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.

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.

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 molecule, 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.wibr) 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 2.8 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.

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 OD₄₆₀ 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 of the bacteria in Dubos medium with phosphate exceeded the growth of those in the medium without phosphate as determined by OD₄₆₀ measurements. The culture with phosphate was diluted with medium until identical OD₄₆₀ 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-2-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 Kyhse-Andersen (1984). The nitrocellulose sheets were incubated for 2 h at room temperature with a mixture of the anti-Pab mAbs HYT 28, HBT 12 and HAT 2, and the anti-Paa mAb HAT 5, diluted in phosphate-buffered saline (0.5 M-NaCl, 0.15 M-Na₃PO₄, pH 7.2) with 0.05% Tween 20 (PBS/Tween). The nitrocellulose sheets were washed with PBS/Tween three times for 10 min. Bound mAbs were detected by ³⁵S-labelled anti-mouse immunoglobulins from sheep (Amersham). The specific activity was 200 to 700 Ci (mmol immunoglobulin)⁻¹ [7.4 to 25.9 TBq (mmol immunoglobulin)⁻¹]. The labelled immunoglobulins were diluted in PBS/Tween to 0.5 µCi ml⁻¹ (1.85 × 10⁴ Bq ml⁻¹). The labelled anti-mouse immunoglobulins were incubated with the nitrocellulose blots for either 1 h at room temperature or overnight at 4 °C. Unbound immunoglobulins were removed by three washes with PBS/Tween. The nitrocellulose blots were allowed to air-dry before autoradiography (Hyperfilm-MP, Amersham). The films were usually exposed for 4 d.

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

	↓-kp-s gspe--a	60
MKIRLHTLLA	VLTAAPLLLA AAGCGSKPPS GSPETGAGAG TVATTPASSP VTIAETGSTL	
	90	120
LYPLFNLWGP	AFHERYPNVT ITAQGTGSGA GIAQAAAGTV NIGASDAYLS EGDMAAHKGL	
	150	180
MNIALAISAQ	QVNVNLPQVS EHLKLNKVL AAMYQGTIKT WDDPQIAALN PGVNLPGTAV	
	210	240
VPLHRSDGSG	DTFLFTQYLS KQDPEGWGKS PGFGTTVDFF AVPGALGENG NGGMVTCGAE	
	270	300
TPGCVAYIGI	SFLDQASQRG LGEAQLGNSS GNFLLPDAQS IQAAAAGFAS KTPANQAISM	
	330	360
IDGPAPDGYP	IINYEYAIYN NRQKDAATAQ TLQAFHLWAI TDGNKASFLD QVHFQPLPFA	
	374	
VVKLSDALIA	TISS	

Fig. 1. Amino acid sequence of Pab, given in conventional one letter symbols. On the upper line in lower case letters is shown the sequence obtained by amino acid sequencing of native Pab. The lower line gives the sequence deduced from the nucleic acid sequence. The arrow indicates the signal peptidase cleavage site.

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 preferentially a 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-

Fig. 2. Autoradiography of Western blot analyses of *M. tuberculosis* H37Rv grown with (+P) or without (-P) phosphate. Lanes 2 and 3 contain serial twofold dilutions of the samples in lane 1. The samples were separated on a 10% polyacrylamide gel and transferred onto a sheet of nitrocellulose, which was subsequently incubated with a mixture of anti-Pab mAbs HYT 28, HAT 2 and HBT 12 and the anti-Paa mAb HAT 5. When the bands were excised from the nitrocellulose sheet corresponding to the autoradiogram, and their radioactivity determined by scintillation counting, c.p.m. values minus background (with background, 185 c.p.m., obtained from areas of the sheet where no bands could be seen) were as shown. Similar results were obtained in three independent experiments.

nique and compared with the synthesis of Paa – 'the 65 kDa protein' – which served as an internal standard in this experiment. *M. tuberculosis* Paa exhibits significant homology to the heat shock protein GroEL of *E. coli* and is not involved in phosphate metabolism (Shinnick *et al.*, 1988). Fig. 2 shows that the production of Pab is greater under phosphate starvation while the production of Paa is unaltered. The enhanced synthesis of Pab cannot, therefore, be attributed to a general stress response.

PstS of *E. coli* exerts its function in the periplasm. There are no reports of a true periplasmic space in mycobacteria. The appearance of Pab in CF is not sufficient evidence for Pab being an excreted, extracellular protein as during the culture period (5 weeks), some bacteria will probably have lysed. If the function of Pab is to ensure phosphate uptake during phosphate-limited conditions, we believe that the protein has to remain attached to the bacterium once transported across the cell membrane, either directly anchored in the cell membrane or somehow associated with the cell wall. Further studies are needed to reveal the exact location of the molecule.

In conclusion, we have established the N-terminal 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*.

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