

Full Length Research Paper

Interaction of *Mycobacterium tuberculosis* ESAT-6 protein with ADAM9 protein

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Accepted 19 November, 2022

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, how ESAT-6 protein interact with host protein is still unclear. In order to study the function of the *M. tuberculosis* protein ESAT-6 in the infection process, we searched for host proteins that interact with this secreted mycobacterial protein. Using a yeast two-hybrid system we identified the ADAM9 (a disintegrin and metalloprotease) protein as a candidate to interact with ESAT-6. This interaction was further confirmed by protein overlay and surface plasmon resonance binding assay using recombinant ESAT-6 and ADAM9, and by GST pull-down analysis of the mycobacterial expressed ESAT-6 and ADAM9. The interaction domains were localized by yeast two-hybrid studies using truncated derivatives of ESAT-6 protein. The C-terminus of ESAT-6 binds to the ADAM9. Thus, the host protein ADAM9 represents a possible cellular receptor for the mycobacterial protein ESAT-6. This is the first report demonstrating the interaction of ADAM9 with a structural protein of *M. tb*.

Key words: ESAT-6 protein, ADAM9 protein, *Mycobacterium tuberculosis*, yeast two-hybrid, GST pull-down assay, surface plasmon resonance binding.

INTRODUCTION

Pathogenic mycobacteria, in particular *Mycobacterium tuberculosis*, the causative agent of tuberculosis, have the remarkable capacity to circumvent destruction within one of the most hostile cell types of a vertebrate host: the macrophage. The ability of pathogenic mycobacteria to survive inside macrophages has been known for more than 30 years; yet, only recently have advances in molecular genetics, biochemistry, immunology, as well as global analysis of gene expression, started to unravel that *M. tb* infection modifies the gene expression of the host macrophage, which results in a highly sophisticated interaction between the pathogen and its host (James et al., 2007; Durrant et al., 2010) therefore, better understanding of the molecular mechanism of interaction between the pathogen and its host is important for effective control and prevention of TB (Yew et al., 2008; Rao et al., 2007).

The 6-kDa early secretory antigenic target (ESAT-6) from *M. tb* is a dominant target for T cells in the early phases of infection (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). A knockout of ESAT-6 in *Mycobacterium bovis* results in decreased virulence of the pathogen (Wards et al., 2000), indicating that the two molecules may play important roles in immunopathogenesis and virulence, 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.

MATERIALS AND METHODS

Strains, general techniques

The strain of *Saccharomyces cerevisiae* used in this study was AH109 from Clontech company. Yeast cells were cultured at 30°C either in a complete YPD medium (1% yeast extract, 1% peptone,

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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. 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 ESAT-6 was PCR amplified from *M. tb* H37Rv genomic DNA and cloned into the pMD18-T vector (Takara, China). The full-length ESAT-6 gene was subjected to DNA sequencing, and the inserts were verified against the corresponding region of the complete genome H37Rv. The full-length ESAT-6 gene was excised from the pMD18-T-ESAT-6 construct using the restriction enzymes EcoRI and BamHI, and ligated into the pGBKT7 vector to generate an N-terminal in-frame fusion with the GAL4 activation domain (BD). To identify the putative domain of amino acid sequence required for ADAM9/ESAT-6 interaction, different fragments ESAT-6 of gene were prepared by PCR. PCR-amplified DNA fragment for the N-terminal 48 residues (1-48) of ESAT-6 and C-terminal 47(49-95) residues were subcloned into the yeast two-hybrid vector pGBKT7 in order to identify the putative domain of amino acid sequence required for ADAM9/ESAT-6 interaction. The ADAM9 gene was obtained by PCR from the human lung cDNA library, and subcloned into the yeast vector pGADT7. In addition, the plasmids for glutathione S-transferase (GST) fusions PGST-ESAT-6 was described previously (Lwabuchi et al., 2004). The recombinant ADAM9 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 surface plasmon resonance binding assay, ADAM9 was subcloned into pENTR/D-TOPO (Invitrogen) according to the manufacturer's protocol. The cloned gene was transferred to pDEST17 by LR recombination according to the Gateway protocol (Invitrogen). Using the same method, the gene encoding ESAT-6 was cloned into pENTR/D-TOPO and then transferred into pDEST17. 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, AH109 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 – 40 μ M 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°C for 1–8 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 \pm 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-ESAT-6 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.

GST pull-down assay

GST pull-down assay was performed using the ProFoundeTM Pull-Down GST Protein: Protein Interaction Kit (Pierce). The purified GST-ESAT-6 was adsorbed onto 60 μ l Glutathione-Sepharose beads, equilibrated with BupHTM TBS buffer (25 mM Tris-HCl and 0.15 M NaCl, pH 7.2) in a HandeeTM 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 μ l 1:1 wash solution of TBS: ProFoundeTM lysis buffer for 4 times. After addition of 0.5 mg of the purified ADAM9 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 μ l of wash solution as mentioned above. The bound proteins were eluted by 100 Mm of glutathione elution buffer. The eluted samples were analyzed by SDS-PAGE.

Surface plasmon resonance binding assay

Direct interaction of ESAT-6 and ADAM9 was monitored using a BIACORE 3000 biosensor (Biacore AB, Sweden). Recombinant His-tagged ESAT-6 (dialysed against 10 mM acetate buffer, pH 3.5) was coupled to the flow cells of a sensor chip (carboxymethylated dextran chip CM5, Biacore AB) using 100 mM N-hydroxysuccinimide and 400 mM N-ethyl-N'-(dimethylaminopropyl) carbodiimide until an appropriate level of coupling (3500 resonance units) was reached. Deactivation after immobilization was achieved using 1 M ethanolamine (pH 8.5). All binding studies were performed in 75 mM phosphate buffered saline, pH 7.5 using a flow rate of 5 μ l min⁻¹ at 25°C. A control flow cell was prepared under identical conditions in the absence of protein using acetate buffer. Recombinant His-tagged ADAM9 was dialysed against running buffer and injected in different concentrations into the flow cell coupled with ESAT-6.

RESULTS

Identification of ADAM9 as an ESAT-6 interacting protein by yeast two-hybrid system

The ESAT-6 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-ESAT-6, verified by auto sequencing assay, was used to transform yeast AH109. Yeast cells containing pGBK-ESAT-6 were transformed with a human lung cDNA library. Approximately 4.8×10^6 transformants were screened for His-Ade-Trp-Leu independent growth and blue colony formation in the β -gal assay. Thirty-four positive clones were obtained. As some AD-cDNA fusion products can activate reporter gene transcription without interacting with the BD-ESAT-6 fusion protein, this false-positive clone can be identified

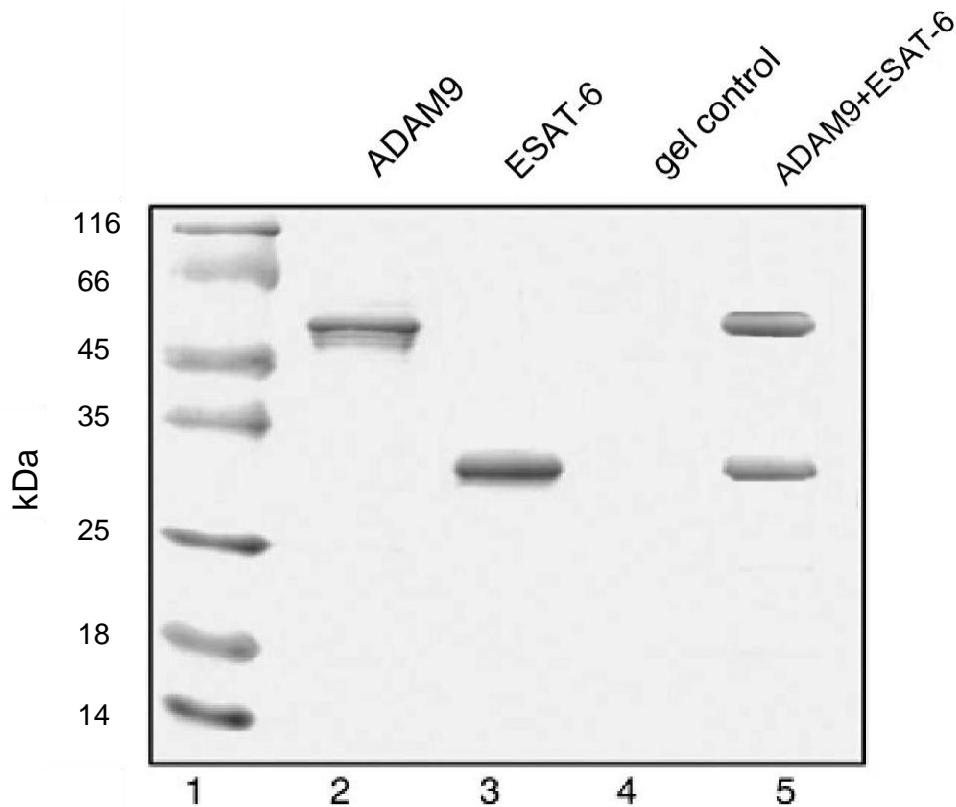


Figure 1. ESAT-6/ADAM9 interaction was determined by GST pull-down. Samples were analyzed on a 12% SDS–polyacrylamide gel, and the band was visualized with Coomassie brilliant blue. Components in each lane are shown at the top. Lane 1, molecular mass marker; lane 2, purified His-tagged ADAM9; lane 3, purified GST-tagged human ESAT-6; lane 4, agarose gel control; lane 5, ESAT-6 and the pull-down human ADAM9.

using the technique of segregation analysis. Only 4 of 34 clones survived all genetic tests and were considered to be genuine positive clone, DNA sequence analysis of the fragment revealed that the four cDNA fragments inserted have a high identity with four genes in the GeneBank database. One of these clones was identified as ADAM9 protein, As shown in Figure 3, the protein encoded by the pGAD-ADAM9 clones interacted specifically with the ESAT-6 protein.

GST pull-down assay directly revealed ESAT-6/humanADAM9 interaction

In order to identify the interaction between ESAT-6 and ADAM9, the recombinant human GST-ADAM9 protein was over-expressed in *E. coli*, followed by purification using Glutathione–Sepharose affinity beads. ESAT-6 /ADAM9 interaction was then determined by GST pull-down assay, in which the purified human GST- ESAT-6 (Figure 1, lane 3) protein was immobilized on the Glutathione–Sepharose beads as a bait protein according to the kit. The His-tagged ADAM9 protein as the prey was

purified through a Ni affinity column (Figure 1, 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 1, ADAM9 protein could be also detected on the SDS–PAGE, suggesting that GST-ESAT-6 was eluted by glutathione together with ADAM9 protein. ESAT-6 binding to human ADAM9 could be considered to be specific because the bead itself did not pull down any ADAM9 protein as shown in lane 4. All these results thereby determined that ESAT-6 protein could specifically bind to human ADAM9 *in vitro*.

Surface plasmon resonance revealed ESAT-6/humanADAM9 interaction

The binding of syntenin-1 to ESAT-6 was confirmed using a surface plasmon resonance technique. Recombinant ESAT-6 protein was immobilised on a CM5 Biosensor chip and syntenin-1 was injected into the fluid phase. ADAM9 bound dose-dependently to ESAT-6 and this binding was specific as no interaction occurred to the

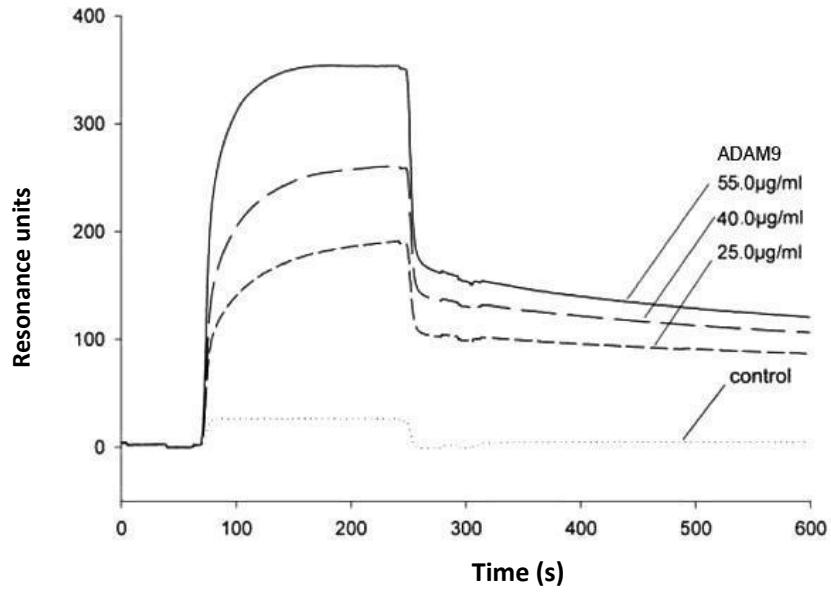


Figure 2. Binding of syntenin-1 to ESAT-6 determined by surface plasmon resonance. 6X His-tagged ESAT-6 was immobilized onto a CM5 Biosensor chip. 6X His-tagged ADAM9 was injected at concentrations of 55, 40, and 25 µg/ml, respectively.

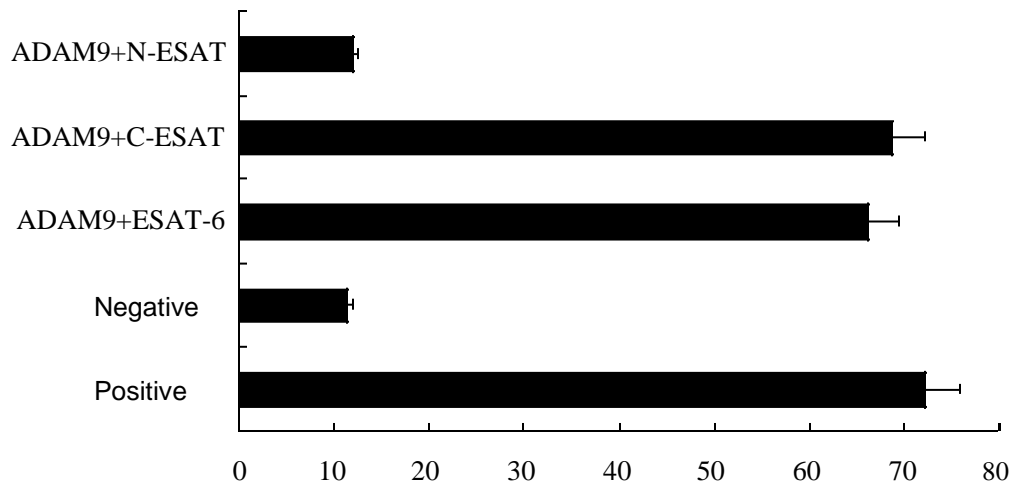


Figure 3. Map the interaction domain of ESAT-6. The empty vectors pGBKT7 and pGADT7 co-transformed were used as the negative control and the pGBKT7-53 and pGADT7-T co-transformed were used as the positive control. Every experiment was repeated for at least three times and the data were obtained by average. The error bars represent standard error of the mean.

control cell (Figure 2).

Mapping the ADAM9 binding region of the ESAT-6 protein by yeast two-hybrid assays

To map the involved regions of ESAT-6 protein in the ESAT-6/ADAM9 interaction, different fragments ESAT-6

of gene were prepared by PCR. PCR-amplified DNA fragment for the N-terminal 48 residues(1-48) of ESAT-6 and C-terminal 47(49-95) residues were subcloned into the yeast two-hybrid vector pGBKT7, and then co-transformed respectively with pGADT7-ADAM9 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 3, the C-terminal 47(49-95) residues domain of the ESAT-6 protein is responsible for the majority of the binding to ADAM9, which suggest that carboxyl terminus of ESAT-6 in *M. tb* play a key role in the binds to ADAM9 protein.

DISCUSSION

In the present study, by employing a series of biochemical and biophysical methods, we have firstly reported that ESAT-6 protein has a specific binding affinity to human ADAM9, and the further yeast two-hybrid assay demonstrated that the C-terminus of ESAT-6 probably contribute to the ESAT-6/human ADAM9 interaction. In addition, the yeast two-hybrid result has clearly indicated that only C-terminus of ESAT-6 is required for ESAT-6/human ADAM9 interaction, which is in line with that ESAT-6 binding to syntenin-1 by C-terminus of ESAT-6 (Schumann et al., 2006), suggesting that C-terminus of ESAT-6 might play a potent role in *M. tb* infection.

The interaction between ESAT-6 and ADAM9 was verified in the present study. ADAM9, one of the first ADAM proteins to be identified and characterized, is a membrane-anchored metalloproteinase an N-terminal prodomain followed by a metalloprotease domain, a disintegrin domain and cysteine-rich region, an epidermal growth factor (EGF) repeat, a transmembrane domain, and a cytoplasmic tail with potential SH3 ligand domains (Weskamp et al., 1996). ADAM9 is catalytically active in both biochemical and cell-based assays and can cleave several membrane proteins (Peduto et al., 2005; Roghani et al., 1999; Horiuchi et al., 2007). In addition, ADAM9 is thought to participate in cell-cell interactions by binding to integrins (Nath et al., 2000). Therefore, we presume that ADAM9 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, we have identified an interaction between ESAT6, an early secreted protein of *M. tb* and ADAM9, an intracellular host protein. The knowledge of protein-protein interactions of the virulence factor ESAT-6 is of importance to understand the role of ESAT-6 during the infection process. However, the pathophysiological significance of the interaction between ESAT-6 and ADAM9 is largely unknown; elucidation of these questions will depend on further studies. Moreover, the disruption of interaction between ESAT-6 and ADAM9 proteins using RNA interference technology may provide further clues to the specific function of ESAT-6 and ADAM9 protein.

ACKNOWLEDGEMENTS

This work was supported by the foundation of Yancheng

Agricultural Technology Development Projects (YK2009066) and Yancheng Institute of Technology (Natural Science Research Open Project(XKY2010002, 2009008).

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