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Mapping of the Delayed-Type Hypersensitivity-Inducing Epitope of Secreted Protein MPT64 from *Mycobacterium tuberculosis*

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The gene encoding the immunogenic protein MPT64 found in culture filtrates of *Mycobacterium tuberculosis* H37Rv was expressed in *Escherichia coli* K-12 and purified as a recombinant protein. The purified recombinant MPT64 elicited delayed-type hypersensitivity (DTH) in outbred guinea pigs sensitized with *Mycobacterium bovis* BCG Tokyo. The skin reactions were comparable to those obtained with native MPT64. No skin reactions were observed when either recombinant MPT64 or native MPT64 was used in guinea pigs sensitized with *M. bovis* BCG Danish 1331. Amino- and carboxy-terminal deletion mutants of MPT64 were purified as fusion proteins for the mapping of DTH-inducing epitopes on recombinant MPT64 by use of the guinea pig skin test model. The part of the molecule responsible for the biological activity was located at the carboxy-terminal end. Further studies with overlapping synthetic peptides have pinpointed the biological activity at a single DTH-inducing epitope consisting of 15 residues between amino acids Gly-173 and Ala-187. Screening by PCR of 56 clinical isolates of *M. tuberculosis* from Danish and Tanzanian patients demonstrated the presence of *mpt64* in all of the strains. These results point to MPT64 as a possible candidate for a skin test reagent specific for diagnosis of human tuberculosis.

Tuberculosis, caused by the slow-growing *Mycobacterium tuberculosis*, is still a world health problem. The World Health Organization estimates that there are 8 million new cases and 2 to 3 million deaths resulting from tuberculosis every year (37). For many years, the incidence of tuberculosis dropped steadily in developed countries. However, the disease continued to exact a high mortality rate in the developing world (25), and during the last few years we have witnessed a revival of tuberculosis in the United States (2, 30) and elsewhere. These facts have led to renewed efforts to improve the existing methods in the control of the disease. Two of the major products presently used for this purpose, the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine and the skin test reagent tuberculin purified protein derivative (PPD), both have shortcomings: the efficacy of BCG remains a matter of controversy (31), and the diagnostic value of tuberculin PPD is weakened because the sensitivity is induced by a broad range of mycobacteria. In particular, the fact that successful BCG vaccination as well as infection with *M. tuberculosis* leads to high-level tuberculin PPD reactivity complicates the interpretation of tuberculin test results. Biochemical, immunological, and molecular biological characterization of *M. tuberculosis* has led to the identification of several antigens which may be useful in the development of improved diagnostic methods and/or vaccines (40). The proteins actively secreted by *M. tuberculosis* during growth have attracted special attention for several reasons. One of the predominant proteins is MPT64, a 24-kDa protein initially isolated from culture filtrates of *M. bovis* BCG Tokyo (10). The protein is produced and secreted by the strains of the tuberculosis complex (*M. tuberculosis*, *Mycobacterium africanum*, and *M. bovis*, including *M. bovis* BCG) (1, 11). However, it was recently demonstrated that not all substrains of *M. bovis* BCG carry the gene (19, 27).

Recent studies by Hasløv et al. (16) described the systematic analysis of secreted antigens from *M. tuberculosis* for improved diagnostic skin test reagents for tuberculosis. The antigens in the molecular mass region giving a specific delayed-type hypersensitivity (DTH) reaction were further characterized; MPT64 showed superior specificity compared with various secreted antigens and with tuberculin PPD in outbred guinea pigs infected with *M. tuberculosis* H37Rv or *M. bovis* BCG Tokyo but not in *M. bovis* BCG Danish 1331-infected animals (16). The distinction between tuberculin PPD sensitivity conferred by an *M. tuberculosis* infection from that induced by a BCG vaccination or an infection with mycobacteria not belonging to the tuberculosis complex would be extremely useful in the control of tuberculosis.

Presently, we report the use of recombinant MPT64 (rMPT64) as a skin test reagent in outbred guinea pigs, the mapping of a putative DTH-inducing epitope by the use of recombinant deletion mutants of MPT64, and a fine mapping by the use of synthetic peptides. Furthermore, evidence for the presence of *mpt64* on genomic *M. tuberculosis* DNA isolated from Danish and Tanzanian tuberculosis patients is presented.

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MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strain used in this study was XL1-Blue (4). The mycobacterial strains used in this study were *M. bovis* (BCG) Danish

1331 (Statens Seruminstitut, Copenhagen, Denmark), *M. bovis* BCG Tokyo (World Health Organization International Laboratory for Biological Standards, Statens Seruminstitut), and clinical isolates from 30 Danish and 26 Tanzanian tuberculosis patients identified by standard diagnostic methods at the Mycobacteria Department, Statens Seruminstitut.

The mycobacterial genomic DNA was prepared as described elsewhere (34). The pMAL-*p* expression vector (New England Biolabs, Beverly, Mass.) was used for the expression studies.

DNA technology. Standard procedures were used for the preparation and handling of DNA (21).

The cloning of *mpt64* from *M. tuberculosis* H37Rv chromosomal DNA and the construction of the deletion mutants have been described previously (27).

The primers (MPT64-4 and MPT64-3) were synthesized on the basis of the nucleotide sequence from *mpt64*, and the PCRs were carried out as described elsewhere (27).

Antigens. Purified native MPT64 was kindly provided by S. Nagai, Osaka, Japan (26). Tuberculin PPD RT23 was prepared at Statens Seruminstitut (20).

Guinea pigs. The outbred guinea pig strain Ssc:AL (Statens Seruminstitut) was used throughout the study. Female guinea pigs weighing 300 to 400 g at sensitization were used.

Preparation and purification of rMPT64. Recombinant antigen was prepared as described in instructions provided by New England Biolabs. Briefly, single colonies of *E. coli* harboring the pTO14 plasmid (27) were inoculated into Luria-Bertani broth containing 50 μg of ampicillin per ml and 12.5 μg of tetracycline per ml and grown at 37°C to 2×10^8 cells per ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.3 mM, and growth was continued for an additional 2 h. The pelleted bacteria were stored overnight at −20°C in column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). Cells were broken by sonication (20 times for 10 s with intervals of 20 s). After centrifugation at 9,000 × *g* for 30 min at 4°C, the maltose-binding protein–MPT64 fusion protein (MBP-rMPT64) was purified from the crude extract by affinity chromatography on an amylose resin column. MBP-rMPT64 binds to amylose. After extensive washes of the column, the fusion protein was eluted with 10 mM maltose. MBP-rMPT64 was concentrated to approximately 1 mg/ml with an Amicon (Beverly, Mass.) cell with a YM10 membrane and then dialyzed extensively against 10 mM Tris-HCl [pH 8.0]–100 mM NaCl. Subsequently, the rMPT64 was cleaved from MBP with 1% (vol/vol) factor Xa at 4°C for 48 h. After cleavage, the product was dialyzed extensively against 10 mM Tris [pH 8.5], and rMPT64 was separated from MBP by fast protein liquid chromatography (FPLC; Pharmacia, Uppsala, Sweden) over an anion-exchange column (Mono Q) with 10 mM Tris [pH 8.5] with a 0 to 500 mM NaCl linear gradient. Fractions containing rMPT64 partly separated from MBP were pooled and dialyzed extensively against column buffer; this was followed by another separation of rMPT64 from MBP by affinity chromatography on an amylose resin column as described above.

Aliquots of the fractions were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE). Fractions containing purified rMPT64 were pooled and concentrated 30 times with an Amicon cell with a YM10 membrane. Subsequently, rMPT64 was dialyzed extensively against physiological saline before use.

Protein concentration was determined by the bicinchoninic acid method (Pierce Chemical Co., Rockford, Ill.).

Preparation and purification of rMPT64 deletion mutants. Three C-terminal and three N-terminal deletion mutants were expressed in the *E. coli* pMAL-*p* expression vector system as described above. The fusion proteins were purified by affinity chromatography on an amylose resin column followed by FPLC over an anion-exchange column (Mono Q) as described above. Aliquots of the fractions were analyzed by SDS–10% PAGE. Fractions containing purified recombinant fusion proteins were pooled and dialyzed extensively against physiological saline before use. Protein concentration was determined by the bicinchoninic acid method.

PAGE and Western blotting (immunoblotting). Samples containing 2 μg of protein were separated by SDS–10% PAGE before being silver stained (3) or transferred onto nitrocellulose sheets by electroblotting.

Protein standards of known molecular masses were obtained from Bio-Rad Laboratories, Richmond, Calif. Nitrocellulose sheets were soaked in phosphate-buffered saline (PBS; pH 7.6) containing 0.5% Tween 20 as the blocking agent. PBS (pH 7.6) containing 0.05% Tween 20 was used for dilution of antibodies and for washing. The nitrocellulose-bound samples were probed with the monoclonal antibody (MAb) C24b1 as described earlier (1). The detecting antibodies were horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (P260; DAKO A/S, Glostrup, Denmark). Color reaction was obtained with 3.3′,5.5′-tetramethylbenzidine as the substrate.

Selection of peptides. On the basis of the guinea pig skin test results obtained with the truncated versions of rMPT64, synthetic peptides were produced. To cover the region from amino acid residue Gln-112 of the MPT64 molecule to the amino-terminal residue Ala-205, one 24-residue peptide, thirteen 25-residue peptides, and one 26-residue peptide all overlapping with 20 amino acids were synthesized and tested in the guinea pig model. The peptides were designated P1 to P15 (Table 1).

Peptide synthesis. Solid-phase peptide synthesis was performed with the fluorenylmethoxycarbonyl (Fmoc) strategy by use of multiple-column peptide synthesis developed in our laboratory as described previously (17, 24). All peptides were synthesized with Fmoc amino acid pentafluorophenyl esters (Milligen) except for Asp in the case of Asp-Ser and Asp-Thr sequences and except for Arg, which were coupled as Fmoc-Asp(Ada)-OH (Calbiochem-Novabiochem) and Fmoc-Arg(Pmc)-OH (Milligen), respectively, with *O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethyluronium tetrafluoroborate (TBTU; Milligen) and 1-hydroxybenzo-triazole hydrate (HOBT; Milligen) as coupling agents. In all cases, 0.22 M solutions (dimethylformamide at five times excess) were used for the couplings with an acid-labile PepSyn KA resin with the first amino acid attached (S, ~0.1; Milligen/Biosearch). Dimethylformamide was distilled in vacuo prior to use. The peptides were cleaved from the resin with trifluoroacetic acid (TFA)-H_2O-ethanedithiole-thioanisole (90:5:3:2, vol/vol/vol/vol) at room temperature for 2 h and then washed with TFA-H_2O (95:5, vol/vol). The combined TFA washes were concentrated in vacuo, and the peptide was precipitated and washed in ether, dried, purified by gel filtration on a Sephadex G-15 column (Pharmacia), and freeze-dried. High-pressure liquid chromatography (HPLC) was performed on a Waters HPLC system with a C_{18} reversed-phase column (Waters Rad-Pak Delta-Pak C_{18}; 15 μm, 100 Å [10 nm], 8 by 100 mm; flow rate, 1.5 ml/min for analytical separations) with a buffer consisting of 0.1% TFA and a buffer consisting of 0.1% TFA and 10% water in acetonitrile. Amino acid analyses were performed by the PICOTAG system (Waters). All compounds more than 80% pure were used for the skin test studies.

TABLE 1. Sequence of overlapping synthetic peptides

Peptide	Residues	Sequence
P1	113–138	QAYRKPITYDTLWQADTDPLPVVFPI
P2	118–142	PITYDTLWQADTDPLPVVFPIVQGE
P3	123–147	TLWQADTDPLPVVFPIVQGELSKQT
P4	128–152	DTDPLPVVFPIVQGELSKQTGQQVS
P5	133–157	PVVFPIVQGELSKQTGQQVSIAPNA
P6	138–162	IVQGELSKQTGQQVSIAPNAGLDPV
P7	143–167	LSKQTGQQVSIAPNAGLDPVNYQNF
P8	148–172	GQQVSIAPNAGLDPVNYQNFAVTND
P9	153–177	IAPNAGLDPVNYQNFAVTNDGVIFF
P10	158–182	GLDPVNYQNFAVTNDGVIFFFNPGE
P11	163–187	NYQNFAVTNDGVIFFFNPGELLPEA
P12	168–192	AVTNDGVIFFFNPGELLPEAAGPTQ
P13	173–197	GVIFFFNPGELLPEAAGPTQVLVPR
P14	178–202	FNPGELLPEAAGPTQVLVPRSAIDS
P15	183–205	LLPEAAGPTQVLVPRSAIDSMLA

Immunization of guinea pigs. Groups of guinea pigs were immunized intradermally on the abdomen four times with 0.1 ml of the chosen vaccine, which was either freeze-dried BCG Danish 1331 containing approximately 4×10^6 CFU/ml or BCG Tokyo containing approximately 24×10^6 CFU/ml of the reconstituted preparations.

Skin tests. Guinea pigs were shaven on the back and given intradermal injections of 0.1 ml of the preparations in question in physiological PBS (pH 7.4). The reactions were read blind after 24 h by two independent readers, each measuring two transverse diameters of the erythemas. Reaction diameters are given as means corresponding to single diameters.

RESULTS

Expression of recombinant MPT64 in *E. coli*. The gene *mpt64* was expressed in *E. coli* with the pMAL-*p* expression vector developed at New England Biolabs. The recombinant proteins were fused to MBP and encoded by *malE*, and the expression was regulated by the strong IPTG-inducible P_{tac} promoter. A specific recognition site for the protease, factor Xa, allows the protein of interest to be cleaved from MBP after purification on the amylose-affinity column. Figure 1A shows the SDS-PAGE analysis of the extract of cells expressing rMPT64 both as a fusion protein and as a purified fusion protein before and after cleavage with factor Xa and subsequent purification. On average, 15 to 25 mg of fusion protein was obtained per liter of culture. Both the fusion protein and purified rMPT64 reacted strongly with MAb C24b1 as shown by Western blotting (Fig. 1B).

Furthermore, the purified rMPT64 was, by Western blotting, shown to bind the MAb C24b1 at the same mobility position as

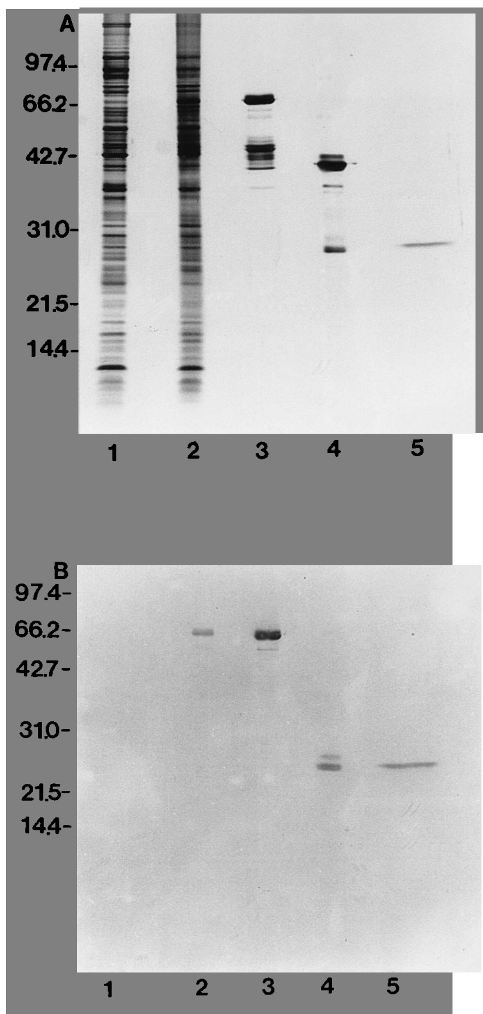

FIG. 1. SDS-PAGE analysis of rMPT64. A silver-stained SDS-polyacrylamide gel (A) and a Western blot (B) probed with the MAb C24b1 of *E. coli* extract containing recombinant plasmid (pTO14) before and after induction with IPTG (lanes 1 and 2), purified fusion protein (MBP-rMPT64) (lane 3), fusion protein cleaved with the protease factor Xa to release MBP and rMPT64 (lane 4), and purified rMPT64 separated by FPLC and affinity chromatography after cleavage (lane 5) are shown. Numbers at the left indicate molecular masses (in kilodaltons).

purified native MPT64 and as MPT64 found in *M. tuberculosis* H37Rv short-term culture filtrate (Fig. 2).

Skin testing of guinea pigs with rMPT64. Because of our interest in the immunological potential of MPT64 as a skin test reagent, groups of outbred guinea pigs were vaccinated with either BCG Danish 1331 (absence of *mpb64*) or BCG Tokyo (presence of *mpb64*) (27). All guinea pigs were skin tested with 0.1 μg of purified rMPT64, MPT64, and 2 tuberculin units of tuberculin PPD RT23. The skin reactions in the guinea pigs are shown in Fig. 3. As expected, rMPT64 elicited delayed-type hypersensitivity (DTH) reactions in the guinea pigs sensitized with BCG Tokyo only. The sizes of the skin reactions are comparable to those obtained with native MPT64; no skin reactions were seen with guinea pigs sensitized with BCG Danish 1331. The skin reactions were of the erythematous type, whereas very little induration resulted from skin tests with rMPT64 and MPT64 (data not shown).

DTH-inducing epitope mapping on the rMPT64 fusion proteins by skin tests in guinea pigs. To localize the antigenic domains recognized by the DTH-inducing cells involved in skin reactions, a series of deletion mutants was constructed (27). The deletion mutants were expressed in the *E. coli* pMAL-*p* expression vector. By manipulating the gene encoding the fusion protein rather than the nonfused protein, it was possible by use of polyclonal antibodies raised against the MBP to monitor the production of the truncated molecules. Furthermore, it was possible to monitor whether any proteolytic degradation of the products occurred. In total, six of the truncated versions of rMPT64, three with C-terminal and three with N-terminal deletions, were purified as fusion proteins (Fig. 4). Furthermore, the complete rMPT64 as fusion protein (TO14) and the MBP alone, purified similarly, were used as positive and negative control preparations, respectively, for the skin

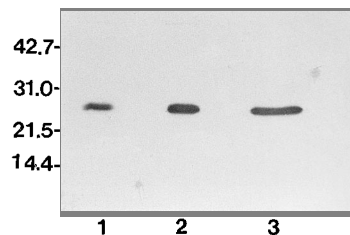

FIG. 2. Western blot analysis of *M. tuberculosis* H37Rv short-term culture filtrate, native MPT64, and rMPT64 separated by SDS-PAGE and probed with the MAb C24b1. Lanes: 1, *M. tuberculosis* H37Rv short-term culture filtrate; 2, purified native MPT64; 3, purified rMPT64. Numbers at the left indicate molecular masses (in kilodaltons).

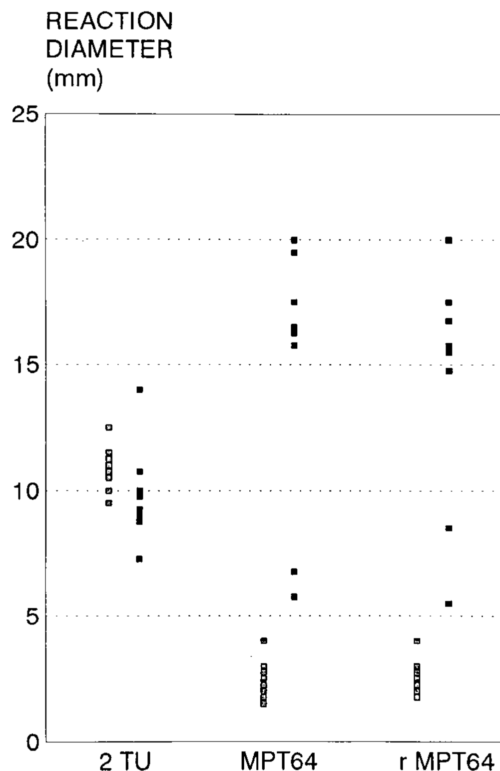

FIG. 3. Measurement of reactions of outbred guinea pigs skin tested with 0.1 μg of antigen or 2 tuberculin units (2 TU) of tuberculin PPD RT23. Skin testing was performed 4 weeks after vaccination with either BCG Tokyo (■) or BCG Danish 1331 (□).

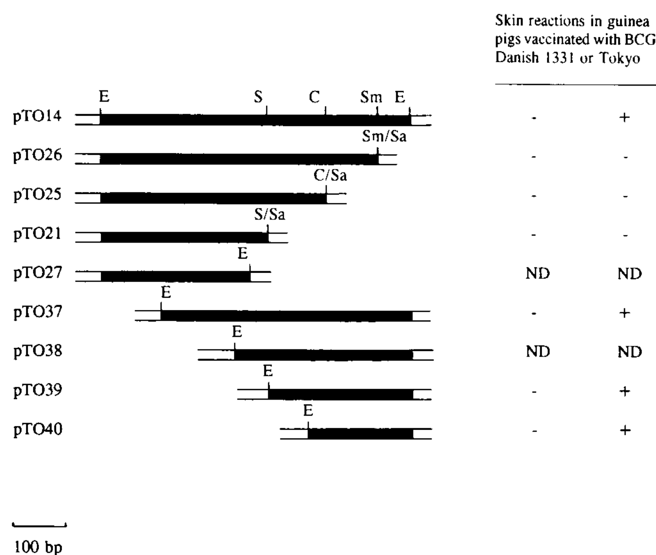

FIG. 4. Physical map of recombinant plasmids expressing various regions of *mpt64* and skin test reactivity in outbred guinea pigs to 0.1 μg of purified fusion proteins expressed by the plasmids. (Left) Symbols: open bars, vector DNA; closed bar, *mpt64*. The transcription of the gene is from left to right. (Right) Reactivity by skin testing of outbred guinea pigs to purified recombinant fusion proteins was established by DTH analysis. Each group contained eight animals. Symbols: +, skin reactions larger than 8 mm (two times mean value plus standard error of the negative control preparation, i.e., purified MBP); −, skin reactions smaller than 4 mm (mean value plus standard error of the negative control preparation, i.e., purified MBP); ND, not determined. Restriction site abbreviations: C, *Cla*I; E, *Eco*RI; S, *Stu*I; Sa, *Sal*I; Sm, *Sma*I.

test experiments. For the DTH-inducing epitope mapping, groups of outbred guinea pigs were vaccinated with either BCG Danish 1331 or BCG Tokyo. The results of the skin reactions in the guinea pigs are summarized in Fig. 4. The negative control preparation, purified MBP, gave no significant DTH reactions in the guinea pigs (data not shown).

From the skin test experiments with the truncated recombinant fusion proteins, it can be concluded that BCG Tokyo-sensitized guinea pigs recognize a DTH-inducing epitope encoded by the plasmids pTO37, pTO39, and pTO40, which express N-terminally truncated versions of the protein. The ability to give a DTH reaction is abolished when 25 or more of the C-terminal residues are removed as shown by the fact that the gene product of pTO26 gave no DTH reaction. However, it cannot be excluded that at least a part of the pTO26-encoded molecule is included in the epitope.

DTH-inducing epitope mapping with synthetic peptides. To precisely locate the epitope responsible for the DTH reaction, a series of synthetic peptides was produced. The peptides were constructed on the basis of the DTH reactions obtained with the fusion proteins encoded by the plasmids pTO37, pTO39, and pTO40, which express N-terminally truncated versions of the protein. Each peptide overlapped the next by 20 amino acids. Guinea pigs were vaccinated with either BCG Danish 1331 or BCG Tokyo and subsequently skin tested with 1 μg of the peptides. The results show that BCG Tokyo-sensitized guinea pigs recognize the peptides P11, P12, and P13 (Fig. 5). Thus, it can be concluded that a putative DTH-inducing epitope is located in the overlapping part of these three peptides, namely within the 15 amino acids from Gly-173 to Ala-187 (Fig. 5).

Presence of *mpt64* in *M. tuberculosis* strains isolated from tuberculosis patients. If MPT64 has a potential as an alternative skin test antigen for use in humans, it is important to verify that the *mpt64* gene is present in a high proportion of clinical isolates. We therefore examined whether *mpt64* or a part of *mpt64* was present on the chromosome of *M. tuberculosis* isolated from tuberculosis patients. The PCR technology described previously (27) was used for this investigation of 56 patient samples. A set of PCR primers which produced a fragment of 176 bp was used; both primers were situated in the interior of *mpt64* (27). All 56 samples (26 Tanzanian and 30 Danish) were found to be positive, and no unspecific reactions were observed (data not shown).

The results indicate that patients from Denmark and Tanzania infected with *M. tuberculosis* all contain *mpt64* or a part of *mpt64*. Therefore, the gene encoding MPT64 is with high probability present in all tuberculosis patients. If the gene is constitutively expressed, it may thus be expected that tuberculosis patients are sensitized by the gene product and, accordingly, that MPT64 or rMPT64 skin reactivity may appear.

DISCUSSION

Tuberculin PPD has been and still is an extremely useful diagnostic and epidemiological tool. The diagnostic value of tuberculin PPD is, however, limited by the fact that sensitivity is induced by a broad range of mycobacteria. Therefore, the distinction between tuberculin PPD sensitivity conferred by an *M. tuberculosis* infection from that induced by a BCG vaccination or an infection with mycobacteria not belonging to the tuberculosis complex would be extremely useful. Over the years, many attempts have been made to develop new species-specific skin test reagents. Lately, purified and semipurified antigens, synthetic peptides, and recombinant proteins have been tested in animal models (1, 7, 10, 14, 15, 26, 28, 38) and in clinical trials (5, 32, 35).

One antigen, MPT64, has attracted special attention because it is able to elicit a strong DTH reaction in guinea pigs infected with *M. tuberculosis* H37Rv or BCG Tokyo but not BCG Danish 1331 (10, 16). This point has been explained lately by genetic analyses which have shown that the gene *mpt64* is present on chromosomal DNA from *M. tuberculosis* isolates and from some but not all of the BCG substrains. Thus, the gene is present in the Tokyo but not the Danish 1331 substrain (27).

If MPT64 is going to be useful as an alternative skin test antigen, it is important to verify that the gene is consistently present in clinical isolates. In the present study, screening of a fairly large number of clinical isolates of *M. tuberculosis* by PCR demonstrated the presence of *mpt64* in all of the strains. The primers used for the PCR are *mpt64* specific in the sense that no homologies to other DNA sequences have been found by database searches. The results obtained in the present study confirm the results described previously (22). It is therefore possible that T lymphocytes from tuberculosis patients will be exposed to and be able to recognize the gene product, MPT64, and that a DTH reaction will be seen in the patients when skin tested with the antigen.

The guinea pig skin reactions to MPT64 observed in the present study were of the erythematous type with very little induration. In contrast, tuberculin PPD gave skin reactions with erythema as well as with induration. This confirms the recent findings of Haga et al. (9). Nevertheless, histological examination of skin reactions does not demonstrate differences in the cellular infiltrations caused by MPT64 and tuberculin PPD (9, 13). Preliminary human skin test data from a clinical trial have demonstrated similar levels of induration caused by MPT64 and tuberculin PPD, indicating that different immuno-

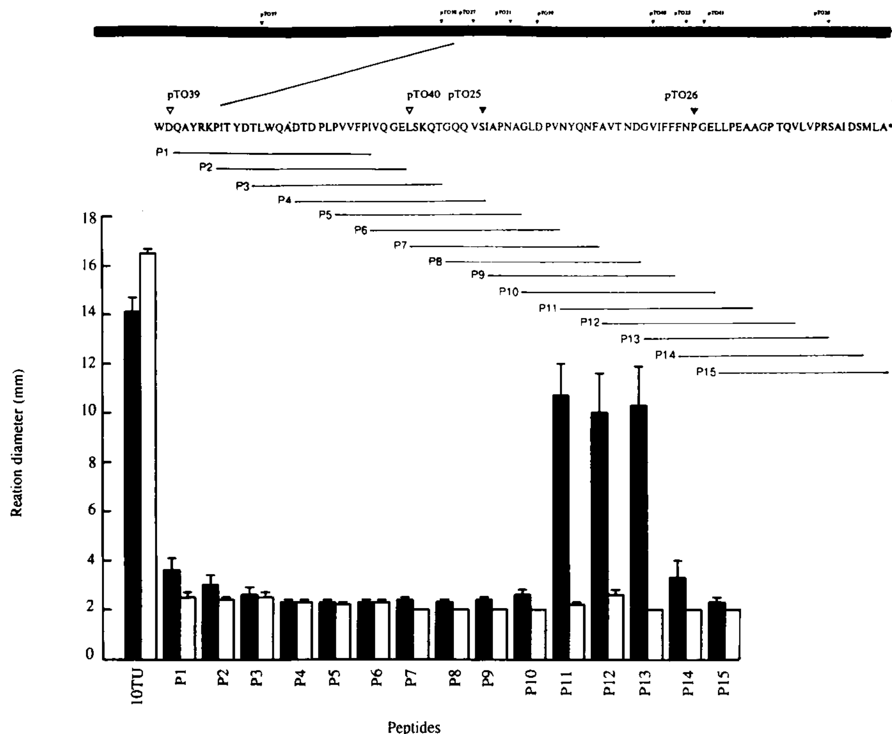

FIG. 5. (Top) Physical map of the carboxy-terminal region of rMPT64 shown as the deduced one-letter amino acid sequence. The start (▽) and end (▼) positions of the different deletion mutants are shown. (Middle) Synthetic peptides covering the amino acid residue Gln-112 to amino acid residue Ala-205 overlapping each other with 20 residues. (Bottom) Outbred guinea pigs were skin tested with 1 μg of antigen (P1 to P15) or 10 tuberculin units of tuberculin PPD RT23 (10TU). Skin testing was performed 4 weeks after vaccination with either BCG Tokyo (■) or BCG Danish 1331 (□). The mean value plus standard error corresponding to eight animals in each group is shown.

logical mechanisms may be operative in guinea pigs and humans (13).

In the present study, the recombinant molecule of MPT64 was found to be as suitable as native MPT64 as a skin test reagent in the guinea pig model. Our results support and extend a recent study in which recombinant MPT64 was used as a skin test reagent (9) with the finding that rMPT64 as well as MPT64 has a specificity superior to that of tuberculin PPD (Fig. 3). Several approaches have been used lately for mapping T-cell and DTH-inducing epitopes of mycobacterial antigens, e.g., synthetic peptides, truncated fusion proteins used in either in vitro or in vivo studies (for examples, see references 12, 18, 35, 36, and 39). To our knowledge, an in vivo study taking advantage of the use of both recombinant truncated molecules and overlapping synthetic peptides for DTH-inducing epitope mapping has not been undertaken before. The use of synthetic peptides in in vivo analysis is a powerful and informative approach and a useful supplement to the in vivo experiment using recombinant proteins. The DTH reactions seen with the synthetic peptides confirm the results obtained with the recombinant truncated versions of MPT64. For example, the gene product of pTO40 gave a DTH reaction because the epitope is included in the molecule, whereas the gene product of pTO26 gave no DTH reaction probably because the molecule is truncated in the middle of the putative DTH-inducing epitope. The DTH reaction may be restricted by the major histocompatibility complex (MHC) class II molecules (33), and peptides associated with murine MHC class II molecules have been found to be 13 to 17 amino acids long (29). We have in the guinea pig skin test model localized a putative DTH-inducing epitope within 15 amino acids. Further in vivo studies using synthetic peptides will reveal the final motif recognized by the MHC class II molecule in the guinea pig model.

Other studies have described mycobacterial antigens as possessing few DTH-inducing stimulatory epitopes. The 38-kDa antigen from *M. tuberculosis* was shown to have two possible epitopes (36); one of these has been found to be specific (35). In vitro studies of a 21.5-kDa *Mycobacterium avium* antigen identified two epitopes when recombinant fusion proteins and synthetic peptides were used (39).

It was previously believed that T-cell epitopes had certain characteristics in common which might be predicted by computer algorithms. One such method was described by Margalit et al. (23). However, the DTH-inducing epitope described in this study did not fulfill the criteria outlined by this algorithm.

The present experiments suggest that rMPT64 may be a suitable skin test reagent for diagnosis of tuberculosis because it has a specificity superior to that of tuberculin PPD in guinea pigs. A crucial point will be the MHC-determined distribution of reactors and nonreactors in a human population with tuberculosis at various stages. Other studies (for example, see reference 6) indicate that a single antigen may not effectively replace the unfractionated tuberculin PPD preparation for use in in vitro diagnostic tests. A combination of several purified antigens or a combination of several DTH-inducing epitopes selected for their species specificity may be required. Only human clinical trials using MPT64, rMPT64, truncated versions of them, and synthetic peptides can clarify this point and will demonstrate whether the antigen is in fact suitable as a diagnostic tool to differentiate infection from vaccination. A pilot study in a human population in Copenhagen is in progress.

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