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Method Article

An inducible expression system for high-level expression of recombinant proteins in slow growing mycobacteria

Lisa Leotta, Joanne M. Spratt, Carlyn U. Kong, James A. Triccas *

Microbial Pathogenesis and Immunity Group, Department of Infectious Diseases and Immunology, Sydney Medical School, The University of Sydney, NSW 2006, Australia

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ABSTRACT

A novel protein expression vector utilising the inducible *hspX* promoter of *Mycobacterium tuberculosis* was constructed and evaluated in this study. High-level induction of three mycobacterial antigens, comprising up to 9% of bacterial sonicate, was demonstrated in recombinant *Mycobacterium bovis* BCG when grown under low-oxygen tension, which serves to enhance *hspX* promoter activity. Recombinant proteins were efficiently purified from bacterial lysates in a soluble form by virtue of a C-terminal 6-histidine tag. Purification of the immunodominant *M. tuberculosis* Ag85B antigen using this system resulted in a recombinant protein that stimulated significant IFN-γ release from Ag85B-reactive T cells generated after vaccination of mice with an Ag85B-expressing vaccine. Further, the *M. tuberculosis* L-alanine dehydrogenase (Ald) protein purified from recombinant BCG displayed strong enzymatic activity in recombinant form. This study demonstrated that high levels of native-like recombinant mycobacterial proteins can be produced in mycobacterial hosts, and this may aid the analysis of mycobacterial protein function and the development of new treatments.

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1. Introduction

An essential requirement for study of the biological and immunological function of mycobacterial proteins is their production in a recombinant form that resembles that of their native counterpart. Early studies demonstrated that recombinant proteins obtained from fast growing mycobacterial hosts, such as *Mycobacterium smegmatis*, displayed superiority in terms of structure, function and immunogenicity compared to same protein purified from *Escherichia coli* expression systems (Garbe et al., 1993; Roche et al., 1996; Triccas et al., 1996). Subsequently *M. smegmatis* has become a useful host for the expression and purification of recombinant mycobacterial proteins in a native form, and this has included the development of tagged vectors to facilitate protein purification (Daugelat et al., 2003; Triccas et al., 1998a) as well as the development of Gateway cloning system (Goldstone et al., 2008). Modifications have also be made to *M. smegmatis* that facilitate protein purification, such as mutation of the histidine rich *M. smegmatis* GroEL1 protein to reduce its affinity for nickel affinity beads and thus improve the purity of polyhistidine-tagged recombinant proteins (Noens et al., 2011).

However there are limitations on the use of *M. smegmatis* as a host for the production of native-like proteins of slow growing mycobacteria. Post-translational modification of proteins can differ between *M. smegmatis* and *Mycobacterium tuberculosis* (Arya et al., 2013), and such modifications are known to have a profound effect on mycobacterial protein function (Horn et al., 1999; Temmerman et al., 2004). For example, the Apa protein of *M. tuberculosis* is a strong stimulator of T cell responses, and the native version of the protein purified from *M. tuberculosis*, *Mycobacterium bovis* or BCG displays a complex mannosylation pattern (Horn et al., 1999). However, the recombinant antigen expressed in *M. smegmatis* revealed a different mannosylation pattern and

* Corresponding author at: Department of Infectious Diseases and Immunology, Charles Perkins Centre (D17), The University of Sydney, NSW 2006, Australia. Fax: +61 2 9351 4731.
E-mail address: jamie.triccas@sydney.edu.au (J.A. Triccas).

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was markedly less potent in terms of T cells stimulation compared to the native proteins. This suggests that mycobacterial protein immunogenicity may be more accurately studied using recombinant hosts that closely resemble the source of the native protein.

We have previously described use of the *M. tuberculosis hspX* promoter (P*hspX*) to drive protein expression within recombinant (r) BCG (Kong et al., 2011). *HspX* is part of the DosR regulon, a set of coregulated genes that controls the balance between aerobic and anaerobic respiration of *M. tuberculosis*, important for the shift to latency during infection of the host (Leistikow et al., 2010). P*hspX* is highly induced upon entry of *M. tuberculosis* into cultured macrophages (Yuan et al., 1998), under conditions of hypoxia and (Yuan et al., 1998) during stationary phase of growth of aerated *M. tuberculosis* cultures (Yuan et al., 1996). We determined that P*hspX* is rapidly induced in vivo and can be used to modulate antigen-specific immunity after vaccination with rBCG (Kong et al., 2011). We surmised that the high level induction of P*hspX* in BCG may be exploited to produce recombinant proteins from slow growing mycobacteria in their native form. In this report, we describe the development of an expression system based on P*hspX*, and detail the purification and function of the recombinant proteins produced.

2. Materials and methods

2.1. Construction of hspX expression plasmids

In order to construct *hspX*-based expression vectors, the coding region of the *M. tuberculosis* Ag85B gene (*fbpB*) was amplified from *M. tuberculosis* H37Rv genomic DNA using the primers 85HIS.for (5′TACAAAAGCTTGGATCCATGACAGACGT GA 3′) and 85HIS.rev (5′ATAGTTAACTCAGTGGTGGTGGTGGT GGTGGAATTCCAGCTGGGATTCGCCGGCGCCTAACGAACT 3′) and inserted into the pMV306:GFP mycobacterial/*E. coli* shuttle vector (Yuan et al., 1998) to generate pJEX88. The *hspX* promoter region (P*hspX*) plus the entire *fpbB* gene was excised from pJEX88 using *Xba*I/*Hpa*I and ligated with *Xba*I/*Hpa*I-digested pMV261 (Stover et al., 1991) to yield pJEX92. Digestion of pJEX92 with *Bam*HI removed the *fbpB* insert to result in the expression vector pJEX93. Expression of the *M. tuberculosis* alanine dehydrogenase gene (*ald*) was achieved by amplification of the coding region from *M. tuberculosis* H37Rv (95 °C 1 min, 55 °C 1 min, 72 °C 1 min, 30 cycles) using primers Ald.for (5′AGGTATGATCACGCTCGGTATTCC 3′) and Ald.rev (5′TCCGAGAATTCGGCCAGCACGCTGG 3′). For expression of the *Mycobacterium leprae* 35 kDa protein, the gene was amplified from *M. leprae* genomic DNA (95 °C 1 min, 55 °C 1 min, 72 °C 1 min, 30 cycles) with primers 35.for (5′TAGGGAT CCATGACGTCGGCT 3′) and 35.rev (5′CTAGAATTCTCACTTG TACTC 3′) and inserted in pJEX93.

2.2. Expression and purification of recombinant histidine-tagged protein from m. bovis BCG

Expression plasmids were introduced into *M. bovis* BCG Pasteur strain by electroporation as described previously (Triccas and Ryan, 2009) and kanamycin resistant colonies selected on Middlebrook 7H11 medium (Difco Laboratories) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). Selected colonies were grown in Middlebrook 7H9 medium (Difco Laboratories) supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% albumin-dextrose-catalase (ADC) (rolling, 37 °C for 5 days) after which cultures were either left standing or grown rolling for a further 14 days. Cells were then harvested and sonicated 4 times for 1 min, after which sonicates were analysed for protein expression by SDS-PAGE and immuno-blotting with the anti-HIS monoclonal antibody (mAb) HIS.H8 (AbCam) using the ECL detection system (GE Healthcare, Buckinghamshire, UK). For protein purification, the sonicate was applied to Ni-NTA resin (Qiagen Inc., CA, USA) and bound protein was washed consecutively with 5 mM, 20 mM and 40 mM imidazole in sonication buffer (1xPBS, 5% glycerol, 0.5 M NaCl and 5 mM $MgCl_2$). Protein was eluted with 200 mM imidazole in sonication buffer and dialysed against PBS.

2.3. Protein capture ELISA

ELISA plates were coated with the murine anti-*M. leprae* 35 kDa mAb ML03 (50 μg/ml); (Morris and Ivanyi, 1985) and mycobacterial sonicates were added at a concentration range of 0.1 mg/ml to 100 mg/ml. Plates were blocked with 3% BSA, washed, and anti-rabbit 35 kDa protein polyclonal antibody (1:1000) added. Binding was visualised using alkaline phosphatase conjugated anti-rabbit IgG and n-nitrophenyl-phosphate (NPP) (1 mg/ml). Protein amount was determined by comparison with purified *M. leprae* 35 kDa protein concentration standards (Triccas et al., 1996).

2.4. IFN-γ ELISPOT assay

Female C57BL/6 mice (Animal Resources Centre, Perth, Australia, 5 mice per group) were vaccinated intramuscularly three times with 100 μg of the DNA-85B vaccine construct (Palendira et al., 2002) and 4 weeks after the last immunization the spleen was collected from immunized mice and single cell suspensions prepared in complete RPMI media, supplemented with 2 mM glutamate, 50 mM 2-mercaptoethanol and 10% FCS. The number of interferon gamma (IFN-γ)-producing cells was determined by enzyme-linked immunosorbent spot (ELISPOT) analysis, as described previously (Palendira et al., 2002) using Ag85B at a concentration of 5 μg/mL, 0.5 μg/mL or 0.05 μg/mL. ConA was used at a concentration of 3 μg/mL. All experiments were performed under approval from the University of Sydney Animal Ethics Committee.

2.5. Alanine dehydrogenase assay

BCG-derived recombinant Ald protein tested was tested for Ald enzyme activity using the method of Andersen et al. (1992). Briefly, 10 μg of protein was mixed with 50 mM glycine K–OH, pH 10.2 and 100 μl of substrate containing 50 mM L-alanine, 0.625 mM NAD+, 0.624 mM phenazine methosulphate and 0.24 mM nitroblue, and incubated for 10 min at 37 °C. The absorbance was read at 540 nm. Ald purified from *Bacillus subtilis* (Sigma-Aldrich) was used a positive control (10 μg).

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