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Expression and purification of *Mycobacterium tuberculosis* ESAT-6 and MPT64 fusion protein and its immunoprophylactic potential in mouse model

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Abstract

The completion of *Mycobacterium tuberculosis* genome sequence has opened a new way for the identification and characterization of bacterial antigens, such as ESAT-6, CFP10, MPT64, and Ag85 complex, which are helpful for tuberculosis control. In this work, genes of ESAT-6 and MPT64 were fused and expressed in *Escherichia coli* in form of inclusion bodies with a histidine tag. The expressed fusion protein was purified by nitrilotriacetic acid (Ni–NTA) affinity chromatography under denaturing conditions, and the yield was 18 mg/L of culture. In mice, the purified ESAT-6–MPT64 fusion protein elicited stronger humoral response, greater splenic lymphocyte stimulated index, and higher levels of IFN-γ and IL-12 production than that of the single MPT64 inoculation group, and rendered modest protection on the experimental tuberculosis mouse models. In short, the ESAT-6–MPT64 fusion protein might be a potential candidate vaccine for tuberculosis.

Keywords: *Mycobacterium tuberculosis*; ESAT-6; MPT64; Expression; Vaccine

It is estimated that nearly one-third of the world population is infected with *Mycobacterium tuberculosis*, the causative agent of human tuberculosis (TB) [1]. The current vaccine against *M. tuberculosis, M. bovis* Bacillus Calmette–Guérin (BCG),[2] has been extensively evaluated and demonstrated variable protective efficacies ranging from 0 to 85% in different field trials [2]. The availability of the complete genome sequence of *M. tuberculosis* has opened up new hopes for the identification of antigens useful in the development of new vaccines and diagnostic reagents to control TB [3]. Comparative genomics has shown that 16 regions, designated as RD1–RD16 (RD, region deleted), are present in virulent and clinical strains of *M. tuberculosis* and *M. bovis*, but deleted in *M. bovis* BCG vaccine strains [4,5]. These RD region proteins including ESAT-6, MPT64, CFP10, MPB83, KatG and so on, have been reported to be effective against *M. tuberculosis* infection in animal models when used individually or in combination [6,7].

Owing to the complexity of the host immune response against tuberculosis and the genetic restriction imposed by major histocompatibility complex molecules, it has become clear that an effective subunit vaccine or diagnostic reagents containing multiple epitopes may be required to ensure a broad coverage of a genetically heterogeneous population [8,9]. ESAT-6, a member of the RD1 region, which is the

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[2] *Abbreviations used:* BCG, *M. bovis* Bacillus Calmette–Guérin; TB, tuberculosis; *E. coli*, *Escherichia coli*; CFP, Culture filtrate protein; CFU, Colony forming unit.

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first region absent from BCG substrains derived from the original BCG Pasteur strains, shares striking immuno-dominance in the human immune response against *M. tuberculosis* [10]. MPT64, the best characterized antigen from RD2, which is the second region absent from the original BCG strains, can distinguish guinea pigs immunized with *M. tuberculosis* from those immunized with other nonpathogenic mycobacteria, i.e., BCG Danish 1331 and *M. avium* [11,12]. ESAT-6 and MPT64 are both encoded by genes in *M. tuberculosis* but absent from most or all of *M. bovis* BCG strains, render protection in experimental tuberculosis animals, and elicit delayed type hypersensitivity responses in sensitized animals [12,13]. They both contain numerous well-characterized epitopes, which recognized by T cells from patients at early *M. tuberculosis* infection, and are now under trial as vaccine and diagnostic reagents [14,15].

In the transition from a research-based test to a commercial-used test, one of the primary hurdles is the development of an efficient and scalable production system for the test [6]. Some immuno-dominant antigens of *M. tuberculosis* are difficult to express in various *E. coli* expression systems for some reasons such as: high GC content in average of 65.6% in the whole genome, different enzymatic systems of mycobacterium from *E. coli* expression systems, multiple transmembrane domains toxic to the host cells, and some other reasons unknown [16]. In a previous work in our laboratory, we observed that the expression of MPT64 in *E. coli* expression systems was low and the expressed protein was insoluble in *E. coli* [17], whereas the recombinant ESAT-6 was expressed at very high level. We also observed that fusion expression with ESAT-6 might increase the expression of some antigen of *M. tuberculosis* in the same system. However, the expression level and solubility of the ESAT-6 fusion proteins had been evidenced to differ with identity of the fusion partner.

In this study, ESAT-6 and MPT64, two immunodominant antigens of *M. tuberculosis* were cloned, sequenced, and expressed as a fusion protein in *E. coli*. In addition, the fusion protein was inoculated in mice to observe its immunogenicity, and the humoral-mediated and cell-mediated immune responses of vaccinated mice were also observed.

Materials and methods

Materials, strains and vectors

All chemical reagents were from commercial sources and of analytical grade. PGEM-T-easy cloning kit and T4 DNA ligase were purchased from Promega Corporation (Madison, WI, USA). Ni–NTA resin was purchased from Invitrogen (Carlsbad, USA). *E. coli* DH5α and *M. tuberculosis* H37Rv strains were from our own stock. BCG Danish 1331 was obtained from Lanzhou Institute of Biological Products (Lanzhou, China). Recombinant ESAT-6 and MPT64 protein were produced in our lab [17]. Mycobacterium culture filtrate proteins (CFPs) were prepared as described previously [12]. Specific-pathogen-free female C57BL/6J (H-2b) mice were purchased from the Laboratory Animal Research Centre of Fourth Military Medical University (Xi'an, China).

Cloning of esat-6 and mpt64 genes

The *esat-6* and *mpt64* genes were amplified by PCR using genomic DNA from *M. tuberculosis*. The *esat-6* gene was amplified using the following primers: 5′-GC GGA TCC ATG ACA GAG CAG CAG TGG AAT-3′ and 5′-GC AAG CTT TGC GAA CAT CCC AGT GAC-3′ (BamHI and HindIII restriction sites are underlined, and there is no stop codon in the reverse primer). The *mpt64* gene was amplified using a pair of primers: 5′-GC AAG CTT *GGT GGC TCA GGT GGC TCC GGT GGA GGC GGA AGC GGC GGT GGA GGA TCA* GTG CGC ATC AAG ATC TTC -3′ (the forward primer has a 48 bp linker nucleotide in italics, HindIII restriction site is underlined), and 5′-GC GAA TTC TCC GCC GCC CCA AAT CCG -3′ (EcoRI restriction site is underlined). PCR was performed under standard conditions (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, 30 cycles). The 297 bp PCR product containing *esat-6* gene was subcloned into pGEM-T-easy to yield pGEM-T-E and transformed into *E. coli* strain DH5α. Unfortunately it was found that the 789 bp DNA fragment of *mpt64* was difficult to obtain because of its long upstream primer (74 bp), so we built pGEM-T-M containing *mpt64* gene and identified it by DNA sequencing. The two plasmids pGEM-T-E and pGEM-T-M were digested with BamHI/HindIII, and HindIII/EcoRI, respectively to generate *esat-6* and *mpt64* genes, which were incorporated into pProEX HTb expression vector pre-digested with BamHI and EcoRI and later transformed into *E. coli* strain DH5α. Positive clones by ampicillin (25 μg/ml) screening were further verified by restriction enzyme mapping as well as by DNA sequencing, designated pProEX HTb-EM. *E. coli* culture, plasmid purification, and transformation were performed using standard protocols.

Expression of fusion protein

Several clones harboring the *esat-6* and *mpt64* fused gene were cultured in 5 ml LB medium (1% bacto-tryptone,0.5% yeast extract, and 85 mM NaCl) with ampicillin (25 μg/ml), shaking (220 rpm) overnight, and then an aliquot of 1 ml culture of the clone was incubated to new culture medium to the optical density of 0.6 (OD 600 nm). Fusion protein expression was induced with 1 mM isopropyl-D-thiogalacto-pyranoside (IPTG) for 4 h at 37 °C. The cells were harvested by centrifugation at 6000*g* for 15 min at 4 °C, the supernatant was discarded, and the cell pellets were frozen at −70 °C.

Analysis by SDS–PAGE and immunoblotting

The expression products were subject to 12% SDS–PAGE, stained with Coomassie blue, and the image analy-