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Full Length Research Paper

The vascular endothelial growth factor interacts with *Mycobacterium tuberculosis* ESAT-6

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Mycobacterium tuberculosis **continues to be a leading cause of human deaths due to an infectious agent. The ESAT-6 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, little is still known about the host factors that interact with ESAT6. Therefore, a yeast two-hybrid screening of a human lung complementary deoxyribonucleic acid (cDNA) library, using ESAT6 as bait, was performed to identify ESAT-6 interaction with host protein. A vascular endothelial growth factor (VEGF) was found to interact specifically with the ESAT6 protein. Subcellular localization and glutathione S-transferase (GST)-pull down assays were used in an attempt to further confirm the interaction of VEGF and esat-6. All our data suggest that the interaction of VEGF with a structural protein of *M. tuberculosis* may be important for modulation of virulence of *M. tuberculosis*.**

Key words: ESAT-6 protein, VEGF protein, *Mycobacterium tuberculosis,* Yeast two-hybrid.

INTRODUCTION

As one of the world's most devastating infectious diseases, tuberculosis (TB) is responsible for approximately 2 million deaths per year (Abebe and Bjune, 2006; Delogu et al., 2002). The situation has become even more precarious due to the emergence of multidrug resistant strains of *Mycobacterium tuberculosis* and lethal combination of TB and human immunodeficiency virus (HIV) infections (North et al., 1999; Ordway et al., 2007). Therefore, better understanding of the molecular mechanism of latent tuberculosis infection (LTBI) is important for effective control and prevention of TB (Yew and Leung, 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.

ESAT-6, the 6 kDa early secretory antigenic target of *M. tuberculosis*, is a secretory protein and potent T cell antigen (Aagaard et al., 2003; Skjqt et al., 2000; Sorensen et al., 1995). Furthermore, ESAT-6 has recently been demonstrated to induce protective immunity when administered as either a subunit (Brandt et al., 2000) or a DNA vaccine (Kamath et al., 1999, Harboe et al., 1996). Thus, in this study, we use ESAT-6 protein as the bait to screen for interacting proteins from a human lung cDNA library by the yeast two-hybrid assay system.

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Abbreviations: M.tb, *Mycobacterium tuberculosis*; **cDNA,** complementary deoxyribonucleic acid; **VEGF,** vascular endothelial growth factor; **TB,** tuberculosis; **LTBI,** latent tuberculosis infection; **SD,** synthetic defined; **PCR,** polymerase chain reaction; **GST,** glutathione S-transferase; **YPD,** yeast/peptone/dextrose; **HIV,** human immunodeficiency virus; PBS, phosphate-buffered saline; **TBS,** tris buffered saline.

MATERIALS AND METHODS

Strains, general techniques

The strain of *Saccharomyces cerevisiae* used in this study was AH109 from Clontech company (USA). Yeast cells were cultured at 30°C either in a complete yeast/peptone/dextrose (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* JM109 was used for general cloning. Human lung cDNA library came from Clontech company (USA).

Construction of plasmids

For yeast two-hybrid assays, recombinant expression plasmids were constructed using pGBK (BD Clontech, USA). In this vector, the inserted genes are expressed in-frame with a GAL4 DNA-binding domain (GAL4-BD). To construct plasmid pGBK- ESAT-6, the gene coding for the ESAT-6 was amplified by polymerase chain reaction (PCR) using M.tb H37Rv genomic DNA as template. After digestion with *EcoRI* and *BamHI*, the resulting fragment was cloned into the pGBK *Eco*RI/ *BamHI* sites. In addition, the plasmids for GST fusions PGST-ESAT-6 was described previously (Lwabuchi et al., 2004). The recombinant VEGF protein of M.tb was cloned, expressed and purified according to the published procedure reported by Luo et al. (2004) for GST pull-down assay. For mammalian cell expression, the full-length ESAT-6 gene and VEGF were subcloned into the fluorescence vector pEGFP-N1and pDsRed-N1, respectively.

Yeast two-hybrid assay

The Matchmaker GAL4 Two-hybrid system (BD Clontech) was used to screen for proteins that interact with ESAT-6 from a human lung cDNA library (BD Clontech). Yeast two hybrid screening was performed using materials and protocols provided by the manufacturer. Briefly, *S. cerevisiae* AH109 strain pre-transformed with bait plasmid pGBK-ESAT-6 were mated with an Y187 strain pre-transformed with a lung cDNA library expressing fusions of GAL4 activation domain. The mating mixture was plated on synthetic medium lacking tryptophan, leucine, histidine and adenine and then assayed for α-galactosidade activity (SD/-Trp/-Leu/-Hist/-Ade/X-α, -gal medium). Plasmids isolated from positive colonies were used to transform *E. coli* JM109 cells and then sequenced. The obtained DNA sequences were then subjected to a BLAST search in the GeneBank database.

Glutathione S-transferase (GST) pull-down assay

GST pull-down assay was performed using the ProFounde™ Pull-Down GST Protein: Protein Interaction Kit (Pierce). The purified GST-ESAT-6 was adsorbed onto 100 µl glutathione–sepharose beads, equilibrated with BupH™ tris buffered saline (TBS) buffer (25 mM Tris–HCl and 0.15 M NaCl, pH 7.2) in a Handee™ Mini-spin column, and served as bait proteins in the subsequent steps. After 4 h at 4°C, the beads carrying GST fusion ESAT-6 were resuspended in 400 µl 1:1 wash solution of TBS: ProFounde™ lysis buffer for 4 times. After addition of 0.5 mg of the purified VEGF as prey protein, the mixtures were gently shaken for 3 h at 4°C and left for an additional 30 min without mixing. The rinsing was repeated for 5 times using 400 µl of wash solution as mentioned previously. The bound proteins were eluted by 100 Mm of glutathione elution buffer. The eluted samples were analyzed by 12% SDS–PAGE.

Subcellular localization

293T cell were cultured as monolayers in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (invitrogen) and grown at 37°C in a humidified atmosphere containing 5% CO_2. 293T cells were grown on coverslips in a 6-well chamber and simultaneously transfected with the recombinant fluorescence plasmids pEGFP-ESAT-6 and pDsRed-VEGF. After 24 h transfection, the cells were washed with phosphate-buffered saline (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 ESAT-6 protein and VEGF was observed under a Leica confocal microscope (Germany).

RESULTS

Identification of ESAT-6 interacting proteins in a yeast two hybrid screening

In order to identify proteins interacting with ESAT-6, a yeast two hybrid screening was performed. The entire ESAT-6 gene was fused in-frame to the Gal4 DNA binding domain and the resulting construct was transformed into the yeast strain AH109. Using this bait, we screened approximately 10^7 clones from a human lung cDNA library, previously pre-transformed into the yeast strain Y189. Yeast colonies were first selected on medium lacking leucine, tryptophan, histidine and adenine and subsequently assayed for α-galactosidase activity. Plasmids were isolated from 98 positive clones and rescued by transformation into *E. coli*. The identity of the obtained plasmids was established by sequencing allowing the identification of 24 known proteins in the GenBank database. These proteins were found to be involved in different metabolic pathways and functions, namely cell communication and signal transduction, transport, protein metabolism, immune response, and regulation of nucleic acid metabolism. One of these clones was identified as VEGF protein, As shown in Figure 1, the protein encoded by the pGAD-VEGF clones interacted specifically with the ESAT-6 protein.

ESAT6 and vascular endothelial growth factor (VEGF) interact *in vitro by* glutathione S-transferase (GST) pull-down assay

In order to identify the interaction between ESAT-6 and VEGF, the recombinant human GST-VEGF protein was over-expressed in *E. coli,* followed by purification using Glutathione–Sepharose affinity beads. ESAT-6 /VEGF interaction was then determined by GST pull-down assay, in which the purified human GST-ESAT-6 (Figure 2, Lane 3) protein was immobilized on the glutathione–sepharose beads as a bait protein according to the kit.

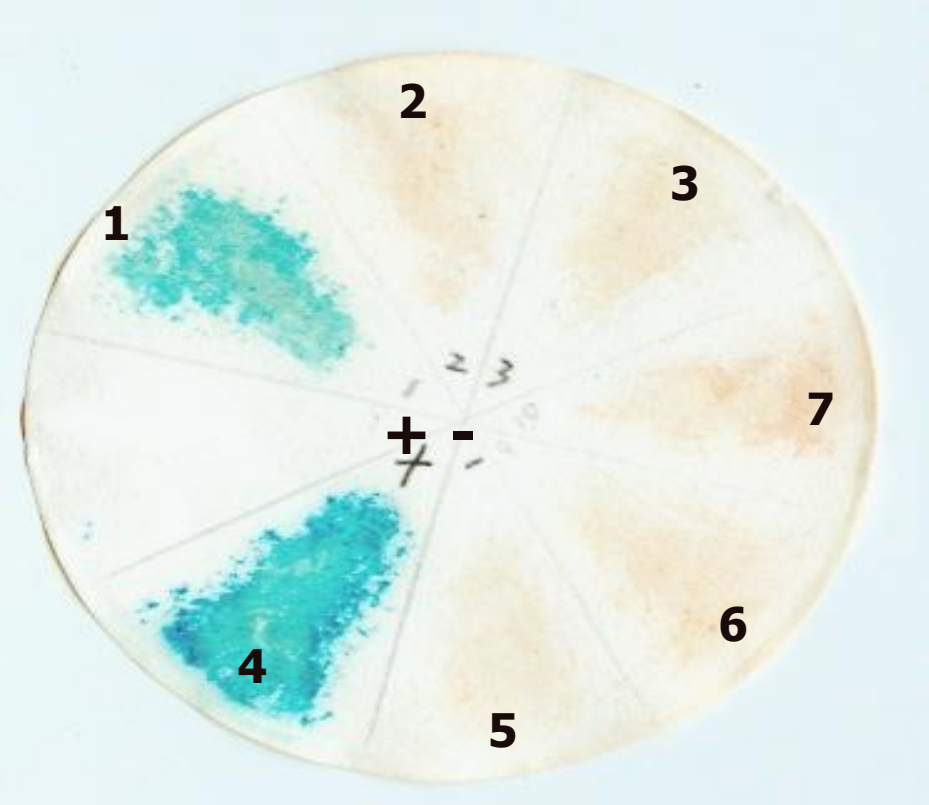

Figure 1. Yeast two-hybrid analysis of the interaction of ESAT6 and VEGF. 1, pGBKT7-ESAT6+pGADT7-VEGF; 2, pGBKT7+pGADT7-VEGF; 3, pGBKT7-Laminc+pGADT7-VEGF; 4, pGBKT7-53+pGADT7-T (postive control); 5, pGBKT7 -Laminc+pGADT7-T (negative control); 6, pGBKT7 +pGADT7; 7, pGBKT7-TLR4+pGADT7.

The His-tagged VEGF protein as the prey was purified through a Ni affinity column (Figure 2, lane 2). After the GST-ESAT-6 and the possible partner were eluted by glutathione, the samples were analyzed by SDS–PAGE. As indicated in lane 5 of Figure 2, VEGF protein could be also detected on the SDS–PAGE, suggesting that GST-ESAT-6 was eluted by glutathione together with VEGF protein. ESAT-6 binding to human VEGF could be considered to be specific because the bead itself did not pull down any VEGF protein as shown in lane 4. All these results thereby conclude that ESAT-6 protein could specifically bind to human VEGF *in vitro*.

ESAT-6 and vascular endothelial growth factor (VEGF) interact *in vivo* by subcellular localization

The localization patterns of the ESAT 6protein and VEGF were investigated in HeLa cells. pEGFP-ESAT6 and pDsRed-VEGF were transfected simultaneously into HeLa cells. HSP40 and MPB64 protein mainly localized in the cytoplasm (Figure 3). The combined results indicated that the ESAT6 protein and VEGF co-localized in the cytoplasm of HeLa cells.

DISCUSSION

Detail molecular pathogenic mechanism of TB remains largely unknown. Nevertheless, previous study showed that ESAT6 protein could involved in the virulence, pathogenesis as well as proliferation of the pathogen. It needs lots of host proteins involved in these function. In current study, using different molecular biology and biochemical approaches, a number of host proteins that interact with the ESAT6 were studied. In this work we made use of a yeast two-hybrid approach to screen 10^7 clones from a human lung cDNA library and identify possible ESAT6 interacting proteins. Using ESAT6 as bait, we initially obtained 98 clones that after subsequent sequence analysis and database search allowed identifying 24 known proteins (data not show). These proteins were reported to be involved in different cellular processes including nucleic acid metabolism, cell communication, transport, protein metabolism, immune response and energy pathways. One of the identified proteins was found to belong to VEGF proteins. Having identified as an ESAT6 interactor in the yeast two-hybrid system, we decided to confirm this interaction *in vitro* using a GST-pull down assay with purified ESAT6 and

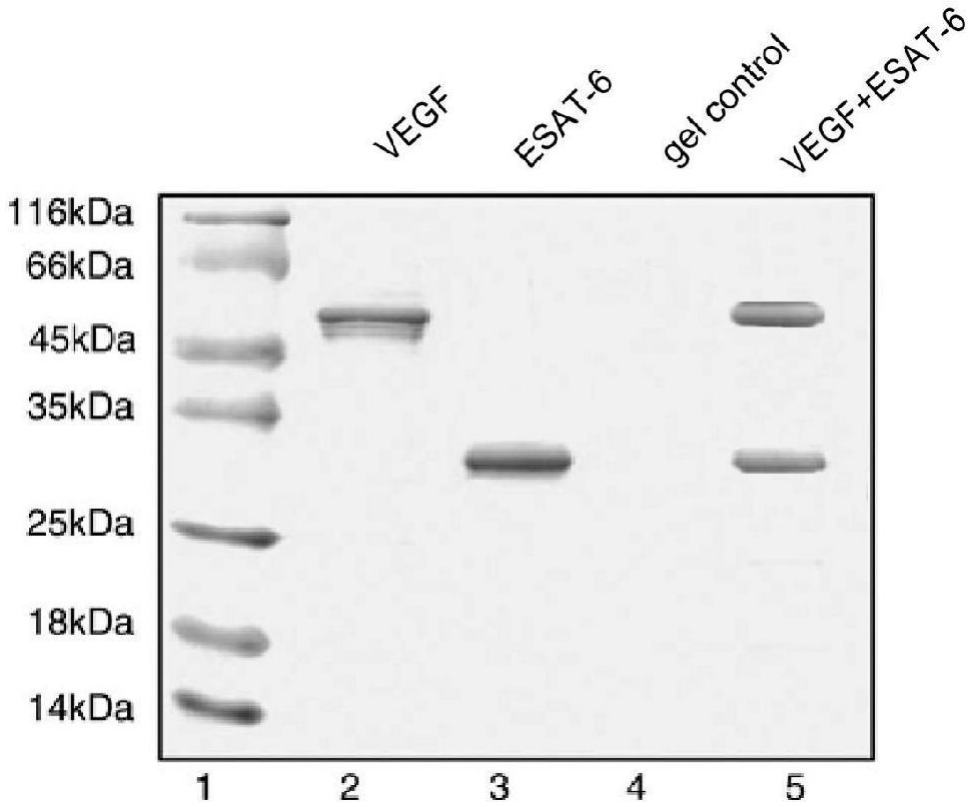

Figure 2. ESAT-6/VEGF interaction was determined by GST pull-down. Samples were analyzed on a 10% SDS–polyacrylamide gel, and the band was visualized with coomassie brilliant blue. Components in each lane are shown at the top. Lane 1, molecular mass marker; lane 2, purified His-tagged VEGF; lane 3, purified GST-tagged human ESAT-6; lane 4, agarose gel control; lane 5, ESAT-6 and the pull-down human VEGF.

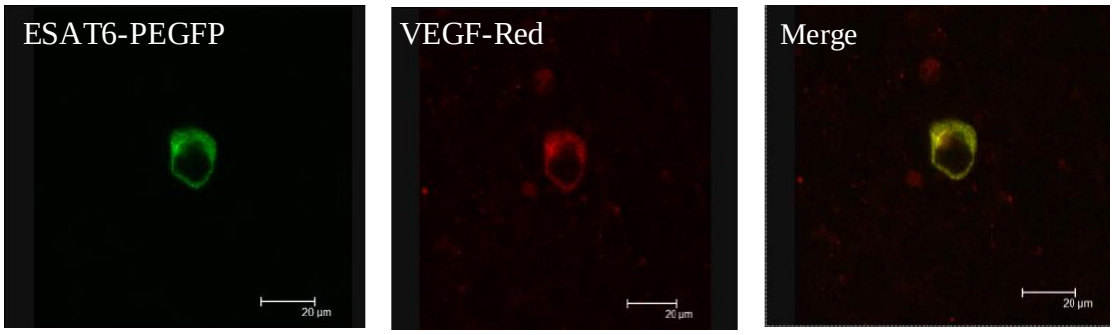

Figure 3. Co-localization of the ESAT-6 protein and VEGF. pEGFP-ESAT6 (green) and pDsRed-VEGF (red) were co-transfected into vero cells. After 24 h, cells were fixed, mounted, and the localization of the proteins was observed with a Leica confocal microscope. As shown, the ESAT6 protein and VEGF were colocalized in the cytoplasm.

increasing amounts of bacterially expressed VEGF. The results indicated that the interaction of the two proteins is specific. Next, we decided to investigate if ESAT6 and VEGF interact *in vivo* in human cells. This was performed using Subcellular localization. The results indicated that the ESAT6 protein and VEGF co-localized in the cytoplasm of HeLa cells. These results showed that ESAT6 and VEGF interact *in vivo and vitro.*

VEGF is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis, which is essential for developmental angiogenesis and plays important roles in adult animals to control vascular

permeability and homeostasis, blood pressure, and pathological angiogenesis associated with wound healing (Ku et al., 1993; Shibuya, 2006; Lee et al., 2007). VEGF also is one of the most potent angiogenic factors, capable of promoting proliferation, migration and survival of endothelial cells and plays the most important role in the initiation of new blood vessel formation (Kayo et al., 2005; Breen., 2007; Leung et al., 1989), therefore, we presume that VEGF might play a crucial role in the virulence, pathogenesis as well as proliferation of the pathogen of M.tb by binding to ESAT-6.

In conclusion, a yeast two-hybrid screening of a human lung cDNA library allowed the identification of ESAT-6 interacting proteins. Among the proteins involved in angiogenic factors VEGF was found to bind *in vitro* and *in vivo* to ESAT6. Although, the pathophysiological signify-cance of the interaction between ESAT-6 and VEGF is largely unknown; elucidation of these questions will depend on further studies. Moreover, the disruption of interaction between ESAT-6 and VEGF proteins using RNA interference technology may provide further clues to the specific function of ESAT-6 and VEGF protein.

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