Characterization of a *Mycobacterium leprae* Antigen Related to the Secreted *Mycobacterium tuberculosis* Protein MPT32

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Secreted proteins may serve as major targets in the immune response to mycobacteria. To identify potentially secreted *Mycobacterium leprae* antigens, antisera specific for culture filtrate proteins of *Mycobacterium tuberculosis* were used to screen a panel of recombinant antigens selected previously by leprosy patient sera. Four potentially secreted antigens were identified by this approach, and one was recognized by antibodies specific for MPT32, a secreted *M. tuberculosis* protein. The DNA coding for the *M. leprae* antigen, which we have designated 43L, was isolated and characterized and found to encode a 25.5-kDa protein that is preceded by a consensus signal peptide of 39 amino acids. The N-terminal amino acid sequence of 43L shows 50% homology with the 20 known N-terminal amino acids of MPT32, and 47% homology was found with the N terminus of a 45/47-kDa antigen complex identified in *Mycobacterium bovis* BCG. These findings indicate that 43L represents an antigen related to MPT32 and the *M. bovis* BCG 45/47-kDa complex and that 43L is likely to be a protein secreted by *M. leprae*. Purified recombinant 43L protein is recognized by antibodies and T cells from healthy contacts and leprosy patients, illustrating that secreted proteins are of importance in the immune response to *M. leprae*.

In recent years, recombinant DNA technology has become an important tool in the identification of *Mycobacterium leprae* antigens that are involved in the spectrum of immune responses that can be induced upon infection with this intracellular parasite (30). Among these antigens are heat shock proteins (HSPs), which are predominantly localized in the cytoplasm. HSPs, a large number of which were selected by using monoclonal antibodies, are prominent antigens in both T-cell and B-cell responses to *M. leprae* (13, 14, 31). In addition to HSPs, secreted antigens have been shown to constitute important target structures in the immune response towards *M. leprae* (8, 10, 25).

Upon infection with *M. leprae*, protective immunity as well as immunopathology leading to disease may be induced, and both processes are believed to be dependent on antigen-specific T-cell recognition. It is still a controversial matter whether particular *M. leprae* antigens may either provide protection or induce immunopathology. A role for actively secreted antigens in protective immunity has been proposed on the basis of the observation that living attenuated mycobacteria provide a higher degree of immunity than heat-killed bacteria (27) and a limited degree of protection with secreted antigens was obtained in an animal model for tuberculosis (18). Recently, it has been demonstrated that live *Mycobacterium tuberculosis* predominantly provokes T-cell responses to antigens secreted by the bacteria during growth (2). Several antigens that could be involved have been identified in culture filtrates of *M. tuberculosis* and have been designated antigen 85 complex, MPT32, MPT51, MPT53, MPT63, and a 3- to 9-kDa protein (3, 15, 28).

Since *M. leprae* cannot be cultured in vitro, it is difficult to analyze putative products that are specifically secreted by

this species. To circumvent this problem, we have used sera that are specific for secreted antigens of *M. tuberculosis* to identify potentially secreted *M. leprae* homologs among a panel of more than 20 different recombinant antigens that were previously selected by using antibodies from leprosy patients (22). DNA clones expressing four different recombinant antigens were identified by the sera directed against secreted *M. tuberculosis* proteins. One of these was shown to encode an antigen related to the *M. tuberculosis* secreted protein MPT32.

MATERIALS AND METHODS

Bacterial strains, phages, plasmids, and cosmids. The bacterial strains, phages, plasmids, and cosmids used in this study are listed in Table 1. Strain POP2136 was used as a host for plasmid pEX2 and derivatives, and strain χ2819 was used as a host for an *M. leprae* genomic library cloned into the cosmid pHC79 (5). Strain JM109 was used as a host for plasmids pUC13 and derivatives. Bacterial strains were grown in LB medium and LB agar (11) supplemented with ampicillin (100 µg/ml) and tetracycline (10 µg/ml).

Antibodies. Serum samples from 21 borderline tuberculoid and tuberculoid (BT/TT) leprosy patients, 17 lepromatous and borderline lepromatous (LL/BL) leprosy patients, and 10 healthy contacts (HCs) were from endemic leprosy regions in Ethiopia. These sera were used in a 1:100 dilution. Polyclonal rabbit antibodies directed against secreted proteins of *M. tuberculosis* were K12 and K13 directed against MPT63, K14 and K15 directed against MPT51, K16 and K17 directed against MPT53, K23 directed against total secreted proteins of *M. tuberculosis*, K95 directed against MPT64, and K180 and K190 directed against MPT32 (15). These sera were used in a 1:200 dilution. Horseradish peroxidase-

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TABLE 1. Bacterial strains, phages, and plasmids used in this study

Strain, phage, plasmid or cosmid	Description	Insert	Reference
Strains			
E. coli POP2136	F⁻ endA thi gsdK malT malPQ λcI857		
E. coli K-12 χ2819	F⁻ lacY1 glnV44 galK2 galT22 (λcI857 b2 red3 S7) recA56 ΔthyA57 metB1 hsdR2		5
E. coli JM109	thi Δ (lac-proAB) (F⁻ tra D36 proAB lacIᵠZΔM15) λ⁻		29
Plasmids and cosmids			
pEX2	Apʳ contains Pr promoter of bacteriophage λ and cro-lacZ gene fusion		24
pEX2/L14	pEX2 recombinant	1.0-kb M. leprae EcoRI fragment of L14	This study
pEX2/L7	pEX2 recombinant	0.5-kb M. leprae EcoRI fragment of L7	25
pGEX-1λT	Apʳ, glutathione S-transferase gene		23
pGEX/L14	pGEX-1λT recombinant	1.0-kb M. leprae EcoRI fragment of L14	This study
pHC79	Apʳ Tcʳ		6
pYA1108	pHC79	M. leprae DNA	This study
pYA1110	pUC13	4.3-kb PstI fragment from pYA1108	This study
Phages			
L2	λgt11 recombinant	1.2-kb M. leprae DNA insert	22
L7	λgt11 recombinant	0.5-kb M. leprae DNA insert	22
L14	λgt11 recombinant	1.0-kb M. leprae DNA insert	22
L24	λgt11 recombinant	1.8-kb M. leprae DNA insert	22
L44	λgt11 recombinant	2.1-kb M. leprae DNA insert	22

labelled anti-rabbit and anti-human immunoglobulins were obtained from Sigma Chemical Co., St. Louis, Mo.

Production of recombinant fusion proteins. Expression of cro-lacZ hybrid genes from pEX2 derivatives was induced as described previously (26). For T-cell proliferation assays, lysates of Escherichia coli were centrifuged (10 min, 12,000 × g) and the pellets, enriched in fusion proteins, were resuspended in Iscove's modified Dulbecco's medium and stored at −20°C until used.

Expression of glutathione hybrid genes from pGEX derivatives was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to a log-phase culture with an optical density at 600 nm of 0.5. The culture was maintained for another 3 h at 37°C. Cells were pelleted by spinning the culture for 30 min at 1,000 × g. Cells were resuspended to an optical density at 600 nm of 5 in lysis buffer (30% sucrose, 50 mM Tris-HCl [pH 8], 100 mM KCl, 1 mM dithioerythritol, 5 mM EDTA). Ten percent sarcosyl solution (10% N-lauryl sarcosine in H₂O) was added to a final concentration of 0.2%. After mixing and sonication (six times for 30 s at 80 W in a Branson sonifier B-12 sonicator), the cell extract was frozen at −20°C until use. In order to purify the fusion protein, we performed affinity chromatography, using a glutathione-binding column according to the manufacturer's instructions (Pharmacia P-L Biochemicals, Inc., Milwaukee, Wis.). Purification of the native 43L antigen after cleavage of the glutathione fusion product with thrombin was performed as described elsewhere (23). Proteins were >90% pure, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were stored at −20°C until use.

SDS-PAGE and immunoblotting. Production of recombinant (fusion) proteins in E. coli lysates was analyzed by SDS-PAGE on 7.5 or 12% acrylamide gels and by Western blotting (immunoblotting). SDS-PAGE was done according to the method of Laemmli (9), and electrophoresis was performed at 70 mA. Either the gels were stained with Coomassie brilliant blue or the proteins were transferred to nitrocellulose membranes (type BA83; 0.2-μm-pore-size filters; Schleicher & Schuell, Dassel, Germany) in a Bio-Rad transfer apparatus according to the recommendations of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Immunoblotting of the membranes with pooled patient sera or monospecific polyclonal rabbit antisera was done as described previously (1). Patient sera were used in a 1:100 dilution. The monospecific polyclonal rabbit antisera were used in a 1:200 dilution.

DNA analysis and sequencing. Restriction endonucleases were used as specified by the manufacturer (Promega Corporation, Madison, Wis.) Standard procedures were used for preparation of plasmid and cosmid DNAs. Southern blotting and hybridization were performed as described previously (11) with nylon membranes (Hybond-N+; Amersham International, Amersham, Buckinghamshire, United Kingdom). Hybridization buffer and hybridization conditions used were as specified for the Hybond-N+ membranes. Washing was performed under conditions of medium stringency, one time for 10 min at room temperature with 2× SSPE (1× SSPE is 10 mM Na₂HPO₄, 0.18 M NaCl, and 1 mM EDTA [pH 7.7]) followed by one time for 10 min at 65°C with 1× SSPE. Probe labelling with α-³²P was done as specified by the manufacturer (Promega Corporation) with the use of a random-primed labelling kit. The EcoRI DNA insert from λgt11 recombinant clone L14 was subcloned into the EcoRI site of the expression vector pEX2 by PCR as described by Thole et al. (25).

Nucleotide sequence analysis was performed on plasmid and cosmid DNAs that had been purified by CsCl-gradient centrifugation (11), by the dideoxy chain-termination method (21) with the T7 sequencing kit (Pharmacia). The synthetic oligonucleotide primer 8625-2 that is complementary to the DNA sequence 12 to 26 bp upstream of the EcoRI site of pEX2 was used to sequence the M. leprae region fused to the cro-lacZ gene in the pEX2/L14 derivative. A set
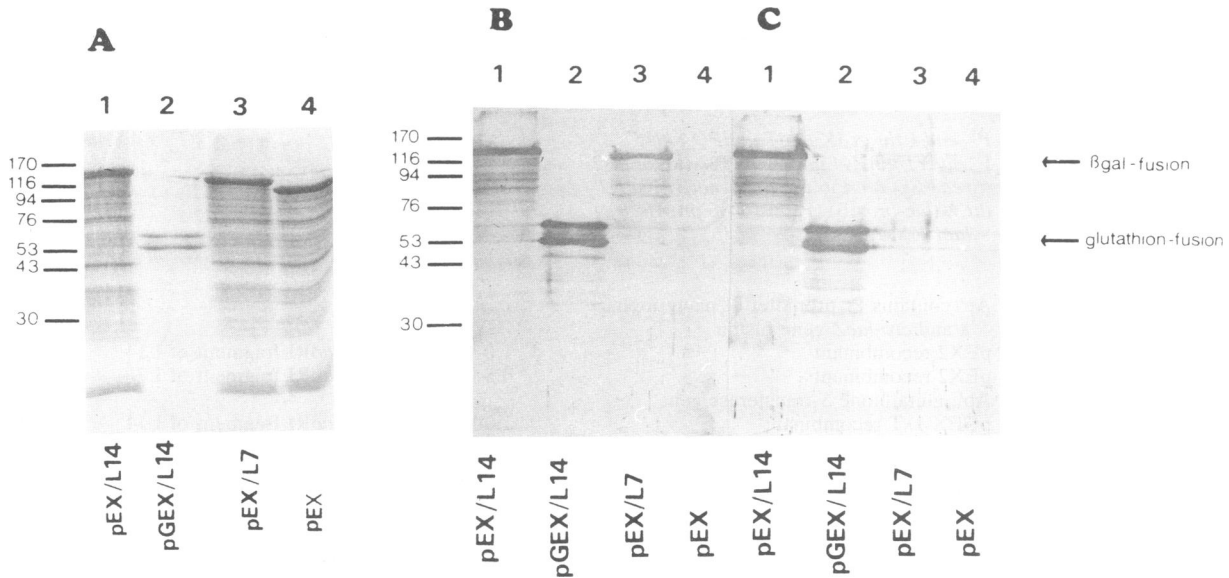
FIG. 1. SDS-PAGE followed by Coomassie staining (A), and followed by Western blot analysis with polyclonal rabbit antiserum K23 directed against an extract of total secreted *M. tuberculosis* proteins (B) or polyclonal rabbit antiserum K190 directed against the MPT32 protein of *M. tuberculosis* (C). Lanes 1, *E. coli* lysate of pEX2/L14; lanes 2, glutathione fusion protein purified from a lysate of the 1.0-kb insert of pGEX/L14; lanes 3, lysate of pEX/L7; lanes 4, lysate of pEX2 (control). Molecular masses are indicated in kilodaltons.

of primers based on the *M. leprae* insert gene sequence was subsequently synthesized to determine the 5' part of the sequence of the clone pEX2/L14. To obtain a cosmid carrying the complete gene corresponding to L14, an *M. leprae* cosmid library (5) was screened by hybridizing with the 1.0-kb *Eco*RI insert of pEX2/L14. *Pst*I fragments from a positive cosmid that hybridized with the 1.0-kb insert of pEX2/L14 were subcloned into pUC13, and a resulting pUC13 derivative (pUC13/43L) containing the complete 43L gene was used to determine the sequence of the 3' part of the gene. To obtain expression of a glutathione fusion carrying the complete gene, unidirectional subcloning into the *Bam*HI and *Eco*RI sites of expression vector pGEX-1λT was performed with a *Bam*HI- and *Eco*RI-digested PCR fragment obtained by using a *Bam*HI site containing a forward primer (CGCGGATTCGATCCCGCGCCCCTGCCGCCA) complementary to the first codons of the mature 43L gene and an *Eco*RI-containing primer (GGCGAATTCCCGCCTACAA CACCGATAACGA) complementary to sequences downstream of the putative stop codon of 43L, with genomic *M. leprae* DNA as a template. All primers were synthesized on a Cyclone DNA synthesizer (Biosearch Inc., Millipore Corporation, Bedford, Mass.).

Proliferation assays. Lymphocyte stimulation tests were performed by testing freshly isolated peripheral blood mononuclear cells. In a 96-well round-bottomed microtiter plate, 100,000 cells per well were cultured in Iscove's modified Dulbecco's medium supplemented with 10% human serum and the optimal concentration of antigen. The antigen used was the *E. coli* lysate extract from pEX/L14 in 1:100 and 1:5,000 dilutions. For testing the recombinant purified native 43L protein, 150,000 to 200,000 cells per well were used and the purified antigen was added at a concentration of 10 μg/ml. Cultures were set up in triplicate and incubated at 37°C in a fully humidified atmosphere containing 5% CO_2 for 6 days. For the last 18 h, 1.0 μCi of [³H]thymidine (specific activity, 5.0 μCi/mmol; NEN, Boston, Mass.) was added to

each well and cells were finally harvested full automatically on glass fiber filters. [³H]thymidine incorporation was assessed by liquid scintillation spectroscopy on a β-plate reader.

RESULTS

Identification of putative *M. leprae* secreted proteins. Previously, 50 recombinant clones were selected from an *M. leprae* λgt11 expression library by pooled leprosy patient serum samples (22). These clones were grouped by hybridization studies and were shown to express at least 22 novel antigenic determinants. By immunoblotting, we tested whether antigens expressed by 25 representative members of this large panel of clones were recognized by antiserum K23 directed against total culture filtrate proteins of *M. tuberculosis*. The gene products of five λgt11 clones (L2, L7, L14, L24, and L44) were recognized (Fig. 1, L7 and L14). This indicated that these clones produce potentially secreted *M. leprae* antigens. The reactivity with antigens produced by L7 and L44 was not unexpected, since these clones express parts of the *M. leprae* B component of the secreted antigen 85 complex (25). To further characterize the antigens expressed by L2, L14, and L24, they were tested for recognition by monospecific polyclonal rabbit antibodies directed to individual secreted proteins of *M. tuberculosis*. This revealed that the antigen expressed by L2 was recognized by antibodies to MPT53, a 15-kDa protein. The antigen expressed by L14 was recognized by antibodies directed to MPT32, a 41-kDa protein, whereas the antigen expressed by L24 was not recognized by any of the tested antisera against individual secreted proteins of *M. tuberculosis*. In this study, we have focused on the protein expressed by L14.

Subcloning of L14 in expression systems. In order to generate large quantities and to purify the antigen expressed by L14, the 1-kb *Eco*RI PCR fragment of L14 was subcloned into the *Eco*RI sites of pEX2 and pGEX-1λT. The insert
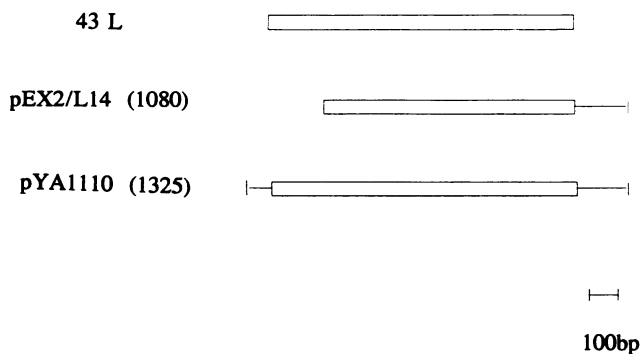
FIG. 2. Schematic presentation of the sequenced *M. leprae* insert DNA of clones pEX2/L14 and pYA1110. Sizes of the inserts are shown in base pairs (parentheses). The position of the ORF is indicated with an open box.

orientation was checked by comparing fusion protein sizes of the cro-β-galactosidase and the glutathione fusion products with the expected protein size from the original λgt11 clone. A characteristic doublet can be seen in both Coomassie-stained SDS-PAGE and in Western blots probed with anti-MPT32 and anti-*M. tuberculosis* total secreted protein (Fig. 1). Recombinant native complete antigen shows a similar doublet band of 27/32 kDa (see the description below and Fig. 4). Although much smaller, these bands could correspond to the observed double bands reported for the corresponding 45/47-kDa complex antigens of *Mycobacterium bovis* BCG (20).

Molecular analysis of pEX2/L14 and derivatives. The complete *M. leprae* DNA insert of pEX2/L14 was sequenced and found to be 1,080 bp long. The analysis of this DNA fragment revealed the presence of an open reading frame (ORF) predicting a protein consisting of 230 amino acids fused to β-galactosidase. To obtain the complete gene, the EcoRI insert of the L14 clone was used as a probe to screen a cosmid library. Two cosmid clones, designated pYA1108 and pYA1109, were isolated. A 4.3-kb PstI fragment from pYA1108 hybridized with the 1.0-kb fragment of clone L14, and a PstI fragment with a similar size was detected in genomic *M. leprae* DNA. The 4.3-PstI fragment from pYA1108 was subcloned into pUC13, resulting in plasmid pYA1110 (Fig. 2). By using this plasmid, the region corresponding to the insert of pEX2/L14, as well as 245 bp at the 5'-terminal end of this region, was sequenced. This resulted in a DNA sequence of 1,325 bp, as shown in Fig. 3. Analysis of this DNA fragment revealed an ORF corresponding with the ORF in pEX2/L14. The ORF starts at position 75 and ends at position 935. An additional start codon in the same reading frame is present at position 165. Computer analysis revealed a putative signal sequence between positions 75 and 191, encoding a 39-amino-acid signal peptide that includes a consensus Ala-Asn-Ala cleavage site at its C terminus (16), which would correspond with its identification as a potentially secreted *M. leprae* protein.

The first 20 N-terminal amino acids showed extensive homology with the N terminus of MPT32 and the N terminus of a 45/47-kDa complex of *M. bovis* BCG (Fig. 4 [15, 20]). The presence of a similar gene in *M. tuberculosis* was confirmed by hybridization of the 1.0-kb EcoRI fragment of clone pEX2/L14 to a homologous band in genomic *M. tuberculosis* DNA (Fig. 5). Given also the reactivity of the anti-MPT32 sera to the product expressed by L14, which

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         10        20        30        40        50        60
CCTAGAAAAACCCCCAACCGCAACATAAGCAAGCCGTCCTTGCGTCGCAGTAGTAACACG

         70        80        90       100       110       120
GTAGGTTCGTCGCCATGAATCAGGTTGACCTGGACTCGACACATCGCAAAGGATTGTGGG
                 M  N  Q  V  D  L  D  S  T  H  R  K  G  L  W

        130       140       150       160       170       180
CGATACTGGCGATTGCCGTGGTGGCCAGCGCCAGTGCCTTTACGATGCCGTTCCGTGCGG
 A  I  L  A  I  A  V  V  A  S  A  S  A  F  T  M  P  F  R  A

        190       200       210       220       230       240
CCGCCAACGCCGATCCCGCGCCCCTGCCGCCATCGACGGCTACGGCAGCTCCCTCACCTG
 A  A  N  A  D  P  A  P  L  P  P  S  T  A  T  A  A  P  S  P

        250       260       270       280       290       300
CGCAGGAGATCATTACACCCCTTCCAGGCGCCCCTGTCTCGTCCGAAGCCCAACCGGGTG
 A  Q  E  I  I  T  P  L  P  G  A  P  V  S  S  E  A  Q  P  G

        310       320       330       340       350       360
ATCCCAATGCGCCGTCGCTCGATCCGAATGCACCATACCCACTTGCAGTCGATCCCAACG
 D  P  N  A  P  S  L  D  P  N  A  P  Y  P  L  A  V  D  P  N

        370       380       390       400       410       420
CCGGCCGAATCACCAACGCTGTCGGTGGATTTAGCTTCGTCCTTCCTGCCGGTTGGGTGG
 A  G  R  I  T  N  A  V  G  G  F  S  F  V  L  P  A  G  W  V

        430       440       450       460       470       480
AGTCGGAGGCTTCACATCTTGACTACGGTTCGGTGCTGCTCAGCAAAGCCATCGAGCAGC
 E  S  E  A  S  H  L  D  Y  G  S  V  L  L  S  K  A  I  E  Q

        490       500       510       520       530       540
CGCCCGTGCTTGGTCAGCCGACGGTGGTCGCTACCGACACCGTATAGTGCTCGGCCGGC
 P  P  V  L  G  Q  P  T  V  V  A  T  D  T  R  I  V  L  G  R

        550       560       570       580       590       600
TGGACCAAAAGCTCTACGCCAGTGCCGAAGCCGACAACATTAAGGCCGCGGTCCGACTGG
 L  D  Q  K  L  Y  A  S  A  E  A  D  N  I  K  A  A  V  R  L

        610       620       630       640       650       660
GCTCGGATATGGGTGAGTTCTACCTGCCATACCCCGGTACGCGGATCAACCAAGAAACCA
 G  S  D  M  G  E  F  Y  L  P  Y  P  G  T  R  I  N  Q  E  T

        670       680       690       700       710       720
TTCCGCTCCACGCCAACGGGATAGCTGGAAGCGCCTCCTACTACGAGGTCAAATTCAGCG
 I  P  L  H  A  N  G  I  A  G  S  A  S  Y  Y  E  V  K  F  S

        730       740       750       760       770       780
ATCCCAATAAGCCAATTGGCCAAATATGTACGAGCGTAGTCGGCTCGGCTCGCCAGCGGCGAGTA
 D  P  N  K  P  I  G  Q  I  C  T  S  V  V  G  S  P  A  A  S

        790       800       810       820       830       840
CCCCTGACGTGGGGCCCTCGCAGCGTTGGTTTGTGGTATGGCTCGGAACCTCGAATAACC
 T  P  D  V  G  P  S  Q  R  W  F  V  V  W  L  G  T  S  N  N

        850       860       870       880       890       900
CGGTGGACAAGGGCGCAGCCAAAGAGCTGGCTGAGTCTATCCGGTCAGAGATGGCTCCGA
 P  V  D  K  G  A  A  K  E  L  A  E  S  I  R  S  E  M  A  P

        910       920       930       940       950       960
TCCCGGCGTCGGTTTCCGCTCCGGCACCTGTTGGATAGGCCATCCGACATCCACTCCGCT
 I  P  A  S  V  S  A  P  A  P  V  G  -

        970       980       990      1000      1010      1020
GCCACTGCGGACCGTGTTTCCTTGACCCGCCGCCCGCAGAGCAAACCACTGTCGACAATA

       1030      1040      1050      1060      1070      1080
GGCACTCATCGGTGTATACCCACATTACAGCCCCGCATCTGCGCTGAGCTATGCCATCGA

       1090      1100      1110      1120      1130      1140
GTGCAAGGAGATTACACATGGACGTCATGGCAGCTACCGAGTACCTCGCCCGCTCAACGA

       1150      1160      1170      1180      1190      1200
CGTTGACTAGTGTTGGCTGGATTGGTTACATCATTATCGGTGGTATAGCGGGCTGGATCG

       1210      1220      1230      1240      1250      1260
CAGGCAAAATCGTTCAAGGCGGCGGATCGGGCATCTTGATGAACATCGTTATCGGTGTTG

       1270      1280      1290      1300      1310      1320
TAGGCGCGTTGATCGGCGGCTTTTTACTGAGCTTCTTCGTTAACACCGCTGCCGGCGTTG

GTGGT
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FIG. 3. Nucleotide sequence of clone pYA1110. The ORF encoding a 25.5-kDa protein is indicated in single-letter amino acid code, and the putative signal sequence is in italics. A putative Shine-Dalgarno ribosomal binding site and possible −10 sequences are underlined. *M*, start codon; −, putative stop codon.

MPT32   D P A P A P P V P X T A A A P P X X A A
         * * * *   * *   * * *   *
43L   D P A P L P P S T A T A A P S P A Q E I
         #   #   # #   # #   # #
45/47 kDa A P E P A P P V P P A A A A P P A

FIG. 4. Homology of the N-terminal amino acid sequences from 43L of *M. leprae*, MPT32 of *M. tuberculosis* (14a), and the 45/47-kDa complex of *M. bovis* BCG. *, homology between 43L and MPT32; #, homology between 43L and the 45/47-kDa complex.

does not contain this N-terminal region, we conclude from these data that we have identified and characterized an *M. leprae* antigen related to MPT32 and the *M. bovis* BCG 45/47-kDa complex. We have designated the *M. leprae* antigen 43L, corresponding to the nomenclature for MPT32 (43T) listed in a recent compilation of mycobacterial protein antigens (30).

To purify native recombinant 43L, PCR-amplified *M. leprae* DNA encoding the mature protein was subcloned into the expression vector pGEX-1λT and purified from the expressed glutathione fusion protein after cleavage with thrombin as described in Materials and Methods. Native mature 43L migrates with a mobility of 27 and/or 32 in SDS-PAGE, corresponding to the calculated molecular mass of the mature protein of 25,553 Da (Fig. 4). The molecular mass of 43L thus is considerably smaller than the estimated molecular masses of 41 kDa for MPT32 and of 45/47 kDa for the *M. bovis* BCG 45/47 kDa complex.

Immunological analysis of the secreted *M. leprae* protein. In order to test the B-cell response to the 43L protein in leprosy
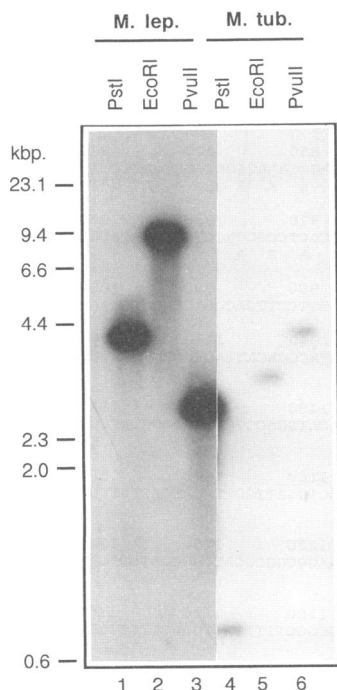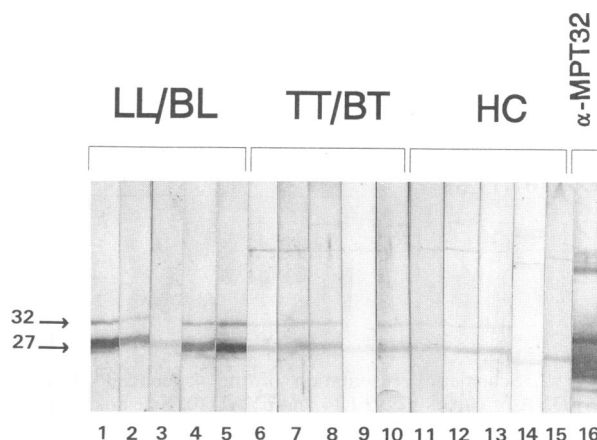

FIG. 6. Reactivity of antibodies in serum samples from leprosy patients and healthy individuals with the purified 43L protein. The Western blots for 5 LL/BL patients (lanes 1 to 5), 5 TT/BT patients (lanes 6 to 10), 5 HCs (lanes 11 to 15), and (as a positive control) anti-MPT32 (α-MPT32) (lane 16) are shown. The arrows indicate the 27- and 32-kDa protein bands.

patient sera, immunoblotting was performed with purified native 43L and serum samples from LL/BL patients, BT/TT patients, and HCs. Of the 17 tested LL/BL patient samples, 15 were shown to strongly recognize recombinant 43L. Eleven of 21 BT/TT patient serum samples gave a moderate reaction, and a weak reactivity with 43L was found in 3 of 10 tested healthy contacts. A representative number of these reactivities are shown in Fig. 6. The data indicate that 43L is recognized by the majority of the tested LL/BL sera but that the reactivity in TT/BT patients and HCs is generally much weaker.

In order to assess T-cell recognition of this antigen, proliferation of peripheral blood mononuclear cells derived from HCs, LL/BL patients, and BT/TT patients was tested in the presence of a semipurified lysate containing the fusion protein expressed by pEX2/L14. Despite the semipurified nature of the antigen preparations, significant proliferation was found in 4 of 21 HCs, in 4 of 29 BT/TT patients and in 1 of 18 LL/BL patients tested (data not shown). In concordance with these data, purified native 43L is recognized by peripheral blood lymphocytes from two of two tested HCs and two of three tested BT/TT or LL/BL patients (Table 2). The T-cell and antibody recognition indicates that antigen 43L is recognized by HCs and by patients throughout the leprosy spectrum.

FIG. 5. Southern blot analysis of total DNA from *M. leprae* (M. lep.) (lanes 1 to 3) and *M. tuberculosis* (M. tub.) (lanes 4 to 6) digested with *Pst*I (lanes 1 and 4), *Eco*RI (lanes 2 and 5), and *Pvu*II (lanes 3 and 6) hybridized with the 1,000-bp insert of clone pEX2/L14. The exposure times for both Southern blots were 5 days. Molecular markers are indicated (in kilobase pairs) by horizontal dashes.

TABLE 2. Proliferation T-cell response to the purified 43L protein by peripheral blood mononuclear cells[a]

Protein source	[³H]thymidine incorporation (cpm)				
	HC A	HC B	BT A	BT B	BL A
Medium	293	612	1,995	738	1,362
M. leprae	22,086	30,855	18,262	2,124	43,266
M. tuberculosis	17,840	19,022	17,745	25,364	52,181
43L	14,953	6,286	12,772	1,323	10,814

[a] Responses from three tested patients (BT and BL) and two HCs are shown. *M. leprae* sonicate, *M. tuberculosis* sonicate, and purified 43L protein were tested at a concentration of 10 µg/ml. For the patients, 200,000 fresh isolated cells were tested and 150,000 cells were tested for the HCs.

DISCUSSION

In this study, we present structural and immunological analyses of the *M. leprae* antigen 43L that show clear structural and immunological relatedness with the secreted *M. tuberculosis* protein MPT32 and structural homology with the *M. bovis* 45/47-kDa complex antigen.

In a search for secreted proteins of *M. leprae*, we have identified a number of *M. leprae* recombinant antigens reacting with rabbit antibodies directed against secreted proteins of *M. tuberculosis*. The antigen 43L has been shown to cross-react with an antiserum against MPT32, a secreted protein of *M. tuberculosis*.

DNA sequence analysis indicates that the gene for 43L encodes a mature protein of 248 amino acids. The N terminus of the mature protein shows 50% homology with the N terminus of MPT32 (15) and 47% homology with the N terminus of the 45/47-kDa complex of *M. bovis* BCG (20). No significant homologies were found in screening the complete protein sequence with other proteins using Gen-Bank/EMBL data banks. Furthermore, the protein contains three Asp-Pro-Asn-Ala repeats at positions 301, 321, and 351. The function of these repeats is unknown. Interestingly, the mature protein is preceded by a signal peptide of 39 amino acids. The presence of a signal sequence strongly supports the notion that 43L is secreted like its *M. tuberculosis* counterpart MPT32.

The appearance of a doublet on SDS-PAGE and immunoblots is consistent with the multiple bands observed for the 45/47-kDa protein complex of *M. bovis* BCG (20). Posttranslational modifications of a protein encoded by a single gene could explain the doublet. An alternative explanation could be partial degradation. A homolog of 43L in *M. tuberculosis*, MPT32, is found to be the most unstable protein of all secreted proteins tested thus far (15). Southern blot analysis of digested total genomic DNAs of *M. leprae* and *M. tuberculosis* indicates that the gene coding for 43L has one copy in *M. leprae* and that a homolog is indeed present in *M. tuberculosis*, as a single-copy gene. The presence of similar but less-related genes, as postulated for the 45/47-kDa complex of *M. bovis* BCG, cannot be excluded (20). The N-terminal sequence of *M. tuberculosis* MPT32 shows a distinct difference from the N-terminal sequence of the *M. bovis* BCG 45/47-kDa protein at residues 1, 3, and 11 (Fig. 4). This is considerably more than usually observed when corresponding amino acid sequences of *M. tuberculosis* and *M. bovis* BCG are compared. This observation of extensive homology but distinct difference between the two N-terminal sequences may also point to the existence of more than one gene.

The size of the 43L protein as deduced from sequence analysis of two independently cloned genes reveals a molecular mass of 25.5 kDa for the mature protein. Correspondingly, the native protein 43L appears on SDS-PAGE as a protein with a molecular mass of approximately 27/32 kDa. This molecular mass is considerably smaller than those reported for MPT32 of *M. tuberculosis* (41 kDa) and for the 45/47-kDa complex of *M. bovis* BCG (15, 20). This difference in molecular mass may be accounted for by differential posttranslational modification, yielding a larger product in the case of naturally expressed protein in *M. leprae* than for the expression in *E. coli*. In this regard, it should be noted that evidence for glycosylation of mycobacterial proteins has recently been presented for the *M. tuberculosis* 19-kDa antigen (7). This issue can be further investigated after expression of 43L in a mycobacterial host (12).

Secreted proteins are believed to be important in the immune response to *M. leprae*. The secreted proteins analyzed so far are shown to elicit a T-cell response and a B-cell response in leprosy patients and HCs. This is shown for the antigen 85 complex (19, 25). Secreted proteins of *M. leprae* have been difficult to identify, largely due to the inability to culture *M. leprae* in vitro. The current study demonstrates that this problem can be circumvented by using antisera directed against secreted proteins of *M. tuberculosis*. This approach is limited by the use of rabbit antisera directed to *M. tuberculosis*. Secreted proteins of *M. leprae* that display less or no homology with *M. tuberculosis* will therefore not be detected. Furthermore, the rabbit's immune system might be unresponsive to certain antigens. Therefore, among the 25 recombinants tested, there may be additional secreted proteins to clones identified in this study.

Immune responses to secreted proteins might be particularly important in the initial phase of infection. *M. leprae* is capable of surviving intracellularly in resting macrophages. Secreted proteins of *M. leprae* may induce immune responses in an early stage because of their accessibility to the immune system. In this early stage, they can be processed and presented in combination with major histocompatibility complex molecules on the surface of macrophages, thereby stimulating T cells that can initiate activation of the resting macrophages and causing removal of intracellular bacteria. The observation that putative secreted *M. leprae* protein 43L indeed elicits T-cell and B-cell responses in a number of HCs, BT/TT patients, and LL/BL patients supports this hypothesis. This study confirms the assumption that secreted *M. leprae* proteins may induce both cellular and humoral immune responses in patients and controls, regardless of the ability of these individuals to respond to total *M. leprae* (17). Several of the most prominent mycobacterial antigens identified thus far are HSPs. Such HSPs are evolutionarily conserved and share homology with eukaryotic HSPs. Consequently, the immune system recognizing mycobacterial HSPs may cross-react with human HSPs. The antigen recognized by T and B cells described in this study may be important in the light of vaccine development, since it would not provoke an autoimmune reaction, possibly as a result of its expression being restricted to mycobacteria.

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