFSL GTGQVIKGWD QGLLGMCEGE KRKLVIPBEL GYGERGAPPK IPGGATLVFE VELLKIERRT EL........
Bovine FKBP             DRNKPFKFVL GKQEVIRGWE BGVAQMSVGQ RAKLTISPDY AYGATGHPGI IPPHATLIFD VELLKLE... ..........
S.cerevisiae FKBP       DRGSPFQCNI GVGQVIKGWD VGIPKLSVGE KARLTIPGPY AYGPRGFPGL IPPMSTLVFD VELLKVN....
N.crassa FKBP           DRGEPLNFTV GQGQVIKGWD BGLLGMKIGE KRKLTIAPHL AYGNRAVGGI IPANSTLIFE TELVGIKGVQ KGE.......
N.meningitidis URF       DRRQPLTITL GVGQVIKGWD BGFGGMKEGG KRKLTIPSEM GYGATRRGGV IPPHATLIFE VELLKVYE.. ..........
C.trachomatis L2protein KNKEPILLPL TK--VIPGFS QGMQGMKEGE VRVLYIHPDL AYGT---AGQ LPPMSLLIFE VKLIEANDDN VSVTE.....
L.micdadei Mip          KTGKPATFKV S--QVIPGWT BALQLMPAGS TWBVYIPSNL AYGPRSVGGP IGPMBTLIFK IHLISVKKSD A.........
P.aeruginosa URF        SAEWFALDS. ----VIEGWR TALRAMPVGA RWRVVIPSAQ AYGHEGAGDL IPPDAPLVFE IDLLGFR... ..........
Consensus sequence       .....*...* ...*VI.G*. .*......G. ...*.*.... *YG....... *.....L*F. ..L*.*.... ..........
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Fig. 6. Comparison of the amino acid sequences of FKBPs exhibiting PPIase activity (*Neurospora crassa*, *Saccharomyces cerevisiae*, human Jurkat T cells [Cy FKBP], human cancer cell line [mem FKBP], and bovine calf thymus cells), and amino acid sequences of the mature Mip-like protein of *L. micdadei*, the C-terminal part of the L2 protein of *Chlamydia trachomatis*, parts of unidentified open reading frames (URFs) of *Neisseria meningitidis* and *Pseudomonas aeruginosa* and the mature *L. pneumophila* (L.p.) Mip protein of strain Philadelphia I. Identical amino acid residues are indicated in bold type. Dashes represent gaps for optimal alignment. Isofunctional replacements are indicated by asterisks. Abbreviations: cy, cytoplasmic; mem, membrane-bound.

the *L. micdadei mip* as well as of the L2 locus also influence the pathogenicity of the respective organisms cannot be excluded.

It has been shown recently that a genetically engineered *mip* mutant of *L. pneumophila* strain Wadsworth was defective in its ability to initiate macrophage infection (Cianciotto *et al.*, 1989; 1990b). Cloning of the *mip* locus from another pathogenic *Legionella* isolate, Philadelphia I (Fig. 2), confirms the view that *mip* is highly conserved among all *Legionella* strains (Cianciotto *et al.*, 1990a). This suggests a common function for the protein in different isolates. The role of Mip enzymatic activity in *Legionella* virulence has yet to be elucidated. It is possible that the Mip protein modifies bacterial cell structures or surface proteins of the phagocytic cell, thereby enhancing their phagocytic capacity. A further possibility is that the Mip protein activity in the phagocytic cell could modify proteins to inhibit phagosome–lysosome fusion and acidification of the vacuole (Horwitz, 1983; Horwitz and Maxfield, 1984). Alternatively, a deregulation of signal-pathway proteins in phagocytes cannot be ruled out.

A large number of extracellular substances with enzymatic activities such as phospholipases, lecitinases, proteases or sugar transferases, some of which are membrane-associated, have been shown to contribute to the pathogenicity of bacteria (see Finlay and Falkow, 1989). The *Legionella* Mip protein, exhibiting PPIase activity reminiscent of that found in FKBPs, is an example of a bacterial virulence factor possessing characteristics normally observed in eukaryotes. A further example of a protein in pathogenic bacteria with enzymatic capacity normally ascribed to eukaryotes was recently detected in *Yersinia*. This genus comprises the species *Yersinia pestis*, the causative agent of plague. The YopH protein of *Yersinia* exhibits tyrosine phosphatase activity which is able to influence the phosphorylation pattern of the eukaryotic cell (Guan and Dixon, 1990; Bliska *et al.*, 1991). Like *Legionella*, *Yersinia* is also able to replicate intracellularly. One may speculate that prokaryotic proteins with enzymatic functions related to eukaryotic cell signal factors represent a new general class of gene products which contribute to survival and dissemination of intracellular bacterial pathogens.

Experimental procedures

Media, enzymes and chemicals

Legionella strains were cultivated on buffered charcoal–yeast

extract (BCYE) agar plates at 37°C in a 5% CO_2 atmosphere for 48 h prior to use. E. coli K-12 strains were grown in L-broth. Radiochemicals were purchased from NEN Research Products. Restriction enzymes, T4 polynucleotide kinase and T4 ligase were obtained from Pharmacia, Boehringer, Biolabs, and Gibco BRL. All other chemicals were obtained from Merck, Difco, BRL, Oxoid, Roth, and Serva. Antibiotics were from Serva or Sigma.

Bacterial strains and plasmids

The genomic library was constructed from L. pneumophila strain Philadelphia I (McDade et al., 1977). For cloning experiments, the E. coli K-12 strain HB101 was used. Plasmid pLAFR2 is a cosmid vector described previously (Knapp and Mekalanos, 1988). For subcloning, vector pBR322 (Bolivar et al., 1977) was used. Antibiotics were added at concentrations of 50 µg ml⁻¹ (ampicillin) and 10 µg ml⁻¹ (tetracycline).

Cosmid cloning procedure

Legionella-specific chromosomal DNA was isolated as described (Ludwig et al., 1991; Wintermeyer et al., 1991). Large (20 kb) chromosomal Sau3A fragments were ligated into the BamHI site of cosmid pLAFR2 and transduced into HB101 with the help of the cosmid packaging system (Hohn and Collins, 1980).

Recombinant DNA techniques

Plasmid DNA was isolated as described (Birnboim and Doly, 1979). For restriction enzyme analysis, DNA was treated with appropriate enzymes and the resulting fragments were separated by gel electrophoresis on 0.8–1.0% agarose gels (Sambrook et al., 1989). DNA fragments were isolated from the agarose gel with the help of the GeneClean kit from Bio101 as described by a protocol of the manufacturer. For cloning, DNA fragments were ligated into suitable vector molecules after heat-inactivation of the restriction endonucleases at 65°C for 10 min (Sambrook et al., 1989). E. coli K-12 strains were transformed by the $CaCl_2$ method (Lederberg and Cohen, 1974).

Oligonucleotide synthesis

Oligonucleo' 'les used for DNA sequencing and DNA–DNA dot blotting wer؛ synthesized with an Applied Biosystems 380A DNA synthesizer using the phosphoramidite method of Beaucage and Caruthers (1981). Oligonucleotides were purified with oligonucleotide purification cartridges from Applied Biosystems.

DNA sequencing

The sequence of the mip-specific DNA region was determined with the help of a sequencing kit from Boehringer, as described by a protocol of the manufacturer.

Computer analysis

The programs used for compiling the nucleotide sequence data were from J. Devereux (University of Wisconsin Genetics Computing Group).

DNA–DNA dot blotting

As a mip-specific probe the oligonucleotide 5'-GGCCCACG-TAGCGTTGGCGGACCTATTGGC-3' (see Fig. 2) was used. The probe was labelled by T4 polynucleotide kinase using [γ³²-P]-ATP. The DNA dot blotting procedure and hybridization were carried out according to Sambrook et al. (1989).

Preparation of antisera

The antigen used for production of the L. pneumophila-specific antiserum was prepared from heat-killed L. pneumophila strains grown on agar plates as described (Hacker et al., 1991). Antiserum was prepared by intravenous injection of the antigen into New Zealand rabbits. The final immunofluorescence titre (IFT) of the antiserum was 1:1024. Antiserum specific for the cloned Mip protein of L. pneumophila was prepared from E. coli K-12 clone HB101(pBLL3039). The final IFT was 1:512. To remove the E. coli K-12-specific antibodies the antiserum was absorbed with a suspension of HB101(pLAFR2).

Immuno-colony blotting

Immuno-colony blots were made as described by Van Die et al. (1985).

Western blotting

For Western blotting, 10 ml of overnight culture of the recombinant E. coli K-12 clones or an equal amount of L. pneumophila cells harvested from agar plates was pelleted by centrifugation at 4°C for 10 min and washed with 10 mM ice-cold HEPES buffer (pH 7.4). Cell pellets were suspended in 1 ml 10 mM HEPES buffer (pH 7.4). The suspensions were passed through a French press (3 ×6210 kPa) and residual cells were removed by centrifugation. Proteins were analysed by SDS–PAGE as described by Laemmli (1979). The Western blots were performed according to the method of Kyhse-Andersen (1984).

Protein sequencing

The protein was subjected to 12.5% SDS–PAGE and blotted on to PVDF membranes (Immobilon transfer; Millipore) as described by Choli et al. (1989). The Mip protein was sequenced in an Applied Biosystem 470 A gas-phase sequencer.

Isolation and purification of the Mip protein

For isolation of the Mip protein, cells from 10 l of overnight cultures of the recombinant E. coli K-12 clone HB101 (pBLL106) were pelleted by centrifugation at 4°C for 10 min at 10 000 r.p.m. and washed with 10 mM ice-cold HEPES buffer (pH 7.4). Bacterial cells were suspended in 50 ml of 10 mM HEPES buffer (pH 7.4), passed through a French press (3 × 6210 kPa) and cell debris were removed by centrifugation for 20 min at 20 000 r.p.m. (in a Kontron T 324 centrifuge; A 8.24 rotor). Polymin P (BASF) was added to the supernatant to

give a final concentration of 0.4%. After being stirred for 30 min, the solution was centrifuged as described above. The supernatant was applied to a DEAE-Sephacel column (2.5 cm i.d. × 35 cm) equilibrated in 35 mM HEPES (pH 7.8). In contrast to *E. coli* PPlase, the Mip protein does not bind to this material. Enzymatically active fractions were applied to a 1.5 × 15 cm Affigel-blue column (Merck) equilibrated with 35 mM HEPES (pH 7.8). Under such conditions the Mip protein adsorbs to the affinity column. Approximately 80% of the protein could be recovered when eluted with 200 ml of 2 mM Tricin (pH 8.5).

Mip activity was eluted by applying a linear gradient (300 ml total) of 0–400 mM KCl in 2 mM Tricine (pH 8.5). Active fractions (at approx. 240 mM KCl) were pooled and KCl was added to a total concentration of 0.5 M to the pool prior to pumping the sample on to a 0.5 × 5 cm phenyl-sepharose column in 2 mM Tricine, 0.5 M KCl, (pH 8.5). The following elution was then carried out using a 50-ml step gradient, starting with 2 mM Tricine (pH 8.5), followed by addition of 0.1% Triton X-100 and ending with 3% Triton X-100. The homogeneity of Mip was verified by silver-staining after SDS–PAGE. The recovery of Mip was about 10% with respect to the Mip enzymatic activity found after separation from the *E. coli* PPlases. For sequencing, salts were diluted out from the protein with water using Centricon C 10 filtration (Amicon).

PPlase assay

Enzymatic activity was measured in 0.035 M HEPES buffer (pH 7.8) at 10°C using the protease-coupled assay described previously (Fischer *et al.*, 1989). Briefly, with respect to the prolyl bond the substrates Succinyl-Ala-Xaa-Pro-Phe-4-nitroanilide (Bachem) exist at pH 7.8 in an equilibrium of about 5–20% *cis* and 80–95% *trans* conformer. Alpha-chymotrypsin cannot readily split off the 4-nitroaniline residue in the *cis* conformer. In the presence of 0.5–1.0 mg ml^{-1} α-chymotrypsin to perform rapid cleavage of the *trans* substrate it remains uncleaved in the solution. Because the *cis* form is subject to a slow isomerization reaction leading to the cleavable *trans* substrate, consumption of the total amount of the chromogenic peptide is achieved after several minutes. In the presence of PPlase activity the slow kinetic phase of appearance of 4-nitroaniline following the *trans* cleavage is a composite of the uncatalysed (k_1) and the PPlase-catalysed (k_{enz}) *cis* to *trans* interconversion. Thus, the first-order kinetics obtained can be described by the rate equation $v = k_{obs}$ [*cis*]; $k_{obs} = k_1 + k_{enz}$.

For all substrates used in the assay the range of total substrate concentrations was limited to 0.06–0.02 mM. Under the reaction conditions described above, the following rate constants of the uncatalysed *cis* to *trans* isomerization (k_1) have been determined: Xaa = Ala (0.0076 s^{-1}); Phe (0.0043 s^{-1}); Leu (0.0073 s^{-1}); Lys (0.0055 s^{-1}); Gly (0.0060 s^{-1}); His (0.0059 s^{-1}); Glu (0.0034 s^{-1}). Utilizing reaction conditions at high Mip-PPlase concentrations ($k_1 \ll k_{enz}$) and rapid monitoring of the slow phase of the reaction (first data points after 5 s) it could be shown that the time-course of the 4-nitroaniline appearance is strictly first-order in rate throughout the reaction. The result shows that the relationship $k_{obs}/$[PPlase] $= k_{cat}/K_m$ holds true for all of the substrates. This was more directly indicated for bovine FK506-sensitive PPlase since the Michaelis constant, K_m (0.52 mM *cis* isomer) of Suc-Ala-Leu-Pro-Phe-4-nitroanilide could be determined by measuring the V/S

dependence at high substrate concentrations (Kofron *et al.*, 1991).

The PPlase assay was performed by adding a 1 μl aliquot of the peptide dissolved in dimethylsulphoxide (DMSO) to 1.1 ml of the solution of α-chymotrypsin (Merck) in the buffer containing Mip-PPlase, too. The concentration of Mip was determined by the Bradford procedure (1976). Stock solutions of the inhibitors were made in EtOH/water (50%/50%, v/v). For the kinetic runs a Hewlett Packard 8452 diode array UV/VIS spectrophotometer was used for monitoring the time course of the difference in absorbance at 390 nm and 520 nm. First-order rate constants (k_{obs}) were calculated on the basis of 100–500 data points covering at least two half-lives of the reaction.

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