African Journal of Microbiology Research Vol. 5(4), pp. 394-398, 18 February, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR10.821
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

Interaction of *Mycobacterium tuberculosis* MPB64 protein with heat shock protein 40

Lei Chen*, Xiaowei He, Xihong Zhao and Jianyu Su

Department of Food Science and Technology, South China University of Technology, 510642, Guangzhou, P.R. China.

Accepted 29 December, 2010

The MPB64 protein of *Mycobacterium tuberculosis* (*M.tb*) is an important structural and functional protein, which has been known to be involved in the virulence, pathogenesis as well as proliferation of the pathogen, however, how MPB64 protein interaction with host protein is still unclear. To identify cellular proteins that interact with the MPB64 protein and to elucidate the possible involvement of MPB64 protein in *M.tb* pathogenesis, a human lung cDNA library was screened using a yeast two-hybrid system assay. HSP40, a molecular chaperone facilitating protein the folding and assembly, was found to interact specifically with the MPB64 protein. The interaction between MPB64 and HSP40 was verified by colocalization experiment and coimmunoprecipitation of HeLa cell lysates expressing both proteins. The mapping studies localized the critical MPB64 sequences for this interaction to amino acid 171-206. Based on these results, we speculate that HSP40 is a functional target of *M. tuberculosis* MPB64 protein in cells. This is the first report demonstrating the interaction of HSP40 with a structural protein of *M. tuberculosis,* indicating a new drug target for *M.tb.*

Key words: MPB64 protein, HSP40 protein, *Mycobacterium tuberculosis,* yeast two-hybrid, co-immunoprecipitation, protein-protein interaction.

INTRODUCTION

Despite progress in the past decades, tuberculosis (TB) remains a major public health problem worldwide. About one-third of the world population is latently infected with Mycobacterium tuberculosis, and 10% of those infected persons develop disease during their lifetime. Therefore, better understanding of the molecular mechanism of latent TB infection (LTBI) is important for effective control and prevention of TB (Yew et al., 2008; Rao et al., 2007). The search for detail molecular mechanism of TB that can reduce the length of therapy as well as address the problem of resistance is, therefore, an urgent problem.

As a starting point for our own efforts towards addressing this urgency, we decided to identify potent protein-protein interactions that must take place between the secretory proteins of *Mycobacterium tuberculosis (M.tb)*, the carrier of TB, and their human counterparts at the primary site of infection, the human lung, for the infection to either take root, or, as in many cases, be cleared (Feist et al., 2009; Breitling et al., 2008). A few of such interactions have also been studied in detail (Kumar et al., 2009; Schumann et al., 2006; Peake et al., 1993; Abou-Zeid et al., 1988); however, a comprehensive demonstration of the infection process is yet to emerge.

In this study, we report our investigations with one such secretory protein, the MPB64 protein that has been implicated in the virulence and pathogenesis of *M. tb* (Harboe et al., 1996; Pym et al., 2002; Hsu et al., 2003; Majlessi et al., 2005). We therefore use MPB64 protein as the bait to screen for interacting proteins from a human lung cDNA library by the yeast two-hybrid assay system.

*Corresponding author. E-mail: chenlei1514@163.com. Tel: +86-020-8711-3252; Fax: +86-020-8711-3252.

MATERIALS AND METHODS

Strains, general techniques

The strain of *Saccharomyces cerevisiae* used in this study were AH109 and Y187 from Clontech company (USA). Yeast cells were cultured at 30℃ either in a complete YPD medium (1% yeast extract, 1% peptone, 2% glucose) or in a synthetic defined (SD) medium supplemented with required essential nutrients. Plates contained 2% agar. Transformation of yeast cells was performed by the lithium acetate (Gietz et al., 1995). *Escherichia coli* KC8 was used for general cloning. Human lung cDNA library came from Clontech Company (USA). DNA manipulation was performed according to established protocol (Sambrook et al., 2001).

Plasmids and construction of recombinant vectors

To generate bait plasmids, the DNA sequence encoding full-length MPB64 was polymerase chain reaction (PCR) amplified from *M. tb* H37Rv genomic DNA and cloned into the pMD18-T vector (Takara, China). The full-length MPB64 gene was subjected to DNA sequencing, and the inserts were verified against the corresponding region of the complete genome H37Rv. To identify the putative domain of amino acid sequence required for HSP40/MPB64 interaction, different fragments MPB64 of gene were prepared by PCR. The six truncated mutants ΔM1-ΔM6 (constructs and putative functional domains were shown in Figure 3A) were subcloned into the yeast two-hybrid vector pGBKT7. The HSP40 gene was obtained by PCR from the human lung cDNA library, and subcloned into the yeast vector pGADT7. For mammalian cell expression, the full-length MPB64 gene and HSP40 were subcloned into the pCMV-Myc vector (Clontech, USA) and pCMV–HA, and fluorescence vector pEGFP-N1and pDsRed-N1, respectively. All DNA manipulations were performed as described by Sambrook et al. (2001). All constructs were verified by restriction digestion and sequencing.

Screening of the lung cDNA library by the yeast two-hybrid system

Yeast two-hybrid experiments were performed as described in the Clontech manual for the MATCHMAKER GAL4 two-hybrid system and in the Clontech yeast protocols handbook (Clontech, USA). Screening for interaction of fusion proteins was performed by plating transformants onto plates lacking leucine, tryptophan, histidine and uracil. In short, Y187 was transformed with a cDNA prey library and the resulting cells were mated with AH109 cells transformed with the bait constructs. Resulting diploid cells were selected for interaction by plating on leucine-, tryptophan- and histidine- deficient media containing 10 to 40 μM 3-AT. The fresh growing clones were assayed for β-gal activity by replica plating the yeast transformants onto Whatman filter papers; the filters were snap-frozen in liquid nitrogen for 10 s twice and incubated in a buffer containing 5-bromo-4-chloro 3-indolyl-β-D-galactopyranoside solution at 30℃ for 1 to 8 h. Positive interactions were detected by the appearance of blue clones. The liquid β-galactosidase activity was determined using the substrate ONPG as described standard protocols handbook (PT3024-1, Clontech, USA). Data for quantitative assays were collected for yeast cell number and are the mean ± S.E.M. of triplicate assays. Appropriate positive/-negative controls and buffer blanks were used. The positive pGAD-cDNA plasmids were isolated from positive yeast transformants by culture in leucine-deficient medium, which resulted in spontaneous loss of the plasmid pGBK-MPB64 and transformed into *E. coli* KC8 for sequence analysis. Auto sequencing assay was performed in Takara Company (China) and the resulting sequence was analyzed in the database of EMBL\Gene Bank by the BLAST program.

In vivo co-immunoprecipitation and western blotting

To reaffirm the results observed from yeast two-hybrid assays, another independent assay, co-immunoprecipitation was carried out. HeLa cells were co-transfected with the plasmids expressing pCMV-Myc-HSP40 and pCMA-HA-MPB64 using the lipofectamine™ transfaction reagent (Invitrogen, USA). At 48 h post-transfection, cells were washed with PBS and then lysed in lysis buffer. Cell lysate was then mixed with anti-HA magnetic microbeads for 30 min on ice. 100 μl of 10% suspension of protein A–Sepharose was then added to the samples. The mixture was allowed to shake for 1 h at 4℃, after following which the beads were washed four times in lysis buffer, and protein was eluted in 2 x SDS dye by boiling the sample for 5 to 10 min. Samples were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked using 0.5% BSA in PBST for 1 h, and incubated overnight with anti-Myc antibodies (3:1000; Clontech, USA). The blot was then washed three times in PBST, incubated with anti-mouse IgG HRPO for 1 h and washed three times in PBST and the proteins were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The HeLa cells transected with the pCMV-Myc and pCMV-HA vectors were used as negative controls.

Subcellular localization

HeLa cells were grown on coverslips in a 6-well chamber and simultaneously transfected with the recombinant fluorescence plasmids pEGFP-MPB64 and pDsRed-HSP40. After 24 h transfection, the cells were washed with PBS three times and fixed in 4% paraformaldehyde for 20 min at room temperature. The coverslips were then washed with PBS and mounted. Intracellular localization of the MPB64 protein and HSP40 was observed under a Leica confocal microscope (Germany).

RESULT

Identification of HSP40 as an MPB64 interacting protein by yeast two-hybrid system

The MPB64 was subcloned into plasmid pGBK to be expressed as a fusion protein with the DNA-binding domain of the Gal-4 protein. The newly constructed plasmid, pGBK-MPB64, verified by auto sequencing assay, was used to transform yeast AH109. Yeast cells containing pGBK-MPB64 were transformed with a fowl kidney cDNA library. Approximate 5.6 x 10^6 transformants were screened for His-Ade-Trp-Leu independent growth and blue colony formation in the β-gal assay. Thirty-eight positive clones were obtained. As some AD-cDNA fusion products can activate reporter gene transcription without interacting with the BD-MPB64 fusion protein, this false-positive clone can be identified using the technique of segregation analysis. Only 3 of 38 clones survived all genetic tests and were considered to be genuine positive clone, DNA sequence analysis of the fragment revealed that the three cDNA fragments inserted have a high identity with three genes in the GeneBank database. One of these clones was identified as heat shock protein 40 (HSP40), which encodes a 337-amino acid protein, as

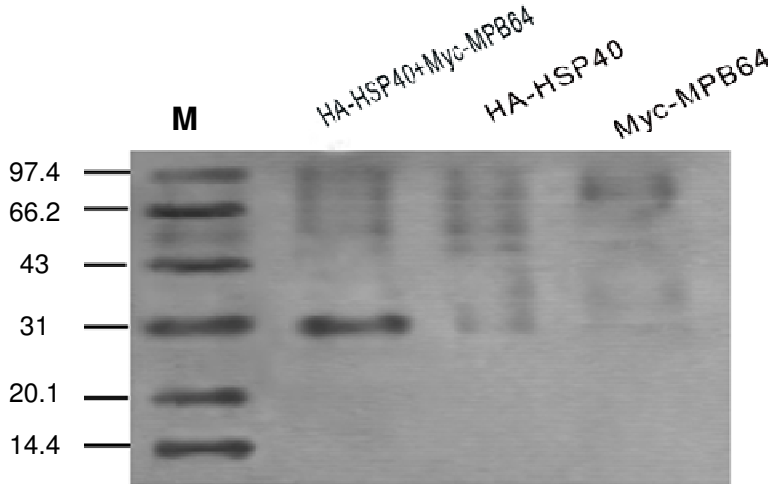

Figure 1. HSP40 protein immunoprecipitated with the MPB64 protein. Indicated plasmids were simultaneously transfected into Vero cells. Twenty-four hours after transfection, coimmunoprecipitation was performed using anti-HA magnetic microbeads. The proteins immunoprecipitated (IP) were assayed with an anti-myc monoclonal antibody.

shown in Figure 3B, the protein encoded by the pGAD-HSP40 clones interacted specifically with the MPB64 protein.

Co-immunoprecipitation determined the interaction of the MPB64 protein and HSP40

To further examine the interaction of the MPB64 protein and HSP40, co-immunoprecipitation was performed. The MPB64 protein was fused at the amino terminus with a Myc-tag, and HSP40 was fused at the carboxyl terminus with a HA tag. The two plasmids were cotransfected into HeLa cells and immunoprecipitated. The immunoprecipitated complexes were separated on SDS–PAGE, and analyzed by Western blot with anti-myc monoclonal antibodies. As shown in Figure 1, about 30 kD protein in accordance with MPB64 protein, was obtained from co-transfected cell, which showed that the Myc-fused HSP40 protein immunoprecipitates with HA-MPB64. Moreover, HA-MPB64 alone and Myc-HSP40 alone could not be immunoprecipitate. These experiments confirm the results that the MPB64 protein can interact with HSP40 protein in mammalian cells.

Co-localization of the MPB64 protein and HSP40

The localization patterns of the MPB64 protein and HSP40 were investigated in HeLa cells. pEGFP-MPB64 and pDsRed-HSP40 were transfected simultaneously into HeLa cells. HSP40 and MPB64 protein mainly localized in the cytoplasm (Figure 2). The combined results indicated that the MPB64 protein and HSP40 co-localized in the cytoplasm of HeLa cells.

Mapping the HSP40 binding region of the MPB64 protein by yeast two-hybrid assays

To map the involved regions of MPB64 protein in the MPB64/HSP40 interaction, four truncated fragments of MPB64 protein were generated (Figure 3A). These fragments, designated from ΔM1 to ΔM6, were cloned into pGBKT7 vector, and then co-transformed respectively with pGADT7-HSP40 into the AH109 yeast cell. The liquid β-galactosidase activity was determined using the substrate ONPG as described standard protocols handbook (PT3024-1, Clontech, USA). As indicated in Figure 3B, the β-galactosidase activity containing ΔM1, ΔM2 and ΔM3 showed viability compared to the negative control, while β-galactosidase activity involving ΔM4, ΔM5 and ΔM6, did not show viability; moreover, the β-galactosidase activity of ΔM3 (171-206) fragment is much higher than the activity of ΔM2 (141–206) fragment. These results imply that the ΔM3 (171-206) domain of the MPB64 protein is responsible for the majority of the binding to HSP40, which suggest that the MPB64 protein binds to HSP40 protein with its carboxyl terminus in *M.tb.*

DISCUSSION

Mycobacterium tuberculosis is a pathogen that persists intracellularly in host macrophages and can cause chronic disease in susceptible individuals. The prevention of phagosome maturation and activity represents a major survival strategy of *M. tb* (Schumann et al., 2006; Pieters

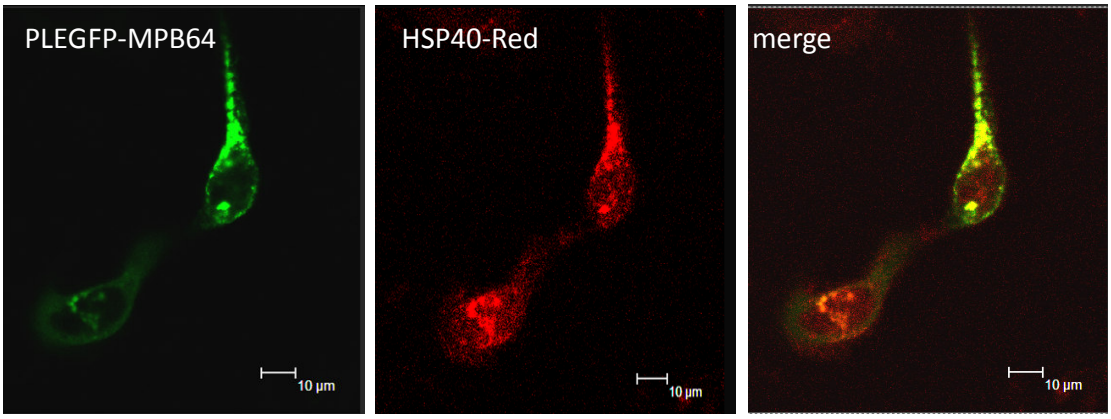

Figure 2. Co-localization of the MPB64 protein and HSP40. pEGFP-MPB64 (green) and pDsRed-HSP40 (red) were co-transfected into vero cells. After 24 h, cells were fixed, mounted, and the localization of the proteins was observed with a Leica confocal microscope. As shown, the MPB64protein and HSP40 were colocalized in the cytoplasm.

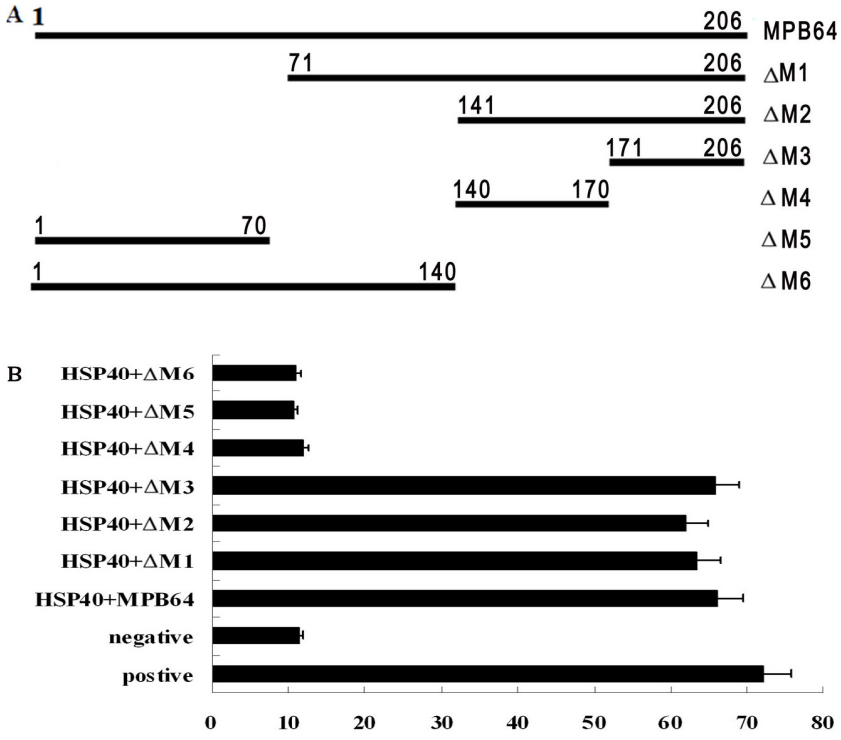

Figure 3. Map the interaction domain of MPB64. Schematic description of the truncated fragments (A) and the yeast two-hybrid assay results for MPB64 and HSP40 interactions in their truncated and non-truncated forms (B). The empty vectors pGBKT7 and pGADT7 co-transformed were used as the negative control and the pGBKT7-53 and pGADT7-T co-transformed were used as the postive control. Every experiment was repeated for at least three times and the data were obtained by average. The error bars represent standard error of the mean.

and Gatfield, 2002). Experiments using *in vitro* infection of macrophages have demonstrated a profound inhibitory influence of *M. tb* cells on their cellular host. Upon infection, *M.tb* modifies the gene expression of the host macrophage, which results in a highly sophisticated interaction between the pathogen and its host. The MPB64 protein of *M.tb* is an important structural and functional protein, which have been known to be involved

in the virulence, pathogenesis as well as proliferation of the pathogen, therefore, Better understanding of the host protein interaction MPB64 protein of *M.tb* may contribute significantly to the development of prophylactic therapeutic, and diagnostic reagents as well as help in TB infection control.

In order to study the function of the MPB64 protein of *M.tb* during the infection process, we searched for host proteins that interact with the MPB64 protein. Using a yeast two-hybrid system, we identified the chicken HSP40 protein as a candidate to interact with MPB64 protein. This interaction was confirmed *in vivo* by co-immunoprecipitate and co-localization technique. The mapping studies localized the critical MPB64 sequences for this interaction to amino acids 171 – 206 by yeast two-hybrid assay. These results showed that the C-terminus of MPB64 could bind to HSP40 protein and the host protein HSP40 is a functional target of MPB64 protein in cells.

The interaction between MPB64 and HSP40 was verified in the present study. Heat shock proteins are molecular chaperones facilitating protein folding, assembly and intracellular transport, and thus are essential for cellular functions (Hightower et al., 1991; Nagata et al., 1996; Soti et al., 2005). Their synthesis is increased greatly in response to a variety of stressful stimuli. Among the HSP members, HSP40, a 40-kD glycoprotein, has the ability to function as a molecular chaperone in the endoplasmic reticulum (Nagata et al., 1996) and as a molecular chaperone-like function under stress conditions. Therefore, we presume that HSP40 plays a crucial role during the folding, maturation and secretion of MPB64 protein.

In conclusion, we have identified an interaction between MPB64, a early secreted protein of *M.tb* and HSP40, an intracellular host protein. The knowledge of protein-protein interactions of the virulence factor MPB64 is of importance to understand the role of MPB64 during the infection process. However, the pathophysiological significance of the interaction between MPB64 and HSP40 is largely unknown; elucidation of these questions will depend on further studies. Moreover, the disruption of interaction between MPB64 and HSP40 proteins using RNA interference technology may provide further clues to the specific function of MPB64 and HSP40 protein.

ACKNOWLEDGEMENTS

We are grateful to students from Department of Respiratory, the First Affiliated Hospital of Changchun University in technical assistance.

REFERENCES

Abou-Zeid C, Ratliff TL, Wiker HG, Harboe M, Bennedsen J (1988).Characterization of fibronectin-binding antigens released by Mycobacterium tuberculosis and Mycobacterium bovis BCG. Infect. Immun., 56: 3046-3051.

Breitling R, Vitkup D, Barrett MP (2008). New surveyor tools for charting microbial metabolic maps. Nat. Rev. Microbiol., 6: 156-161.

Feist AM, Herrgard MJ, Thiele I, Reed JL, Palsson BO (2009). Reconstruction of biochemical networks in microorganisms. Nat. Rev. Microbiol., 7: 129-143.

Gietz RD, Schiestl RH, Willems AR (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast., 11: 355-336.

Harboe M, Oettinger T, Wiker HG, Rosenkrands I, Andersen P (1996) Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG. Infect. Immun., 64: 16-22.

Hightower LE (1991). Heat shock, stress proteins, chaperones, and proteotoxicity, Cell., 66: 91-197.

Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AJ (2003). The primary mechanism of attenuation of bacillus Calmette–Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc. Natl. Acad. Sci.USA., 100: 12420-12425.

Kumar K, Tharad M, Ganapathy S, Ram G, Narayan A (2009). Phenylalanine-Rich Peptides Potently Bind ESAT6, a Virulence Determinant of Mycobacterium tuberculosis, and Concurrently Affect the Pathogen's Growth. PLoS. ONE, 11: 1-12.

Majlessi L, Brodin P, Brosch R, Rojas MJ, Khun H (2005). Influence of Esat-6 secretion system 1 (RD1) of Mycobacterium tuberculosis on the Interaction between mycobacteria and the host immune system. J. Immunol., 174: 3570-3579.

Nagata K (1996). Hsp47: a collagen-specific molecular chaperone. Trends. Biochem. Sci., 21: 22-26.

Peake P, Gooley A, Britton WJ (1993). Mechanism of interaction of the 85B secreted protein of Mycobacterium bovis with fibronectin. Infect. Immun., 61: 4828-4834.

Pieters J, Gatfield J (2002). Hijacking the host: survival of pathogenic mycobacteria inside macrophages. Trends. Microbiol., 10: 142-146.

Pym AS, Brodin P, Brosch R, Huerre M, Cole ST (2002). Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. Mol. Microbiol., 46: 709-717.

Rao A, Ram G, Saini AK, Vohra R, Kumar K (2007). Synthesis andselection of de novo proteins that bind and impede cellular functions of an essential mycobacterial protein. Appl. Environ. Microb., 73: 1320-1331.

Sambrook J, Russell DW (2001). Molecular cloning: A laboratory manual. 3rd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

Schumann G, Schleier S, Rosenkrands I, Nehmann N, Halbich S (2006). Mycobacterium tuberculosis secreted peotein Esat-6 interacts with the human proteinSyntenin-1. Centr. Eur. J. Biol., 1: 183-202.

Soti C, Pal C (2005). Molecular chaperones as regulatory elements of cellular networks. Curr. Opin. Cell. Biol., 17: 210-215.

Yew WW, Leung CC (2008). Updates in tuberculosis 2007. Am. J. Respir. Crit. Care. Med., 177: 479-485.