A highly immunogenic putative *Mycobacterium kansasii* lipoprotein

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The resurgence of tuberculosis, the emergence of multiple drug resistant tuberculosis, and the increasing prevalence of mycobacterial disease in AIDS patients have increased the importance of defining new mycobacterial antigens that can be utilized in the development of improved diagnostic reagents and more effective vaccines. In this report, a highly immunogenic *Mycobacterium kansasii* protein (MK35) and the gene encoding this antigen were characterized. MK35 gene probes reacted with genomic DNA from *M. avium*, *M. bovis* BCG, *M. intracellulare* and *M. tuberculosis* but not with DNA isolated from nine other mycobacterial species. Nucleotide sequence analysis showed that the MK35 gene encodes a 26 kDa protein which contains a consensus bacterial lipoprotein processing sequence. In addition, detergent-phase separation studies strongly suggested that MK35 is a lipoprotein. Skin test assays demonstrated that MK35 elicited a strong response in guinea pigs sensitized with *M. kansasii* but did not react in *M. tuberculosis*-sensitized guinea pigs. These results further suggest that mycobacterial lipoproteins are immunogenic antigens that should be considered in the development of new mycobacterial vaccines and diagnostic reagents.

Keywords: *Mycobacterium kansasii*, lipoproteins, mycobacterial antigens

INTRODUCTION

Mycobacterium kansasii is an environmental organism which has been isolated from soil, house dust, and tap water and had been commonly identified in western Europe and the United States (Davies, 1994). Infections with *M. kansasii* can cause a chronic, progressive, and sometimes fatal pulmonary disease in normal hosts (Lillo *et al.*, 1990). Moreover, disease resulting from *M. kansasii* infections is extremely serious and life-threatening in patients with advanced immunosuppression (Shearer *et al.*, 1986; Shafer & Sierra, 1992). Hospital-based studies have indicated that *M. kansasii* is the second most common nontuberculous mycobacterial infection of AIDS patients in the United States (Horsburgh & Selik, 1989). In fact, two reports from hospitals in Baltimore and Los Angeles

have concluded that *M. kansasii* was isolated more frequently than *M. tuberculosis* among their patients with documented HIV infection (Young *et al.*, 1986; Levine & Chaisson, 1991).

The increasing incidence of mycobacterial diseases in immunocompromised individuals and the emergence of the highly progressive and rapidly fatal multiple drug resistant tuberculosis (MDRTB) have increased public awareness about the inadequacy of current protocols to prevent and clinically manage mycobacterial infections (Dooley *et al.*, 1992; Reilly, 1993). An improved mycobacterial vaccine is clearly needed because the current vaccine, the bacillus Calmette–Guérin (BCG), has questionable effectiveness (Fine & Rodrigues, 1990). Colditz *et al.* (1994), using meta-analysis of data from relevant BCG efficacy trials, have recently concluded that the overall efficacy of BCG is only 50%. More rapid and specific methods for diagnosing mycobacterial diseases are also essential for improving patient care. Current procedures for detecting and speciating mycobacterial infections, such as tuberculin skin testing and mycobacterial cultivation, are clearly inadequate. For example, because the radiographic manifestations, pathology and clinical

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Abbreviation: DTH, delayed-type hypersensitivity.

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features of *M. kansasii* and *M. tuberculosis* infections are similar, standard methodologies cannot rapidly distinguish between these infections (Wallace *et al.*, 1990). The differentiation between *M. kansasii* and *M. tuberculosis* bacilli by cultivation and biochemical analysis currently requires several weeks. In an era of *M. tuberculosis* and the HIV pandemics, the failure to rapidly identify mycobacterial disease can have an important impact on the implementation of proper public health control measures.

The development of improved methods to prevent and detect mycobacterial disease in the future will probably be linked to the isolation of new mycobacterial antigens and the further characterization of previously identified mycobacterial immunogens. In this paper, we describe the identification and characterization of a putative mycobacterial lipoprotein, the MK35 protein from *M. kansasii*. The immunoreactivity of MK35 as demonstrated by serological studies and skin test assays further suggests that mycobacterial lipoproteins are immunodominant mycobacterial antigens.

METHODS

Preparation of mycobacterial DNA and antigens. The *M. kansasii* Bostrom strain was obtained from the culture collection at the George Washington University School of Medicine. The following mycobacterial strains were obtained from the American Type Culture Collection (Rockville, MD, USA): *M. asiaticum* (25274), *M. avium* (35714), *M. bovis* (27291), *M. bovis* BCG (27291), *M. chelonae* (19977), *M. fortuitum* (6841), *M. intracellulare* (13950), *M. marinum* (927), *M. phlei* (11758), *M. scrofulaceum* (19981), *M. smegmatis* (14468), *M. terrae* (15755), *M. tuberculosis* H36Ra (25177), *M. tuberculosis* H37Rv (27294), *M. vaccae* (15483) and *M. xenopi* (19250). The mycobacterial antigen preparations used in these experiments were sonic extracts of actively proliferating mycobacteria grown in Long's synthetic medium (with 1%, w/v, glucose) at 37 °C for three weeks. At late-exponential phase, the cells were harvested by centrifugation, washed in deionized water, sonicated and centrifuged as described previously (Rouse *et al.*, 1990). For DNA extractions, the strains were grown in the same medium, the cells were washed, and total DNA was extracted using published protocols (Morris *et al.*, 1988).

Production and screening of monoclonal antibody (mAb) 3869. The protocols for generating and screening anti-mycobacterial monoclonal antibodies have been previously described (Rouse *et al.*, 1990). Briefly, Balb/c mice were injected intraperitoneally three times in one week with 10 µg per injection of an ammonium sulfate precipitate of a sonic extract from the *M. kansasii* Bostrom strain. Sequential bleedings of these animals indicated that serum antibody levels remained high for several months. After 5 months, the immunized mice were given an intravenous booster of 1 µg of the *M. kansasii* ammonium sulfate precipitate. Cell fusions were performed 3 d later by a modification of the method of Kohler & Milstein (1975). All hybridomas generated were initially screened with preparative immunoblots using the Miniblotter 28 apparatus (Immunitics). To determine whether hybridomas recognized protein-associated epitopes, mycobacterial sonic extracts were digested to completion with proteinase K and pronase prior to the screening assay.

To evaluate the specificity of mAb 3869, the following mycobacterial sonic extracts were resolved by SDS-PAGE: *M. kansasii*, *M. avium*, *M. bovis* (BCG), *M. chelonae*, *M. fortuitum*, *M.*

intracellulare, *M. marinum*, *M. phlei*, *M. scrofulaceum*, *M. smegmatis*, *M. tuberculosis*, *M. vaccae*, *M. xenopi* and *M. vaccae*, plus *Escherichia coli*. Immunoblots prepared from these gels were incubated with mAb 3869 and developed as previously described (Morris *et al.*, 1991).

Production and screening of a *M. kansasii* λ gt11 expression library. Preparation of the expression library was based on established protocols (Morris *et al.*, 1988). This methodology yielded approximately 10^6 individual recombinant bacteriophages. DNA restriction analyses indicated that nearly 90% of these phages contained insert fragments. Immunoreactive bacteriophages were detected with monoclonal antibodies using protocols that were utilized to identify and isolate recombinant bacteriophages from the *M. intracellulare* expression library (Morris *et al.*, 1990).

Purification of the MK35 recombinant protein. The *M. kansasii* recombinant protein was overproduced using the pGEMEX system (Promega), a T7-polymerase-based expression system (Studier & Moffat, 1986). Recombinant clones were overexpressed as described by the manufacturer with the modification that the BL21(pLysS) *E. coli* host strain was substituted for the manufacturer's recommended host strain. Specifically, bacteriophages expressing a recombinant antigen recognized by mAb3869 were purified using LambdaSorb phage absorbent as described by the manufacturer (Promega). The mycobacterial insert fragment was isolated from low-melting-point agarose after *Eco*RI digestion and cloned into the pGEMEX vector. The ligation mixtures were then transformed into BL21(pLysS). The *M. kansasii* antigen was overexpressed by first growing the recombinant pGEMEX BL21 transformants at 37 °C to an OD₆₀₀ of 0.5 in LB medium supplemented with 30 µg chloramphenicol ml⁻¹ and 100 µg ampicillin ml⁻¹. The cells were induced with 5 mM IPTG and incubated with aeration for an additional 3–4 h at 37 °C. After the induction period, the cells were centrifuged, resuspended in 0.05 vol. 50 mM Tris (pH 7.4), 2 mM EDTA, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF) and frozen at -70 °C.

The BL21 lysates containing the overexpressed MK35 protein were resolved using standard SDS-PAGE techniques. To visualize the separated proteins, the gels were then soaked in a fluorescent dye solution that had been prepared by diluting 1 ml of a stock solution (10 mg ml⁻¹ in methanol) of 3,3'-dipentyl-oxacarbocyanine iodide (Molecular Probes) in 100 ml 0.1 M NaHCO₃, 0.1% SDS. After 10 min, the gels were destained in water for another 10 min. The gels were exposed to UV illumination and the overexpressed protein band was excised from the gels. The recombinant protein was electroeluted from the acrylamide slices using the Elutrap System (Schleicher and Schuell). Electroeluted *M. kansasii* recombinant protein was precipitated with 50% saturation ammonium sulfate, resuspended in 10 mM sodium phosphate (pH 7.0) and dialysed against the same buffer at 4 °C. Eluted recombinant protein was purified by hydrophobic interaction chromatography on a Hewlett Packard 1090 series M liquid chromatograph equipped with a photodiode array detector. The column used was a Hewlett Packard phenyl 5PW (7.5 × 7.5 mm) equilibrated with 2 mM ammonium sulfate in 10 mM sodium phosphate (pH 7.6). Protein solutions were adjusted to 2 M ammonium sulfate and were filtered through a 0.45 µm filter before injection. Protein was eluted with a 15 min gradient from 2 mM ammonium sulfate in 10 mM sodium phosphate (pH 7.6) to 10 mM sodium phosphate. The flow rate was set at 0.5 ml min⁻¹. Protein peaks were detected by absorbance at 280 nm.

Skin testing with purified MK35. Hartley strain guinea pigs (350 g) were sensitized by injecting into the nuchal area 2 mg of heat-inactivated bacilli from the *M. kansasii* Bostrom strain, the

M. intracellulare type strain, an *M. avium* serovar 4 AIDS-associated strain, or the *M. tuberculosis* H37Rv strain. Each preparation was suspended at a 10 mg ml⁻¹ concentration in Drakeol 6VR prior to the sensitization. The guinea pigs were skin tested by subcutaneous injection of 3 µg of the purified *M. kansasii* recombinant protein or the gene 10 protein that had been dissolved in 0.1 ml of 0.85 % NaCl, 0.001 % Tween 80. As positive controls, 0.1 µg of homologous sonic extracts were also tested in the sensitized guinea pigs. Delayed-type hypersensitivity responses were recorded 24 h after the recombinant protein challenge by measuring erythema at the site of injection.

Nucleotide sequence analysis of the MK35 gene. Protein expression experiments demonstrated that the entire MK35 coding sequence was located on a 1.3 kb *EcoRI*-*NruI* subfragment of the initial 3.3 kb phage insert fragment. For nucleotide sequence analysis of the MK35 gene, the 1.3 kb subfragment was cloned into the Bluescript SK vector. The sequence was determined utilizing double-stranded DNA sequencing protocols provided with the *BstI* polymerase sequencing system (Bio-Rad). The Bluescript T3 or M13 universal primers and one of several oligonucleotides generated from the *M. kansasii* sequences within the *EcoRI*-*NruI* fragment were used as sequencing primers. The 1.3 kb subfragment was sequenced completely and independently on both strands.

DNA hybridization studies. The DNA hybridizations were done as described previously (Nair *et al.*, 1993). Southern blots were hybridized to the MK35 probe using reagents and protocols provided in the PHOTOGENE Detection System (Bethesda Research Laboratories). After hybridization at 42 °C for 18 h, the blots were washed twice, 5 min per wash, in 5 × SSC/0.5 % SDS at 65 °C and then twice, 20 min per wash, in 0.1 × SSC/1.0 % SDS at 52 °C. The membranes were washed for 5 min in 2 × SSC followed by a brief wash in TBS [10 mM Tris (pH 7.4), 150 mM NaCl] supplemented with 0.05 % Tween 20 (TTBS). After blocking with 3 % (w/v) bovine serum albumin (Sigma) in TTBS at 65 °C for 1 h, the blots were incubated in streptavidin alkaline phosphatase conjugate for 10 min at room temperature, and washed twice in TTBS. A final wash for 1 h at room temperature was performed using a solution provided by the manufacturer. The membranes were then air-dried and incubated with the detection reagent for 3.5 h. Finally, the blots were exposed to Kodak XAR-2 film and developed.

Triton X-114 phase separations. The Bostrom strain of *M. kansasii* was grown in liquid culture and sonicated as described above. Triton X-114 phase separations of the sonic extracts followed previously described protocols (Bordier, 1981; Nair *et al.*, 1992). The protein concentrations of the separated phases were determined using the Bio-Rad protein assay. Samples were loaded at 20 µg per well on to 4–20 % (w/v) polyacrylamide gels (Integrated Separation Systems), electrophoresed, and then transferred to nitrocellulose. The immunoblots were incubated with mAb 3869 and developed as described above.

RESULTS

Isolation of a recombinant bacteriophage expressing the MK35 antigen

To identify novel mycobacterial antigens, a *M. kansasii* *λgt11* expression library was generated and monoclonal antibodies that recognized *M. kansasii* antigens were produced. mAb 3869 was of particular interest because immunoblot analyses demonstrated that this antibody specifically recognized *M. kansasii* sonic extract antigens

Fig. 1. Immunoblot analysis of *λ* lysogen lysates containing the MK35 *β*-galactosidase fusion protein. Immunoblots were incubated with mAb 3869 and developed as described in Methods. Lanes: 1, *M. kansasii* sonic extract; 2, lysate of a *λ* lysogen expressing the MK35 recombinant protein; 3, lysate of a *λ* lysogen expressing an *M. avium* fusion protein.

and did not react with sonic extracts from 12 other mycobacterial species. Furthermore, protease sensitivity assays indicated that mAb 3869 recognized a protein-associated epitope. Initial immunoblot studies suggested that the molecular mass of the reactive antigen was 35 kDa (hence its designation as MK35) (Morris *et al.*, 1991). Subsequent immunoblot characterizations using gradient SDS-polyacrylamide gels have demonstrated that the apparent molecular mass of the MK35 antigen is 32 kDa.

Since mAb 3869 recognized a specific *M. kansasii* protein-associated epitope, it was utilized to screen the *λgt11* library. A recombinant bacteriophage expressing an antigen detected by mAb 3869 was identified and purified. Lambda lysogens were then generated by infecting *E. coli* strain 1089 with antibody-reactive bacteriophage at a permissive temperature. Two mAb 3869 reactive polypeptides with molecular masses of 150 and 25 kDa were detected in *λ* lysogen lysates by immunoblotting (Fig. 1, lane 2). In subsequent immunoblot studies, the reactivity of the 150 kDa antigen with an anti-*β*-galactosidase antibody showed that this *M. kansasii* recombinant antigen is a *β*-galactosidase fusion protein (data not shown). The 25 kDa peptide that reacted with mAb 3869 is probably a breakdown product of the larger fusion protein. As controls, a lysate from an unrelated *M. avium* *λ* lysogen and *M. kansasii* sonic extract was evaluated. The control *λ* lysogen lysate did not react with mAb 3869 (Fig. 1, lane 3). In contrast, mAb 3869 recognized a 32 kDa antigen in the *M. kansasii* extract preparation (Fig. 1, lane 1).

Immunoreactivity of MK35

To evaluate the capacity of MK35 to induce cell-mediated responses, MK35 was tested in a guinea pig delayed-type

Table 1. Skin test reactivity of the MK35 recombinant protein

The mean skin test reaction $\pm$ the standard deviation (mm) for three guinea pigs is shown. NR, No reaction.

Sensitizing antigen	<i>M. kansasii</i> recombinant protein	Homologous sonic extract
<i>M. kansasii</i>	15 $\pm$ 2	12 $\pm$ 2
<i>M. avium</i>	6 $\pm$ 1	15 $\pm$ 1
<i>M. intracellulare</i>	6 $\pm$ 1	15 $\pm$ 1
<i>M. tuberculosis</i>	NR	15 $\pm$ 0
Controls (not sensitized)	NR	NR

hypersensitivity (DTH) assay. For these skin test assays, MK35-gene 10 fusion proteins were utilized because overexpression of these recombinant proteins facilitated their purification. The MK35-gene 10 protein and a gene 10 control antigen were purified as described in Methods. Three micrograms of the purified MK35 recombinant protein and the gene 10 control were injected intradermally into guinea pigs that had been sensitized with either *M. kansasii*, *M. avium*, *M. intracellulare* or *M. tuberculosis*, and into non-sensitized guinea pigs. Concurrent testing with 0.1 μ g of homologous mycobacterial sonic extract confirmed that the animals had been properly sensitized. As seen in Table 1, the recombinant MK35 protein elicited a 15 mm skin test reaction in *M. kansasii*-sensitized animals. In contrast, the MK35 fusion protein did not evoke a response in control non-sensitized guinea pigs. The recombinant protein also induced a moderate cross-reactive response in animals sensitized with *M. avium* complex (6 mm) but did not elicit a detectable response in those immunized with *M. tuberculosis*. The gene 10 control did not induce a DTH response in either control or sensitized animals. Therefore, the cell-mediated

reactions elicited by the fusion protein resulted from the antigenicity of the *M. kansasii* portion of this gene 10 hybrid protein.

Nucleic acid hybridization studies using MK35 gene probes

An *Eco*RI-*Nru*I fragment of the MK35 coding sequence was labelled and used to probe *Eco*RI-digested DNA from 12 different strains of mycobacteria. As expected, the MK35 probe hybridized strongly to a 3.3 kb restriction fragment in the *M. kansasii* genomic sample (Fig. 2a, lane 7). Hybridization was also detected with a 3.5 kb *M. avium* chromosomal fragment (Fig. 2b, lane 2) and a 7.5 kb *M. intracellulare* fragment (Fig. 2b, lane 6) upon extended exposure. The MK35 probe weakly hybridized to a 3.0 kb restriction fragment present in all *M. tuberculosis* complex strains tested (Fig. 2b, lane 4).

Nucleotide sequence analysis of the MK35 gene

To facilitate characterization of the MK35 protein, the nucleotide sequence of the gene encoding it was determined. The MK35 gene sequence and deduced protein sequence are shown in Fig. 3. The sequence contains a 675 bp open reading frame, initiating at an ATG (bp 19–21) and terminating at a TAA (bp 694–696). The open reading frame encodes a putative polypeptide of 225 amino acids with an estimated size of 26 kDa. Consistent with the high G + C content of mycobacterial genes, the MK35 coding sequence is 66 mol% G + C, with a 87% G + C bias in the third nucleotide of amino acid codons. A potential Shine-Dalgarno ribosome-binding site is positioned 11 bp upstream of the initiation codon. Inverted repeat sequences that have been identified downstream of the termination codon in several mycobacterial genes and that have been postulated to facilitate transcriptional termination (Shinnick *et al.*, 1989; Collins *et al.*, 1990; Morris *et al.*, 1992) were not identified in the MK35 gene sequence. The MK35 gene sequence and the

Fig. 2. Southern blot analysis using an MK35 gene probe. (a) Mycobacterial DNA restricted with *Eco*RI was loaded in the following lanes: 1, *M. asiaticum*; 2, *M. avium*; 3, *M. bovis*; 4, *M. bovis* BCG; 5, *M. fortuitum*; 6, *M. intracellulare*; 7, *M. kansasii*; 8, *M. phlei*; 9, *M. scrofulaceum*; 10, *M. smegmatis*; 11, *M. terrae*; 12, *M. tuberculosis* H37Ra; 13, *M. tuberculosis* H37Rv. *Hind*III-restricted DNA molecular mass markers were loaded in lane M. (b) Hybridization was detected in the *M. avium* (2), *M. bovis* BCG (4), and *M. intracellulare* (6) lanes with a fivefold extended exposure.

1 ttcggagatgcaagtatatgaagcactgaccggtgtagtcgagtcgacggtgagc 60
 aagccctctacgttctatatacttctgtaggactggccatcagcgtcagcgtcgcactcg
 M K H L T A V V A V A A V S
 61 ctggcactggtggttgcggctcaaaagaccgaacacacgtccacgtcgagtcgctcg 120
 gacgtgacgaccaacgagcaggttctggtcttgggtctggaggtcagcgtcgacgagc
 L A L A G C G S K T E T K T S T S T S S
 121 tcgacgagttccacgtcgacaacatccgctctcgcacgacgacacgggagcgcag 180
 agctgctcaaggtcgagctgtttaggcgacgagcgtggtcgctgtggtcgctcgctc
 S T S S T S T T S A S S T S A T P G A Q
 181 gccaaagaccatctccgactatctcaacgagaacacgtccacgaaccccggtccac 240
 cgttctctggttagaggtcgataggttgccttctggtcagtcggttggggcaggtg
 A K K T I S D Y L N E N N V T E T P V H
 241 ctggcgatcccggtcgccgacacatcaatttgcgcatgcccaacggtcgagacggcc 300
 gaccgctagggcgagcgggtgtagttaaaggcgtacgggttgcgacgctctggcg
 L G D P G S P T I N L P M P N G W Q T A
 301 aacgacagcagcacctcaccggcgccatcgtctacagtcagcggcgaccccaaggac 360
 ttgctgctgctggagtagtcggcggttagcagatgtagtcggcggtgggttctctg
 N D S S T S Y G A I V Y S Q P A D P K D
 361 ccgccccaccatctccgactggttccaaactcaccggcaacgtggacccggcgaagatc 420
 ggcgggtggttagagcgtgaccagaggtttagtggtgacgttgcacgtggcgctcttag
 P P T I S A L V S K L T G N V D P A K I
 421 atcgagtagtccccggcgagcgtcgagaactgcccgatcaccaggttccggcgaacggg 480
 tagctcaccagggcgccgctcgacgtcttgacggcgccatggtcccaagccggtgccc
 I E Y A P G E L Q N L P G Y Q G S G N G
 481 tccgctccacgtcagcgggttcaatgcctgacgtggcgcgacacacgtccgacgac 540
 aggcgcaggtgagtcgcaaaagttagcagcgtcgaccccggtggatcgaggcgctg
 S A S T L S G F N A W Q L G G T Y V R D
 541 ggcaagaccgcgccgctgcccagaagcgggtgtagtcccggtcaggacgcccgttac 600
 ccgttctggcgcgccgagcgggtcttgcacacactaggggccagtcctcgccgaatg
 G K T R A V A Q K T V V I P G Q D A V Y
 601 gtgcttcagctcaacgcccgaactcactggaagcagcagggcccggtgagtgagcgcac 660
 cagcaagtcgagtgccggtgagtgaccttctgctgctccggcgaactacacacgtgg
 V L Q L N A D S L E S E Q G P L M D A T
 661 agcgtcatcgacgagcagacacgacatcagcagtaacgagtagctggcgagcgcctggc 720
 tcgagtagctgctgctggtgtagtgctgctcattcgctcagcggcgctcgcgagcgg
 S V I D E Q T T I T Q * R S S W P A P G

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the MK35 gene. The putative initiation codon (bp 19–21) and termination codon (bp 694–696) are shown in bold type. A potential ribosome-binding site is underlined at bp 5–9.

deduced amino acid sequence were also analysed using the GCG software package (Devereux *et al.*, 1984). No significant homologies to known non-mycobacterial genes or proteins were found.

The MK35 gene encodes a mycobacterial lipoprotein

Lipoproteins are among the most immunogenic antigens identified in mycobacteria. We have previously demonstrated that the deduced protein sequences of four known mycobacterial lipoproteins – the 19 and 38 kDa *M. tuberculosis* proteins and the 19 and 27 kDa *M. intracellulare* antigens – have similar amino-terminal sequences (Nair *et al.*, 1992, 1993). A comparison of the MK35 protein sequence with the sequences of the other known mycobacterial lipoproteins revealed the same amino-terminal

1VKRQLTVAVAGAAI.LAAGISGCSGGNKSSTSSASSASSSSSTSA 50
 Mi22VKRQLTVAVAGAAI.LVAGLSGCS.SNKSTTSGSETTTAAGTFA
 Msgant19MKHLTAUVAVAAVSLAAGCGSKTETKTSTSTSSSTSTSTST
 Mk35 MSASCAVPRLTRFAVFAVAGATALSAGCSNKSSTSTSTSTSTSTST
 Mi43VKIRLHTLLAVLTAAPLLLAAGCGSKPPSGSPETGAGAGTVATTPA
 Msgpaba
 51 SASTGGAAG..TKVT.....IDG..KDQNV..... 100
 Mi22 SP..GAASG..PKV.....IDG..KDQNV.....
 Msgant19 ASSTSATPGAQAKT.....ISDYLNENNV.....
 Mk35 SAAPSSTPNAEAKVSGLIASVAGNSIQVTKEFNATAAVNFTSATKITEA
 Mi43 SSFVTLAETGSLTLLYPLNFWGPAFHERYPNVITTAQGTGSGAGIAA
 Msgpaba
 101GSVVCNTAGG..TINIAIGGA..... 150
 Mi22GSVVCNTAAG..NVNIAIGGA.....
 Msgant19ETPVHLGDPGSPITNLMPMPNGWTANDSSTSYGAIVYS
 Mk35VPAGLPDVTTQGSCLIVKPTGSGAPQVPTAAVKIYESVNGTCPKPHE
 Mi43 GTVNIGASDAYLSEGDMAAHKGLMNIALAISAQQVNYNLPVSGEHLKING
 Msgpaba
 151ATGIA.....AVLSD..GNPPOVK.SVGLGNVNGVTILGYTS 200
 Mi22ATGIA.....AVLTD..GNPPEVK.SVGLGNVNGVTILGYTS
 Msgant19 QPADPKDPPTIS.....ALVSKLTGNVDPAPK.IIEYAPGELQNLPGYQ
 Mk35 STPGGASSTPPS.....GSPSPAPAKPAWVRGVSASVSGDTINLTGTD
 Mi43 KVLAAAMYQGTIKTWDDPQIALNPGVNLPGTAVVPLHRSDGSGDTFLFTQ
 Msgpaba
 201 GTGQGNATASKDGNYSYKISGT.....ATG.VDMANFMQP 250
 Mi22 GTGQGNASATKDGSHYKITGT.....ATG.VDMANFMQP
 Msgant19 GSGNGSASTLSGFNAWQLGGTYVRDGTTRAVAKTIVVIGP.QDAVVVLQ
 Mk35 ASGNTTQTITVTDKTKYTKQTANTAEIAPGKCLISARGT.TDSGGALQA
 Mi43 YLSKQDPGEWKGSPGFGTTVDFFPAVFGALGENGNGMVTGCAETPGCVAY
 Msgpaba
 251 VNKPFEINVTCS*..... 300
 Mi22 VNKSFEIEVTC*.....
 Msgant19 NADSESEQPLMDATSVIDEQTTITQ*.....
 Mk35 TSIKLRQAVDVGKCKPKQPGQGG*.....
 Mi43 IGISFLDQASQRLGEALQNLNNGSNFLLPDAQSIQAAAAGFASKTPANQA
 Msgpaba

Fig. 4. Comparison of the predicted amino acid sequence of the MK35 protein with the sequences of the *M. intracellulare* 19 kDa lipoprotein (Mi22), the *M. tuberculosis* 19 kDa lipoprotein (Msgant19), the *M. intracellulare* 27 kDa lipoprotein (Mi43), and the *M. tuberculosis* 38 kDa lipoprotein (Msgpaba). The underlined amino acid residues represent the predicted lipoprotein processing sequences. Dots represent gaps in the protein sequences which facilitate alignment of these sequences.

primary sequence similarities (Fig. 4). Each mycobacterial lipoprotein has a sequence motif similar to the prokaryotic consensus sequence for protein lipidation and modification (Wu & Tokunaga, 1986). For MK35, the proposed lipidation sequence LAGCGS (amino acids 17–22) consists of an invariant cysteine flanked by small neutral amino acids. A hydrophobic region (possibly a signal sequence) precedes the lipidation site in each of these lipoproteins. Eleven of thirteen amino acids in this hydrophobic segment of MK35 (residues 4–16) are either leucine, valine or alanine. The region of the mycobacterial lipoproteins following the consensus lipidation sequence characteristically consists mainly of serines and threonines (Nair *et al.*, 1993a, b). For MK35, 23 of 28 amino acids in this segment (amino acids 23–58) are either serine or threonine.

Triton X-114 extractions of *M. kansasii* sonic extracts were performed to further examine the lipophilic nature of MK35. Proteins strongly associated with membranes (i.e. integral membrane proteins and lipoproteins) will segregate with the Triton X-114 detergent phase upon phase separation. Soluble proteins (i.e. cytoplasmic and loosely associated membrane proteins) will partition into the upper aqueous layer. To determine the detergent

Fig. 5. Immunoblot analysis of Triton X-114 extracted sonic extracts. The following samples were resolved by SDS-PAGE and blotted: lane 1, *M. intracellulare* sonic extract; lane 2, *M. kansasii* Triton X-114 detergent phase; lane 3, *M. kansasii* Triton X-114 aqueous phase; lane 4, *M. kansasii* sonic extract. The immunoblot was then incubated with mAb 3869 and developed as described in Methods.

solubility of MK35, *M. kansasii* sonicates were extracted with Triton X-114 and the phases were separated by incubation at 37 °C. The Triton X-114-soluble and aqueous phases were subjected to SDS-PAGE and then transferred to nitrocellulose. The Western blots were subsequently incubated with mAb 3869. The immunoblot analyses strongly suggested that MK35 is a hydrophobic protein because it partitioned almost completely into the Triton X-114 detergent phase (Fig. 5, lane 2) and was only weakly detected in the aqueous layer (Fig. 5, lane 3). As demonstrated previously, it is apparent from this immunoblot that MK35 migrates anomalously on SDS-polyacrylamide gels (Fig. 5, lane 4). Molecular mass estimates based on these experiments have suggested that MK35 is a 32 kDa protein and not the 26 kDa protein defined by nucleotide sequence analysis of the MK35 gene. This anomalous mobility on SDS polyacrylamide gels is characteristic of mycobacterial proteins modified by lipidation.

DISCUSSION

One prerequisite for developing improved mycobacterial vaccines and diagnostic reagents is the identification and characterization of novel mycobacterial immunogens. Lipoproteins are among the most immunoreactive antigens derived from bacterial pathogens, including mycobacteria. Bacterial lipoproteins are generally highly immunogenic *in vivo* and have been implicated as protective antigens for several diseases (Green *et al.*, 1987; Finke *et al.*, 1990; Fikrig *et al.*, 1992). Thus far, the 19 and 38 kDa *M. tuberculosis* proteins and the 19 and 27 kDa *M.*

intracellulare antigens have been identified as mycobacterial lipoproteins using genetic and biochemical methods (Young & Garbe, 1991; Nair *et al.*, 1992, 1993). Each of these proteins induces humoral and cell-mediated immunological responses. In this report, we describe the isolation and characterization of another immunogenic mycobacterial lipoprotein, the MK35 antigen from *M. kansasii*. We have concluded that MK35 is a lipoprotein based on the following data: the deduced amino acid sequence of MK35 has a consensus prokaryotic motif for lipidation, its amino acid sequence is similar to the sequences of known mycobacterial lipoproteins, MK35 has an aberrant electrophoretic mobility, and the MK35 antigen partitions into the detergent-soluble Triton X-114 extraction phase. Furthermore, we demonstrated that MK35 induced DTH reactions in sensitized guinea pigs. Previously we had shown that MK35 is recognized by sera from AIDS patients with mycobacterial disease (Morris *et al.*, 1991). This immunoreactivity of MK35 in DTH and serological assays is consistent with its classification as a mycobacterial lipoprotein.

Given the limited efficacy of the current mycobacterial vaccine, an international public health priority is the development of a more effective vaccine. The characterization of new mycobacterial immunogens should accelerate vaccine development. Because of the problems associated with a live vaccine, including stability, safety, consistency of production and limited applicability for immunocompromised individuals, it has been suggested that a mycobacterial subunit vaccine would be desirable. Recent studies have shown that a subunit vaccine may be feasible because *M. tuberculosis* culture filtrate proteins are partially protective against an *M. tuberculosis* challenge and vaccination with purified *M. leprae* proteins inhibited *M. leprae* multiplication in mouse footpads (Andersen, 1994; Gelber *et al.*, 1994; Horwitz *et al.*, 1995). The inherent immunogenicity of mycobacterial lipoproteins makes them potential candidates for evaluation as subunit vaccine components. Alternatively, the efficacy of BCG as a mycobacterial vaccine could be enhanced by over-expressing protective antigens in BCG. Stover *et al.* (1993) have shown that expression of target antigens in BCG as membrane-associated lipoproteins increases the potential to induce protective responses. Therefore, testing the protective capacity of mycobacterial lipoproteins, including MK35, as recombinant fusion proteins in BCG or a components of a subunit vaccine is warranted.

Because of the increasing importance of mycobacterial disease, there is also considerable interest in the availability of species-specific skin test or serological reagents. The selective and large DTH reactions elicited by MK35 and the seroreactivity of MK35 suggest that this protein, or peptide derivatives of it, may have potential clinical utility as immunodiagnostic reagents for the detection of *M. kansasii* infections in humans. Recent experiments have demonstrated that synthetic peptides designed from the sequences of mycobacterial lipoproteins can yield species-specific DTH responses (Vordermeier *et al.*, 1992; Estrada *et al.*, 1992; Mackall *et al.*, 1993). These studies suggest that the development of species-specific diag-

nostic reagents consisting of multimeric peptide polymers may be feasible. The availability of species-specific reagents for skin testing or serodiagnosis would possibly permit the rapid differentiation of nontuberculous mycobacterial infections from those caused by *M. tuberculosis*.

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