A 46 kDa integral membrane protein from *Mycobacterium leprae* resembles a number of bacterial and mammalian membrane transport proteins

Linda Oskam,¹ Rudy A. Hartskeerl,¹ Caroline J. Hermans,¹
Madeleine Y. L. de Wit,¹ Guus H. Jarings,¹ Robert D. Nicholls²
and Paul R. Klatser¹

Author for correspondence: P. R. Klatser. Tel: +31 205665441. Fax: +31 206971841.
e-mail: klatser@amc.uva.nl

¹ Royal Tropical Institute,
Department of Biomedical
Research, Meibergdreef
39, NL-1105 AZ
Amsterdam, The
Netherlands

² Case Western Reserve
University, School of
Medicine, Department of
Genetics, 10900 Euclid
Avenue, Cleveland, OH
44106, USA

In this paper we describe the nucleotide sequence of a 3.4 kbp region of the *Mycobacterium leprae* genome. This region contains an open reading frame of 1290 bp with a coding capacity for a protein of 46 179 Da, designated the 38L protein. Using antibodies against part of the 38L protein, we were able to demonstrate that the 38L protein is present in the membrane protein fraction of *M. leprae*. The 38L protein showed significant matches with a number of integral membrane proteins involved in the transport of small molecules through the cellular membrane. Among these are a human and a murine protein involved in melanin biosynthesis. The 38L protein might play a role in the hypopigmentation observed in leprosy patients.

Keywords: leprosy, melanin biosynthesis, membrane protein, *Mycobacterium leprae*, tyrosine transport

INTRODUCTION

Leprosy is still a major health problem in many countries, affecting an estimated 5.5 million people worldwide (Nordeen *et al.*, 1992). The disease manifests itself as a clinical-immunological spectrum. At one end of the spectrum patients with tuberculoid leprosy typify the resistant form with a relatively strong T cell response. At the opposite end of the spectrum patients with lepromatous leprosy show a strong antibody response but they are unable to mount a protective cellular immune response against *Mycobacterium leprae* (Ridley & Joplin, 1966). Thus, the cellular immune response plays a crucial role in the protection and resistance against leprosy.

The causative agent of leprosy, *M. leprae*, is an obligate intracellular parasite which cannot be grown outside an animal host. It is therefore difficult to obtain sizeable quantities of *M. leprae* for analysis. Despite this major drawback, a growing number of protein, carbohydrate and lipid antigens of *M. leprae* have been isolated and characterized over the last decade (for a survey see Young *et al.*, 1992). The recent publication of a genome map of

M. leprae and the availability of an ordered cosmid library (Eiglmeier *et al.*, 1993) are important steps in the direction of an organized effort to elucidate the structure and function of components from this organism.

Cell-envelope-associated proteins are of prime importance to the pathogenesis and immunogenicity of bacterial infection in man (Mehra *et al.*, 1989). The cell envelope of *M. leprae* embodies an outer cell wall and a cytoplasmic membrane. It consists of an intricate network of covalently and non-covalently linked lipids, complex carbohydrates, proteins and peptidoglycans (Gaylord & Brennan, 1987). Notwithstanding this complicated structure, Hunter *et al.* (1990) were able to purify the cell wall, cytoplasmic membrane and cytosolic protein fractions. It was shown that cell envelope protein preparations from *M. leprae* are capable of inducing a potent cell-mediated immune reaction to this mycobacterium (Mehra *et al.*, 1989; Melancon-Kaplan *et al.*, 1988). Strong and specific T cell responses to *M. leprae* cell envelope proteins were also demonstrated in paucibacillary leprosy patients and their contacts, but lack of such responses was observed in multibacillary leprosy patients (Roche *et al.*, 1992).

The characterization of genes encoding proteins present in the cell envelope might give us further insight into both the pathogenesis of *M. leprae* and the way in which

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protective immunity against this organism is induced. Here we describe the identification of an integral membrane protein of *M. leprae*, designated 38L.

METHODS

Bacterial strains, plasmid vectors and genetic libraries.

Escherichia coli DH5 α (*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1*) (Sambrook *et al.*, 1989) was used for all plasmid constructions except those in pQE30 (Diagen), for which *E. coli* M15(pREP4) (*lac ara gal mtl F*⁻) was used, and pCRII (Invitrogen), for which *E. coli* INV α F' (*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 λ F'* deoR⁺) was used. For isolation of the 38L gene use was made of a λ gt11::*M. leprae* library (Young *et al.*, 1985) and a library of the *M. leprae* genome in cosmid pHC79 (Clark-Curtiss *et al.*, 1985).

Culture media and antibiotics. *E. coli* strains were cultured in LB medium (Sambrook *et al.*, 1989). Ampicillin, kanamycin and tetracycline were used at final concentrations of 200, 25 and 10 μ g ml⁻¹, respectively.

Molecular cloning, sequencing and sequence analysis. DNA analysis and transformation of *E. coli* strains were performed as described by Sambrook *et al.* (1989). Nucleotide sequences were determined by the dideoxy chain-termination procedure on alkaline-denatured plasmid DNA. A set of nested deletion plasmids was derived using a nested deletions kit (Pharmacia-LKB).

Nucleotide sequences were analysed using the DNASIS software (Pharmacia-LKB). Nucleotide and amino acid homology computations were performed at the NCBI using the BLAST network service of the GCG software package (Devereux *et al.*, 1984). Protein hydrophathy profiles were determined using the method of Kyte & Doolittle (1982) from the PROSIS software package from Pharmacia-LKB.

Subcloning of 38L. PCR with heat-stable DNA-polymerase from *Thermus aquaticus* (*Taq*, Perkin Elmer) was done essentially as recommended by the manufacturer. Briefly, 1.5 U *Taq* polymerase was added to 50 μ l of a solution of 50 mM NaCl, 5 mM MgCl₂, 10 mM Tris/HCl, 0.2 mg BSA ml⁻¹, pH 9.6, containing 1 mM of each of the deoxynucleotides dATP, dCTP, dGTP and dTTP (Pharmacia-LKB) and 1 ng DNA and 200 ng of each primer. PCRs were performed in a Pharmacia-LKB gene ATAQ controller using 40 cycles. Each cycle consisted of denaturation of the DNA for 2 min at 94 °C, annealing of the primers for 2 min at 58 °C, and elongation for 3 min at 72 °C.

Primers 46III_f (GCGGATCCGTATCCAGTATCATAGAC-AAC) and 46III_r (GCGGATCCGCGGGTGAATTCCCA-GAAC) were used to amplify the region of the 38L gene encoding amino acids 328–404. The resulting product was cloned into the TA cloning vector pCRII. The region encoding amino acids 328–404 of the 38L gene was removed by digestion with *Bam*HI and cloned in-frame into the *Bam*HI site of pQE30, resulting in plasmid pTHL1095. Nucleotide sequencing across the junction sites was performed to check for the correctness of the insert.

Polypeptide overexpression, purification and rabbit immunization. An overnight culture of *E. coli* M15(pREP4) containing pTHL1095 was diluted 1:4 in fresh LB containing ampicillin and kanamycin. After 30 min incubation at 37 °C, IPTG was added to a final concentration of 2 mM. After 4–5 h cells were harvested from 50 ml culture and resuspended in 5 ml buffer B (8 M urea, 0.1 M NaH₂PO₄, 10 mM Tris, pH adjusted to 8.0 with NaOH) and stored at –20 °C until further use.

After thawing, the lysate was centrifuged and 2.5 ml of a 50 % (v/v) slurry of Ni-NTA resin (Diagen) was added to the

supernatant. After 30 min incubation at room temperature the resin was pelleted and washed three times with 5 ml buffer C (8 M urea, 0.1 M NaH₂PO₄, 10 mM Tris/HCl, pH 6.3). Buffer C (1 ml) containing 0.1 M EDTA was added to the resin and incubated for 10 min at room temperature followed by centrifugation. The supernatant was applied to a PD10 column (Pharmacia-LKB) in order to separate the polypeptide from the salts. The polypeptide was subsequently eluted with water and fractions of 1 ml were collected. Fractions with an OD₂₈₀ > 0.2 were pooled, freeze-dried and stored until use.

A female New Zealand White rabbit (4 kg) was immunized with approximately 1 mg polypeptide 38LC in 1 ml water with 1 ml Freund's incomplete adjuvant (Difco) by subcutaneous injection on days 0 and 28. Blood samples were obtained after 0 and 42 d. All sera were stored at –20 °C.

SDS-PAGE, polypeptide gel electrophoresis and Western blotting. *M. leprae* subcellular fractions were provided by Dr P. Brennan (Colorado State University, Fort Collins, USA) and prepared as described by Hunter *et al.* (1990).

Electrophoresis on 13 % (w/v) SDS-PAGE gels was performed as described by Laemmli (1970); the low molecular mass marker set from Pharmacia-LKB was used. For the detection of the 38LC polypeptide we used Swank and Munkres gels (Swank & Munkres, 1971) with the myoglobin molecular mass marker set from Pharmacia-LKB.

After electrophoresis, proteins and polypeptides were either stained with Coomassie brilliant blue or transferred onto BA85 nitrocellulose (Schleicher & Schuell). Antigen was detected by using a 200-fold dilution of 38LC-directed antisera and peroxidase-conjugated goat anti-rabbit immunoglobulins (Diagnostics Pasteur) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Identification and characterization of the putative 38L protein of *M. leprae*

A DNA clone from the λ gt11::*M. leprae* library (Young *et al.*, 1985) was selected on the basis that it gave rise to a protein that reacted with monoclonal antibodies specific for a *M. leprae* 12 kDa antigen. Further analysis including DNA sequencing indicated that the 12 kDa *M. leprae* antigen was not encoded by the selected clone (our unpublished results).

However, the nucleotide sequence revealed an ORF encoding a putative protein with characteristics typical for membrane proteins. This ORF, consisting of 1290 amino acids, is located between positions 2654 and 1365 on the complementary strand. This ORF was designated 38L according to the original clone notation (Young *et al.*, 1992). The deduced amino acid sequence is shown in Fig. 1(a). The 38L ORF was preceded by a potential Shine–Dalgarno sequence, AGGA, 7 bp upstream of the ATG start codon.

The 38L gene has a coding capacity for a protein of 429 amino acids with a calculated molecular mass of 46 179 Da. From the hydrophathy pattern shown in Fig. 1(b) it can be deduced that this 38L protein has a low polarity (i.e. 68 % nonpolar amino acids) with the majority of the nonpolar amino acids present in 12 hydrophobic clusters (indicated by arrows) with a mean length of 23 $\pm$ 5 amino acids which are theoretically capable of spanning the bacterial

(a) MSVIAITVFI VTYALIASDR VNKTAAALTG AAIVVTLPII NSEDVFYSHE TGIDWEVIFL LLSMMIIVSV 70
 LRQTGVFEYV AIWTAKRSHG SPLRILLLLV LVMALGSALL DNVTTVLLIA PVTLLVCERL TINAAPFLMA 140
 EVFASNIGGA RTLVGDPPII IASRAGFSF NDFLIHLTP IIVTVVLSA LLPRLFRGAF AVDPERVADI 210
 MSLNEREAIR DRWLLIKCGV VLLLVFVAFI AHPVLHTGPS LVGMLGAGIL IVISKLESD YLSSVKWETL 280
 LFFAGLFIMV GALVKTDVN QLARATTTLT GGHELLTVVL TLGVSTLVSS IIDNIPYVAT MTPIVSELVA 350
 SMPDQSHTDI LWWALALGAD FGGNLTAUGA SANVVMLEIA KSAGTPISFW EFTRKGIAT VISIALAGIY 420
 LWLRYLVMS 429

Fig. 1. Characteristics of the 38L protein: (a) amino acid sequence; (b) hydropathy profile of the 38L gene product. The 12 hydrophobic clusters long enough to span the cytoplasmic membrane are indicated by arrows.

membrane (Klein *et al.*, 1985). No typical signal peptide sequence could be detected at the N-terminal side of the 38L protein. These combined findings suggest that the 38L protein is an integral membrane protein.

Database comparisons revealed that the 38L protein shares regions of significant [$P < 0.01$] identity with four known integral membrane proteins involved in transport of ions and small organic molecules through the cellular membrane.

First of all, the 38L protein shares identity (36% identity; 65% similarity including conservative substitutions over a stretch of 49 amino acids) with the Na^+/H^+ antiporter encoded by the *nhaB* gene of *E. coli*. This 504 amino-acid-long membrane protein is involved in the active extrusion of sodium ions from the cell by proton flux (Pinner *et al.*, 1992).

Secondly, the 38L protein shares significant identity (25% identity; 55% similarity over two stretches totalling 134 amino acids) with the ArsB proteins encoded by an *E. coli* plasmid (Chen *et al.*, 1986) and with ArsB proteins (29% identity; 60% similarity over a stretch of 74 amino acids) encoded by plasmids isolated from *Staphylococcus aureus* and *Staphylococcus xylosus* (Ji & Silver, 1992). ArsB is part of an arsenic resistance ATPase involved in the active efflux of arsenite and antimonite ions from the cell. The ArsB protein is an integral membrane protein which serves as both the anion efflux protein and the membrane anchor for the ArsA moiety of the protein complex (for a review see Silver *et al.*, 1993).

Quite surprisingly, the highest homology (44% identity; 64% similarity) was found with two eukaryotic proteins: the human and murine P proteins encoded by the pink-

eyed dilution locus (Gardner *et al.*, 1992; Rinchik *et al.*, 1993). This extensive homology is shared between the four stretches totalling 291 amino acids of the 38L sequence and the C-terminal halves of both P proteins. Several lines of evidence indicate (Rinchik *et al.*, 1993) that the P proteins are components of the melanosomal membrane, possibly involved in the transport of tyrosine.

Expression and purification of the C-terminal part of the 38L protein in *E. coli*

In order to obtain antiserum against the 38L protein, we attempted to clone the complete gene using various inducible expression vectors. We did not obtain clones with the correct insert overexpressing the complete protein. This may be explained by the observation that the expression of some cloned membrane proteins may be deleterious to the host cell (for an example see Matsuyama *et al.*, 1990). In case of 38L, the low-level expression that occurs before the induction step may be lethal to the host cell.

As an alternative we chose to overexpress the C-terminal part of the 38L protein, namely the polypeptide encompassing amino acids 328–404. This part of the protein contains relatively few hydrophobic clusters. Fig. 2 shows the results of the overexpression and purification of the 38LC polypeptide encoded by pTHL1095. The 38LC polypeptide shows as a product with an apparent molecular mass of approximately 6800 Da (6.8 kDa) on Swank and Munkres gels. Making use of a simple and fast one-step purification protocol followed by desalting and freeze-drying we were able to isolate the 38LC polypeptide (lanes D and E).

Fig. 2. Swank and Munkres gel electrophoresis analysis of the overexpression and purification of the 38LC polypeptide in *E. coli*. Lanes: A and B, *E. coli* M15(pREP4)(pTHL1095) lysates before and after induction with IPTG, respectively; C, the supernatant after binding of the 38LC polypeptide to the Ni-NTA resin; D, purified 38LC after elution; E, purified 38LC after concentration by freeze-drying. Positions of the molecular mass markers are indicated on the left.

Subcellular localization of the 38L protein in *M. leprae*

A rabbit polyclonal antiserum was raised against the 38LC polypeptide. We used this antiserum to screen for the presence of the 38L protein in whole *M. leprae* sonicate and three subcellular *M. leprae* protein fractions consisting of cell wall proteins, cytosol proteins or membrane proteins. Experiments with serum obtained from the rabbit before immunization with the 38LC polypeptide did not show any bands (results not shown); results with the serum obtained after immunization are shown in Fig. 3. A protein band with an apparent molecular mass of approximately 33 kDa reacting with antisera against the 38LC polypeptide was clearly visible in the fractions containing the cytosol proteins and the cytoplasmic membrane proteins. No band was detected in the cell wall fraction. This suggests that, after synthesis of the 38L protein in the cytosol, the protein is integrated into the cytoplasmic membrane where it remains as an intrinsic integral membrane protein. The fact that no 38L protein was present in the outer cell wall protein fraction was corroborated by the absence of a typical signal sequence at the N-terminus of the 38L protein. No signal peptide has been found for any of the integral membrane transporters, since they appear to insert into the membrane by a different, although poorly understood mechanism.

We observed that on gels containing SDS both the 38L protein and the 38LC polypeptide showed an apparent molecular mass which was significantly lower than

Fig. 3. Determination of the subcellular location of the 38L protein in *M. leprae* by immunoblotting. Lanes A, B and C contain cell wall proteins, cytosol proteins and cytoplasmic membrane proteins, respectively; lane D contains purified 38LC polypeptide. Positions of the molecular mass markers are indicated on the left.

expected. This is probably due to the abnormally high binding of SDS by the nonpolar amino acids that are abundantly present (Yazyu *et al.*, 1984).

Neighbouring genes

Sequence analysis of the nucleotide sequence of a 3378 bp segment containing the *38l* gene revealed the presence of three other ORFs. These ORFs revealed no significant homology with any of the sequences from the major sequence databases. Moreover, these ORFs were not preceded by a recognizable Shine-Dalgarno sequence.

Localization of the *38l* region on the cosmid contig map of *M. leprae*

A DNA fragment, with the code S1150, comprising the *38l* gene was used to locate the position of the gene on the *M. leprae* contig map. The *38l* gene appears to be located on contig 84 of the *M. leprae* genome in the vicinity of the *rpoD* locus (Eiglmeier *et al.*, 1993). Nucleotide sequence homology comparisons revealed that part of the sequence ranging from nucleotides 2382 to 3378 is identical to a

989 bp stretch of cosmid B1912 (D. R. Smith, GenBank accession number L01536), which was indeed located in the same region of the *M. leprae* genome (Eiglmeier *et al.*, 1993).

Possible function of the 38L protein

The precise function of the 38L protein is not known. Based on the findings mentioned above, we hypothesize that the 38L protein from *M. leprae* is an integral membrane protein involved in the transport of small molecules, most likely tyrosine, through the cellular membrane. Tyrosine is a precursor in the biosynthesis of many essential molecules. The presence of a tyrosine uptake system may be explained by the observation that for the availability of a number of essential molecules, *M. leprae* relies on scavenging from the host environment (Wheeler, 1989). It was shown previously that extracellular tyrosine is a precursor in the synthesis of phenolic glycolipid in *Mycobacterium kansasii* and *Mycobacterium bovis* (Gastambide-Odier *et al.*, 1967).

The P proteins have been pinpointed as components of the melanosomal membrane involved in the transport of tyrosine, the primary precursor in melanin biosynthesis. Mutations in the chromosomal region encoding the P protein lead to hypopigmentation (Rinchik *et al.*, 1993). On the basis of the strong similarity between the *M. leprae* 38L protein and the human P protein it is tempting to speculate that the 38L protein is somehow involved in hypopigmentation.

It is well established that hypopigmentation is often the first clinical sign of leprosy (Westerhof, 1977). As the degree of pigment loss parallels the increase in resistance and decrease in bacterial load, it is unlikely that the *M. leprae* 38L protein is directly responsible for hypopigmentation, for example by competition with the P protein for available tyrosine. Previous observations indicate that melanocytes in hypopigmented patches are intact but that the transfer of melanosomes from melanocytes to keratinocytes is disturbed (Shereef, 1992; Westerhof, 1977). A possible explanation might be that the 38L protein, produced by the *M. leprae* bacteria residing in the melanocytes, somehow impairs this melanosome transfer leading to hypopigmentation. Alternatively, it was suggested previously that hypopigmentation might be the result of an autoimmune phenomenon (Parker, 1988). Since hypopigmentation is so much more defined in patients with a relatively strong cellular immune response, it is feasible that some kind of cellular autoimmune reaction is involved. The exact role of the 38L protein in the pathogenicity of *M. leprae* remains to be established.

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