Evidence that protein antigen b of *Mycobacterium tuberculosis* is involved in phosphate metabolism

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Protein antigen b (Pab) of *Mycobacterium tuberculosis* has previously attracted interest because of its immunological and diagnostic relevance. In this study we present evidence that Pab possesses a signal sequence and is secreted from the cytoplasm of *M. tuberculosis*. The synthesis of Pab is enhanced under phosphate starvation indicating that the protein is involved in phosphate metabolism in *M. tuberculosis*.

Introduction

*Mycobacterium tuberculosis* is the causative agent of tuberculosis—a serious infectious disease still far from being eradicated, especially in developing countries. However, little is known about the nature of the virulence of *M. tuberculosis* and other mycobacterial pathogenic species. Many mycobacterial proteins are known to be immunologically reactive; here we study the biological function and possible role in pathogenicity of one of them.

We have previously isolated the gene encoding protein antigen b (Pab; 38 kDa) from *M. tuberculosis*, because of the immunological and diagnostic relevance of this protein (Andersen & Hansen, 1989). The protein possesses species specific B-cell epitopes defined by monoclonal antibodies (mAbs) (Andersen et al., 1986). T-Lymphocytes isolated from immunized mice, guinea pigs or humans proliferate when cultured in the presence of purified Pab (Kadival et al., 1987; Young et al., 1986; Worsaae et al., 1987). A high proportion of humans suffering from active tuberculosis—especially if they are of the HLA type DR2—have been shown to develop antibodies towards Pab (Bothamley et al., 1989).

In this study we have sought evidence that Pab is secreted from the cytoplasm of *M. tuberculosis* and that the protein is involved in phosphate metabolism.

Methods

**Bacterial strains.** The strain *Mycobacterium tuberculosis* H37Rv used in this study originates from a series of mycobacteria submitted by the International Working Group on Mycobacterial Taxonomy, 1966.

*mAbs.* The mAbs HYT 28, HAT2 and HBT 12, all of which bind Pab, were produced as described previously (Ljungqvist et al., 1988; Schou et al., 1985). The mAb HAT 5 was obtained from BALB/c mice immunized with *Escherichia coli* Y 1089 (Young & Davis, 1983). The supernatants from growing hybridomas were tested for reactivity towards concentrated culture filtrate (CF) of *M. tuberculosis* H37Rv in an enzyme-linked immunosorbent assay (ELISA). The clone HAT 5 produced mAbs that bound to a protein of molecular mass 65 kDa. That this protein is the so-called 65 kDa protein (in our laboratory designated protein antigen a, Paa) was verified by experiments in which HAT 5 bound a recombinant version of the molecular, MbaA (kindly provided by J. van Embden, Bilthoven, the Netherlands), and the fusion protein produced by the *λ* phage Y 3143 (Husson & Young, 1987) (obtained through the World Health Organization Immunology of Tuberculosis Programme, reference number WHO 0071) which is known to encode Paa.

**Affinity purification of Pab.** Protein A-purified HBT 12 immunoglobulins were coupled to Mini-Leak vinylsulphone-agarose (Kem-entec). CF of *M. tuberculosis* H37Rv was prepared as described previously (Andersen et al., 1986). Briefly, *M. tuberculosis* H37Rv was grown as a surface pellicle on Sauton’s medium in Fernbach flasks. The bacteria were separated from the growth medium, usually after 5 weeks, by filtration. Antigenic material was recovered from the medium by ammonium sulphate precipitation. Samples of the CF were applied to the column. Bound material was eluted by lowering the pH to 2.1 with 0.1 M-glycine/HCl buffer. After desorption the pH of the eluate was immediately adjusted to 8 by titration with 1 M-Tris/HCl, pH 8.6. The eluted material was dialysed against redistilled water and small samples were frozen at −20 °C until use.

The purity of the product was analysed by Western blotting. Probing with hyperimmune polyclonal rabbit anti-*M. tuberculosis* immunoglobulins gave rise to one band only at the expected position corresponding to a molecular mass of 38 kDa.

Abbreviations: CF, culture filtrate; mAb, monoclonal antibody; Paa, protein antigen a, the 65 kDa protein; Pab, protein antigen b, a 38 kDa protein.

The amino acid sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M30046.
Amino acid sequence analysis. Automatic microsequence analysis was done on a gas-phase sequencer (Applied Biosystems 470A). The phenylthiohydantoin derivatives were analysed on-line (Applied Biosystems 120A). All reagents were obtained from Applied Biosystems. Sequencing was done twice with the same result.

Phosphate starvation experiments. M. tuberculosis H37Rv was inoculated into Dubos medium in Erlenmeyer flasks and grown at 37 °C while rotating. Dubos medium contains the detergent Tween 80 to allow dispersed growth. Two samples of 40 ml were harvested by centrifugation at 6000 g when the culture had reached an OD₆₀₀ of 0.3. The bacteria were suspended in either 50 ml Dubos medium or 50 ml Dubos medium prepared without phosphate. This medium was buffered with Tris/HCl, pH 7.6, to a final concentration of 10 mm. Growth was continued for 24 h. The growth in the bacteria medium with phosphate exceeded the growth of those in the medium without phosphate as determined by OD₆₀₀ measurements. The culture with phosphate was diluted with medium until identical OD₆₀₀ values were obtained. A sample (40 ml) of each culture was harvested and suspended in sample buffer (62 mM-Tris, pH 6.8, 2%, w/v, sodium dodecyl sulphate, 0.7 M-mercaptoethanol), boiled for 15 min and subjected to sonication.

Samples of the disintegrated bacteria were run on a 10% (w/v) polyacrylamide gel and transferred onto nitrocellulose sheets (Schleicher and Schuell) by electroblotting in a semi-dry system, essentially as described by Kyhs-Andersen (1984). The nitrocellulose sheets were incubated for 2 h at room temperature with a mixture of the labelled immunoglobulins. The labelled immunoglobulins were diluted in PBS/Tween to 100 μg/ml. The labelled immunoglobulins were diluted in phosphate-buffered saline (0.5 M-NaCl, 0.15 M-Na₂PO₄, pH 7.2) with 0.05% Tween 20 (PBS/Tween). The nitrocellulose sheets were incubated for 2 h at room temperature with a mixture of the labelled immunoglobulins. The labelled immunoglobulins were diluted in PBS/Tween to 100 μg/ml. The labelled immunoglobulins were diluted in phosphate-buffered saline (0.5 M-NaCl, 0.15 M-Na₂PO₄, pH 7.2) with 0.05% Tween 20 (PBS/Tween). The nitrocellulose sheets were incubated for 2 h at room temperature with a mixture of the labelled immunoglobulins.

Results and Discussion

We have recently published the nucleic acid sequence of the gene encoding Pab of M. tuberculosis (Andersen & Hansen, 1989). The deduced amino acid sequence exhibited 30% homology to PstS (also designated PhoS) of E. coli. PstS is a phosphate-binding protein which is localized in the periplasm and involved in the transport of phosphate, especially under phosphate-limited conditions (Magota et al., 1984; Surin et al., 1984). PstS is synthesized in a precursor form with a 25 amino acid residue signal sequence which is cleaved off when the protein reaches the periplasmic space (Magota et al., 1984; Surin et al., 1984). The N-terminal sequence of Pab deduced from its nucleic acid sequence is that of a typical signal sequence: two charged amino acid residues, a lysine and an arginine residue, followed by a stretch of 22 non-polar residues. To test this prediction we affinity purified native Pab from CF of M. tuberculosis H37Rv, and determined the amino acid sequence of the mature protein as described in Methods. The result is shown in Fig. 1. The sequence derived by amino acid sequencing of the N-terminal residues of the native Pab matches the sequence deduced from the nucleic acid sequence with the first amino acid residue probably being the serine at position 26. The cleavage site would therefore be located at the carboxy-terminal side of the glycine residue at position 25. According to Watson (1984), 88% of 40 well-characterized prokaryotic signal sequences end with either an alanine or a glycine residue. According to Oliver (1985) a conserved sequence, A-X-B, is found adjacent to most cleavage sites, where B is position -1 relative to the cleavage site and A is position -3. B is preferably glycine, alanine or serine residue and A is either one of the same three amino acids or leucine, valine or isoleucine. X can be any amino acid. The cleavage site of Pab fits these described features with the sequence glycine–cysteine–glycine.

Three proteins of M. bovis BCG have signal peptide sequences consistent with their being secreted from the cytoplasm. They are the alpha antigen (Matsuo et al., 1988), MPB70 (Teresaka et al., 1989) and MPB64 (Yamaguchi et al., 1989). Also, sequence data on the 28 kDa protein from M. leprae suggests that this protein is localized in the plasma membrane or cell wall (Cherayil & Young, 1988).

Pab is similar to PstS of E. coli in its sequence homology and the presence of a signal sequence. Thus, we wanted to study whether the functions of the two proteins were also related.

We demonstrated that the synthesis of Pab is induced (or derepressed) under phosphate starvation which may indicate that Pab plays a role in phosphate metabolism. M. tuberculosis H37Rv was grown in Dubos medium, and transferred to fresh medium with or without phosphate in which growth was continued for 24 h.

The amount of Pab synthesized with or without phosphate was analysed by an immunodetection tech-
membrane or somehow associated with the cell wall. Further studies are needed to reveal the exact location of Pab. This sequence confirms the amino acid sequence of Pab. This sequence confirms the amino acid sequence deduced from the nucleic acid sequence. A precursor form of the molecule with a signal sequence must exist. The synthesis of Pab is induced under phosphate starvation indicating that the protein is involved in phosphate metabolism in M. tuberculosis. We wish to thank Vibeke Larsen, Iben Nielsen and Tove Simonsen for excellent technical assistance and Grethe Jensen for typing the manuscript. This work has received support from the Research Centre for Medical Biotechnology in Denmark.

References


