Role Of Human Protein Disulfide Isomerase (PDI) In The Redox Regulation Of Anthrax Receptor 2 (ANTXR2)

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ROLE OF HUMAN PROTEIN DISULFIDE ISOMERASE (PDI) IN THE REDOX REGULATION OF ANTHRAX RECEPTOR 2 (ANTXR2)

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Dedication

To my deceased parents
ROLE OF HUMAN PROTEIN DISULFIDE ISOMERASE (PDI) IN THE REDOX REGULATION OF ANTHRAX RECEPTOR 2 (ANTXR2)

by

NAIMA MOHAMED ALSHRIF, Ms

THESIS

Presented to the Faculty of the Graduate School of The University of Texas at El Paso in Partial Fulfillment of the Requirements for the Degree of

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Abstract

Anthrax is an acute infectious disease caused by *B. anthracis*, a gram-positive and spore-forming bacterium. Anthrax toxin is a tripartite AB toxin composed of a receptor binding/pore-forming moiety, protective antigen (PA), and two catalytic moieties, edema factor (EF) and lethal factor (LF). Anthrax toxin PA binds to cell-surface receptors, where it gets activated by furin, and assembles into a ring-like heptameric structure. The toxin-receptor complex is internalized into endosomes where the acidic pH triggers PA pore formation on the endosomal membrane that translocates the toxin catalytic moieties. Recent study has shown that anthrax toxin receptors play a critical role in anthrax toxin action. Reduction of the disulfides of the R2-Ig domain significantly inhibited the release of K⁺ through the PA pore formed on the liposomal membranes and the release of K⁺ through the plasma membranes of the CHO cells over-expressing ANTRXR2. Disruption of the disulfides in R2-Ig also inhibited the PA-mediated LF₅ translocation across the plasma membrane, suggesting that anthrax toxin cellular internalization and trafficking may be influenced by cellular redox regulators. In this study, we investigated the role of PDI in the refolding of ANTXR2 in vitro. Purified PDI was shown to partially summarize the mis-matched disulfide bonds in the recombinant extracellular domain of ANTXR2, demonstrating that PDI may be involved in the redox regulation of the receptor disulfide bonds in vivo.
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Chapter 1: Introduction

1.1 *Bacillus anthracis*

Anthrax is an acute infectious disease caused by *B. anthracis*, a gram-positive and spore-forming bacterium. The possibility of using *B. anthracis* as a biological weapon is extremely high due to the possibility of the mass preparation and distribution of spores, and the limited number of approved drugs against it (1). *B. anthracis* enters the body by inhalation (inhalational anthrax), ingestion (intestinal anthrax), and direct skin contacts (cutaneous anthrax) (Figure 1.1). *B. anthracis* spores, initially consumed by macrophages, germinate within these cells to become vegetative bacteria and transport to the lymph nodes, where they multiply and produce the toxins (lethal factor, edema factor and protective antigen). Death results from high levels of toxins and bacteria. Lethal factor, a zinc metalloprotease, cleaves the important mitogen-activated protein kinase kinase (MAPKK) and triggers a severe inflammatory condition. Edema factor, a calmodulin-dependent adenylate cyclase, overproduces cAMP (Cyclic adenosine monophosphate) and causes abnormal water retention. Protective antigen is involved in the entry of the two toxins into the host cell (2).
1.2 Anthrax toxin

Anthrax toxin is a tripartite AB toxin secreted by virulent strains of the bacterium, *Bacillus anthracis*, the causative agent of anthrax. The toxin consists of three different protein components: lethal factor (LF), a 90 kDa metalloprotease that cleaves MAP kinase kinases (edema factor (EF), an 89 kDa adenylyl cyclase; and protective antigen (PA), an 83 kDa binding and translocation component. PA mediates the transport of LF and EF into the cytosol of target cells, where they exhibit their activities (3,4).
1.2.1 Protective antigen (PA)

Protective Antigen (83 kDa protein, 735 amino acids in length) consist of four functional domains, each one is required for a particular step in the intoxication process. Domain 1, amino acid residues 1 to 249, contains the proteolytic activation site responsible for the liberation of the 20 kDa N-terminal fragment, yielding a 63 kDa protein capable of oligomerizing into ring shaped heptamers. Domain 2, residues 250 to 487, consists of a β-barrel core containing a large flexible loop (between residues 302 and 325) that is believed to be involved in pore formation. Domain 3, spanning residues 499 to 594, contains a hydrophobic region believed to be involved in protein-protein interactions that functions primarily in self association of PA63. Domain 4, spanning residues 595-735, is involved in receptor binding (5).

1.3 Anthrax receptors

Two cellular receptors that bind to PA$_{83}$ have been characterized, namely, ANTXR1 (tumor endothelial marker-8) and ANTXR2 (capillary morphogenesis protein 2) (6). Most human cells appear to express ANTXR2, while ANTXR1 has been reported to be expressed by human epithelial cells of the lung, skin, intestines, and endothelial cells (26). Both receptors are type-1 transmembrane proteins and share about 60% sequence identity (7). The two cellular receptors are composed of von willebrand factor A domain (VWA), also called integrin-like domain (I domain), which binds to PA, and a newly defined immunoglobulin like domain (Ig domain), a transmembrane domain, and a cytoplasmic domain (8,9).
Figure 1.2: ANTXR2 ectodomain is composed of R2-VWA and R2-Ig. A. Schematic of ANTXR2. ANTXR2 is composed of R2-VWA and R2-Ig, a transmembrane domain, and a cytoplasmic domain. B. The crystal structure of R2-VWA domain (1SHU) as displayed in Swiss-PDB-Viewer. Residues Cys175, the Cys39–Cys218 disulfide bond, and Mn$^{2+}$ ion of MIDAS are shown and labeled. C. Sequence alignment of the ectodomains of R1 and R2. The seven conserved Cys residues are highlighted and numbered in R2. Identical residues are labeled with asterisk (*) (9).
PA binds to VWA domain at metal ion-dependent adhesion site (MIDAS) in the presence of Mn$^{2+}$, Mg$^{2+}$ or Zn$^{2+}$, and ANTXR2 has been shown to also use Ca$^{2+}$ but with a higher affinity when liganded by Mg$^{2+}$. ANTRX2 has a higher affinity for PA binding than ANTXR1. VWA domain binds to PA receptor binding domain (domain 4) and pore forming domain (Domain2), suggesting that the receptor plays roles in PA pore formation. The ANTXR2 ectodomain (VWA domain and Ig domain) has a total of seven cysteine residues with three in the R2-VWA domain (C39, C175, C218), and four in the R2-Ig domain (C230, C255, C279, and C315). It has been found that C39 and C218 form a disulfide bond and C175 remains as a free SH group. It has also been predicted that two disulfide bonds are formed in the Ig domain (9).

1.4 Translocation of LF and EF into cytoplasm

The first step in toxin uptake involves binding of an 83-kDa form of PA (PA$_{83}$) to a cell-surface receptor, where it subsequently undergoes a cleavage by furin to generate a 63-kDa PA subunit (PA$_{63}$). PA$_{63}$ spontaneously assembles into a ring-like heptameric structure. The entire toxin-receptor complex is then internalized into the cells through endocytosis. Within the endosomal compartment, acidification induces conformational changes in the PA heptamer, which converts PA heptamer into a membrane-spanning pore on the endosomal membranes. LF and EF are presumably translocated through the PA pore into the cytosol (8,10).
Figure 1.3: Model of anthrax toxin action. 1. Intoxication starts with the binding of PA to receptor. 2. PA is cleaved by furin into PA$_{63}$ and PA$_{20}$. 3. PA$_{63}$ oligomerizes into heptamer, EF and/or LF binds to the heptamer. 4. The complex is internalized by receptor mediated indocytosis. 5. Travel to early endosome. 6. Acidification at the late endosome low pH triggers conversion of prepore to pore. 7. EF/LF is translocated into the cytosol (11).

Sun et. al. have found that the intact disulfides in the R2-Ig domain are important for anthrax toxin action. Reduction of the disulfides of the R2-Ig domain significantly inhibited the release of K$^+$ through the PA pore formed on the liposomal membranes and the release of K$^+$ through the plasma membranes of the CHO cells over-expressing ANTRXR2. Disruption of the disulfides in R2-Ig also inhibited the PA-mediated LF$_N$ translocation across the plasma membrane (9). The mechanism of anthrax inhibition induced by reduction of the receptor disulfide bonds is not clear yet.
An emerging concept is that disulfide bonds in extracellular domains of cell surface receptors have the potential to function as redox operator switches (breaking, forming, exchanging) (20). Reduction, oxidation, and isomerization of the disulfide bonds are regulated by a number of cellular redox factors, including protein disulfide isomerase (PDI), which is a member of the thioredoxin oxireductase super family (12). It is also unknown whether or PDI interacts with anthrax toxin in vivo during intoxication.

It was demonstrated recently that delivery of diphtheria toxin to the cytosol requires host cellular thioredoxin reductase cell factors. PDI has been implicated in the mediating reduction of the diphtheria toxin interchain (13). Also, reduction of HIV gp120 is required for entry into lymphoid cells (14). The cell surface PDI plays roles in the regulation of integrin-dependent cell adhesion through control of the open and closed conformations of integrin receptors (15, 16). In this study we will study the role of the redox regulator factor, PDI, which may affect anthrax toxin-mediated cytotoxicity through regulating the redox states of anthrax toxin receptors.

1.5 The thioredoxin-like protein family

The name of this protein family is derived from its first member. Thioredoxin was initially identified as the hydrogen donor of ribonucleotide reductase. Later, further functions of thioredoxin were found, such as hydrogen donation to sulfate reductase and the redox control of transcription factors. Thioredoxin-like oxidoreductases share the thioredoxin-like fold, a central four-stranded β-sheet with three flanking α-helices and the active site motif Cys-Xaa-Xaa-Cys (17).

1.6 Oxidative refolding of proteins in vitro

The in vitro studying of disulfide bond formation during protein refolding has provided key insights into the process of oxidative protein folding. A few proteins have dominated the
studies on disulfide-linked protein refolding in vitro such as bovine pancreatic trypsin inhibitor (BPTI), and bovine pancreatic ribonuclease A (RNaseA). Oxidative refolding of reduced, unfolded proteins in vitro without the addition of co-factors are normally slow and, if more than one pair of disulfide bonds can be formed, may lead to the formation of incorrect disulfide. To prevent this from happening, low molecular weight thiols in their reduced and oxidized form (e.g. GSH/GSSG, cysteine/cystine or DTTred/DTTox) have often been added as “Redox shuffling” reagents. “Redox-shuffling” reagents allow thiol-disulfide exchange reactions in both directions and increase yield of proteins with correct disulfide bonds through reshuffling of improper disulfide bonds (18).

1.7 Protein disulfide isomerase (PDI)

Protein disulfide isomerase (PDI) is a ubiquitous redox chaperone belonging to the thioredoxin oxireductase super family (19). There are around 20 PDI homologues and the detailed structure and function of eukaryotic PDIs have been covered in recent excellent reviews (20). PDI is a multifunctional enzyme has been found to be one of the major enzymes that assists protein folding, catalyzes the formation, reduction, and isomerisation of disulfide contained proteins in the ER (19). PDIs are found in all eukaryotic species, and expressed in most tissues and organs (21).

While PDI enzymes are predominantly located in the ER in high concentrations, they are also present on the cell surface and can be secreted extracellulary (12). The mature form of human PDI is 491 amino acids, with a molecular weight of about 56 KDa. The multidomain structure of PDI was recognized from an analysis of its amino acid sequences, which consists of four domains, a, b, b’ and a’ plus a highly acidic C-terminal extension c and a 19 amino acid long inter domain linker between b’ and a’ domains named x. PDI contains two thioredoxin-like
motifs (CXXC) in two separate domains (a and a’) and an ER retention signal KDELI at the C-terminus (12,19).

The a and a’ domains which contains the active sites are 47% identical with each other and 27% to thioredoxin. b and b’ domains share 28% identity to each other, but show a very low sequence relatedness with thioredoxin (21). The active site motifs are involved in catalyzing the thiol-disulphide exchange reaction; the b domain is important for the overall structure of the PDI and b’ domain is involved in substrate recognition and binding (19).

![Domain structure of PDI](image.png)

**Figure 1.4:** Domain structure of PDI. a and a’ are the redox-active thioredoxinlike domain with the active site CXXC, b and b’ are the redoxinactive thioredoxin-like domain and c domain. Human PDI domain architecture. Numbers indicate domain boundaries, with residue numbers representing mature human PDI (28).

PDI acts as a redox-dependent and -independent molecular chaperone (19). PDI interacts with many misfolded proteins, peptides and some folded proteins, and has a significant role in ER-associated degradation (15,22). The essential basis for the redox activity of PDI is the presence of two thioredoxin-like motifs –CGHC and the ability to catalyze the activity in thiol-disulfide exchange reaction (22, 23). To function as catalysts of protein thiol-disulphide exchange, PDI must be able to interact with their substrates. The oxidation of a
substrate requires the conversion of a disulfide -CGHC- site to a dithiol, while the reduction of a substrate requires the conversion of a dithiol active site to a disulfide (19,22). Moreover, all of the thioredoxin-like domains of PDI are required for isomerization reactions that involve substantial changes in structure in the substrate (23). Isomerization by PDI occurs via two possible pathways: through direct isomerisation or through cycles of reduction followed by reoxidation. The presence of two protein-thiol oxidoreductase active sites in PDI probably, at least in part, accounts for the diversity of functions and specificity of the enzyme (22, 23).

Figure 1.5: Mechanisms of catalysis by PDI. The main thiol redox reactions driven by PDI on (protein/peptide) substrate containing thiols (23).
PDI has been suggested to have a molecular chaperone and anti-chaperone activity (24). A chaperone is a protein that can associate with other proteins to facilitate their transit in the cell. A chaperone also assists incorrectly folded or unfolded proteins to attain the native state and the assembly of polypeptide chains into their correct three-dimensional structure (22,23). PDI, as a chaperone, inhibits the aggregation of misfolded proteins, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rhodanese, and lysozym. Both GAPDH and rhodanese do not contain disulfide bridges, confirming the non-redox chaperone activity of PDI (23, 24). The redox-active sites of PDI are not required for its chaperone activity (25, 27).

1.8 Specific aims

Our long term goal is to investigate the roles of cellular redox factors; particularly PDI, in anthrax toxin-mediated cytotoxicity. This will provide us insights into the mechanism of anthrax pathogenesis and may lead to the development of effective drugs against anthrax.

Specific aim 1: characterize the role of PDI in renaturation of the extracellular domain of ANTXR2

In this aim we investigate the roles of PDI in refolding of the extracellular domain of ANTXR2 in vitro. PDI has an isomerase and chaperone activity. we will test if PDI assists in the refolding of ANTXR2 protein in vitro through catalyzing the formation of the disulfide bonds.

Specific aim 2: Test the effects of over-expression of PDI on the receptor-mediated anthrax toxin action.

The gene encoding PDI will be cloned into the expression vector pcDNA3.1 (+) (sigma) to generate a HA-tagged PDI. The construct will be transiently transected into the CHO-K1 cells, and expression of PDI will be monitored by western blotting using anti-HA antibody. Effects of PDI over-expression in CHO-K1 cells will be measured using a cytotoxicity assay, in
which PA translocates the model substrate, $\text{LF}_N$-DTA, into the cytosol, where $\text{LF}_N$-DTA inhibits protein synthesis.
Chapter 2: Materials and methods

2.1 PDI plasmid

The wild type PDI (Swiss Prot ID P07237) was very generously provided by Dr. Narayan.

2.2 Expression and purification of recombinant human PDI

The PDI vector contains a hexa-histidine tag (His-tag) sequence MHHHHHHM. E.coli BL21(DE3) was taken out from -80°C (30 µl aliquots) and thawed on ice for 10 min. 2 µl of PDI plasmid was added into the cells and incubated on ice for 20 min. the transformation tube was placed into a 42°C water bath for 45 seconds and then placed back on ice for 5 min. 200 µl of LB media was added into the cells and incubated at 37°C with shaking at 200 rpm. The cells were collected at 10000 rpm for 1 min. the pellet was resuspended with 50 µl LB media and spread onto LB/carb plates and incubated at 37°C overnight.

The next day, a single colony was picked and grown in 25 ml of LB medium supplemented with 25 µg/ml of Carbenicillin at 37°C with shaking at 200 rpm for 16 hours. 20ml of the startup culture was added to 1L LB media supplemented with 100 µg/ml of Carbenicillin and incubated at 37°C with shaking at 200 rpm, and induced at OD600 of 0.8 for four hours with 0.5 mM isopropyl β-D-thiogalactoside. Cells were harvested by centrifuging at 10,000 rpm for 15 min at 4°C and the pellet was suspended into (20 mM Tris-HCl, 300 mM NaCl, 20 mM Imidazole, pH 8.0 (buffer A), and disrupted with an ultrasonic cell disrupter. Then cells were then centrifuged at 19,000 rpm for 40 mins at 4°C. Supernatants were loaded on an immobilized metal affinity column precharged with Ni²⁺ and equilibrated with buffer A. The purity of eluted protein fractions was analyzed on Coomassie Brilliant Blue stained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).
2.3 Electrophoresis Analysis

(SDS-PAGE) was used to analyze protein samples. Discontinuous gels were cast, consisting of a separating gel at pH 8.8 and a stacking gel at pH 6.8. Typically, 12% acrylamide was used in the separating gel. Samples were mixed with the sample loading buffer (1 M Tris-HCl pH 6.8, 40% glycerol, 0.8% SDS, 0.1% w/v bromophenol blue). A molecular weight marker was used to estimate the molecular weight of each band. Gels were run at 107 V through the stacking gel, then at 200 V until the dye front ran off the gel. Gels were stained with Coomassie Blue.

2.4 Measurement of protein concentration

Purified proteins were quantified using a BCA Protein Assay Kit (Thermo Scientific Pierce).

2.5 PDI isomerase assay

R2-ectodomain oligomers was incubated in the oxidation buffer that contained 20mM Tris, 0.5mM DTTred, 1mM EDTA, and 100mM of DTTox for different time points. To optimize the refolding rate, different molar ratio of PDI and receptor were used. As a control, the same reaction was run without PDI. The reaction was incubated at room temperature and then analyzed by SDS-PAGE.

2.6 Construction of HA-tagged PDI

pcDNA 3.1(+) (Invitrogen) was used for human PDI expression in CHO cells. Nhe1 and Not1 sites were generated in the hPDI cDNA by PCR. Amplification cycle entails an initial denaturation at 95°C for 5 mins, followed by 30 cycles of denaturation at 95°C for 45 Seconds, annealing at 55°C for 45 s, and extension at 72°C for 2 min. HA tag (TATCCTTATGACGTGCCTGACTATGCCAGCCTGGGAGGACCT) was introduced to the C terminus of the PDI to monitor the expression using HA antibody.
Figure 2.1: pcDNA3.1 (+) plasmid (Invitrogen). The hPDI gene is inserted into the multiple clone site (MCS) between NheI and NotI.

2.7 Cell Culture and Media

Chinese hamster ovary–K1(CHO-K1 cell line) (ATCC) was grown in Ham’s F-12 medium supplemented with 10% calf serum, 2 mM L-glutamine, 500 units/ml penicillin, 500 units/ml streptomycin sulfate under a humidified atmosphere with 5% CO₂.

2.8 Transient transfection

The day before the assay, CHO cells were seeded in a 6-well plate to 90% confluency at the time of transfection. 10μl of Lipofectamine® 2000 Reagent was diluted in 250μl DMEM free serum medium. Different concentrations of PDI plasmid were used to check the expression levels. Potential effects of PDI over-expression on cell phenotypes were monitored after 24 hours. 24 hours post transfection; cells were collected using cell scraper, centrifuged at 1200 rpm for 5 min. After determining the concentration of the whole protein, SDS loading sample buffer was added to the lysate, and Boiled for 10 min. Samples were applied to SDS-PAGE, followed by western blotting using HA antibody (Santa Cruz Biotechnology,INC) to monitor the expression of PDI.
2.9 Western blotting

proteins were transferred onto the PVDF membrane in the transfer buffer (25mM Trizma Base, 192mM Glycine, 3.5mM SDS, 20% Methanol) for 45 min at 100 volts. Membrane was incubated with the primary antibody (Anti-HA antibody, Santa Cruz Biotechnology, INC) in 2% milk solution overnight. After three times wash with 0.5% Tween20 TBS buffer (20mM Tris, 150mM NaCl, 0.5% Tween,) the membrane was incubated with secondary antibody, goat anti mouse antibody (Santa Cruz Biotechnology, INC), and then washed five times with TBST buffer. The bands were visualized using SuperSignal West Pico Chemiluminescent Substrate.

2.10 Immunofluorescence of HA-PDI

CHO cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X-100 for 15 mins. The permeabilized cells were incubated with a monoclonal mouse anti-HA antibody. Subsequently, the cells were incubated with the secondary antibodies goat anti mouse conjugated with Alexa Fluor 488 (green), and visualized under confocal microscope.

2.11 PDI subcellular fractionation

Twenty-four hours post-transfection, CHO cells were harvested and suspended in cold buffer 1 (250 mM sucrose, 3 mM imidazole, pH 7.4). The cells were centrifuged at 250 x g for 5 min and resuspended in cold buffer 2 (250 mM sucrose, 3 mM imidazole, pH 7.4, 1% protease inhibitors mixture and 0.5 mM EDTA). The cells were broken by passing 20 times through a 25-gauge 5/8-inch needle in buffer 2 followed by centrifugation at 250 x g for 5 min at 4 °C. The post-nuclear supernatant was centrifuged at 100,000 x g for 30 min at 4 °C. The insoluble fraction (membrane fraction) was dissolved in buffer 2 containing 1% Triton X-100 and mixed with an equal volume of SDS sample buffer. The fractions were boiled for 10 min and subjected to SDS-PAGE followed by Western blot analysis.
2.12 Preparation of PA protein

BL21 (DE3) cells were transformed with pET22b-PA wide type, and grown in a BioFlo/CelliGen 115 Fermentor/Bioreactor 2.5L fermentor at 37°C to OD600 of 1.0. Expression of the recombinant protein was induced by the addition of 1mM IPTG. The cells were grown for an additional 3 h at 30°C and harvested by centrifugation for 10 min at 8000 x g. Proteins were released from the periplasm by osmotic shock. The cells were resuspended in 20 mM Tris, pH 8.0, 30% glucose and 1 mM EDTA, and incubated at room temperature for 10 min with continuous stirring. The cells were again harvested by centrifugation, resuspended in ice-cold 5 mM MgSO4 and incubated at 4°C for 10 min with constant stirring. After the cells were again harvested by centrifugation at 8000 x g the periplasmic extract was decanted. The sample was loaded onto a column packed with Q-Sepharose High Performance (Amersham-Pharmacia, Piscataway, NJ). Bound proteins were eluted with buffer B (20 mM Tris, pH 8.0, 1M NaCl). The PA-containing fractions were determined by SDS-PAGE and concentrated down. The PA-protein was loaded onto a Superdex 200 column at flow rate 0.5 ml/min in the gel filtration buffer (20 mM Tris-HCl, 150 mM NaCl, at a pH of 8.0). Fractions containing PA were determined by SDS-PAGE and stored at –80°C.

2.13 Activation of PA

PA was activated by treatment with trypsin, yielding nicked PA (nPA). Trypsin was added to PA83 (1 mg/ml) at a final trypsin:PA ratio of 1:1000 (w:w). The mixture was incubated at room temperature for 1 hour.

2.14 LF_N- DTA protein purification

pET-15b LF_N DTA vector contains a hexa-histidine tag (His-tag) sequence at the N terminus. The protein was purified as previously stated in PDI purification.
2.15 Anthrax toxicity

One day before the assay, a 96-well plate to 90% confluency 96-well plate CHO-K1 cells will be transiently transfected with PDI plasmid as previously stated. Two wells will serve as a control one will be transfected with pcDNA3.1 (+) empty vector (our negative control), and one is used for mock transfection. Dilutions of purified PA and purified LF_N-DTA in cell culture medium were made to a final concentration of $10^{-7}$ M PA and $10^{-6}$ to $10^{-11}$ M LF_N-DTA, and added to each well, except for the control wells we only added medium alone (no toxin control), and then incubated for 4 h at 37°C in a cell incubator. Cell viability was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation assay as per the manufacturer's instructions.
Chapter 3: Results and discussion

3.1 PDI expression and purification

Recombinant human PDI was expressed using the Escherichia coli strain BL21 (DE3). The plasmid encodes a fusion protein containing the entire human PDI sequence with an N-terminal His6 tag. Recombinant PDI was purified from the soluble fraction of the cell lysate using Ni- CAMTM H Cresin (Sigma), which is a high-capacity nickel-affinity matrix. Bound PDI was eluted using 500 mM imidazole in 50 mM Tris/HCl, and 300 mM NaCl (pH 8.0) and collected in 5.0 ml fractions. PDI protein was further clarified by gel filtration using a Superdex 200 column at flow rate 0.5 ml/min in the buffer containing 20 mM Tris-HCl, 150 mM NaCl, at a pH of 8.0 (Fig 2.4).

Figure 3.1: Expression and purification of human PDI in BL21 (DE3).(A), Lanes 1 is molecular weight marker, lane 2 is whole cell lysate after inducing with IPTG (WCL), lane 3 soluble fraction (SF), lane 4 sample before loading into the Ni+ column, lane 5-8 are different fraction. (B), PDI final purification step (gel filtration). Lane 1 is molecular weight marker, lane 1 is purified PDI.
3.2 PDI facilitates the refolding of anthrax receptor 2 ectodomain

To investigate whether ANTXR2 is a specific substrate for PDI, we first examined the oxidative refolding of ANTXR2 ectodomain with purified PDI. ANTXR2 ectodomain soluble oligomer was incubated in oxidative buffer (20 mM Tris-HCL, and 1mM EDTA) with 0.5 mM DTT red and 100 mM DTT OX in the presence or absence of PDI for different time points (1 h, 2 h, 3 h, and overnight). As shown in (Figure 2.5) PDI facilitates the refolding of ANTXR2 as the accumulation of the monomer in the PDI treated PDI (Figure 3.2, lanes 2, 4, 5, and 6) compared to the non-treated PDI protein (Figure 3.2 lanes 1 and 3). For the time titration, we noticed that 1 hr is enough to see the isomerase activity of PDI. The observation suggests that ANTXR2 is a specific substrate of PDI for catalyzing disulfide bond formation in vitro.

![Figure 3.2](image)

**Figure 3.2:** oxidative refolding of ANTXR2 ectodomain with hPDI. Lane 1 and 3 correspond to R2 ectodomain protein without PDI. Each were incubated for 1hr and 2hr respectively; lanes 2-6, R2 ectodomain in the presence of PDI incubated for 1hr, 2hr, 3hr, and overnight, respectively.
3.3 PDI displayed a concentration-dependent isomerase activity

Second, we tested the oxidative refolding of ANTXR2 ectodomain with different R2/hPDI molar ratio. ANTXR2 ectodomain soluble oligomer was incubated in oxidative buffer (20 mM Tris-HCL, and 1mM EDTA) with 0.5 mM DTT red and 100 mM DTTOX in the absence of PDI or in the presence of PDI with different PDI/R2 molar ratios (0.02, 0.1, 0.2, 0.3, 0.6, 0.8 and 1.0) for 1 hr at room temperature. Figure 3.3 shows that as the concentration of PDI is increasing, the amount of protein monomer is increasing as well. We concluded that PDI catalyzes the oxidative refolding of the ANTXR2 ectodomain in a concentration-dependent manner.

![Figure 3.3: oxidative refolding of ANTXR2 ectodomain with different R2/hPDI molar ratio. Lane 2 corresponds to R2 ectodomain protein without PDI; lanes 3-9, R2 ectodomain in the presence of PDI at PDI/R2 molar ratios of 0.02, 0.1, 0.2, 0.3, 0.6, 0.8 and 1.0, respectively; lane 10, corresponds to PDI protein.](image-url)
3.4 Expression and characterization of HA- PDI in CHO cells

CHO cells were grown in a 6- well plate, and transiently transfected with pcDNA-Ha-PDI using Lipofectamine reagent (invetrogen). According to the manufacture, cells were transfected with 2 µg/ml of the pcDNA-Ha-PDI. Transient transfection with 2 µg/ml of the PDI expression vector elicited cell rounding (Figure 3.4). Due to the Potential effects of PDI over-expression on cell phenotypes when we used a high DNA concentration, we decided to use different concentrations of PDI plasmid (100 ng/ml, 200 ng/ml, 400 ng/ml, 800 ng/ml, 2 µg/ml) to check the expression levels and monitor the cells phenotype effect (cell rounding) after 24 hours. We looked to a good PDI expression with less effect on the cells phenotype. We found that transfection with a high concentration of pcDNA-HA-PDI stimulated a dose-dependent cell rounding (Figure 3.5).

![Figure 3.4: Effects of PDI over-expression on CHO cells phenotype. A, non-transfected CHO cells; CHO cells were transiently transfected with pcDNA3.1(+)-PDI, image was taken 24 hrs post-transfection.](image)
Figure 3.5, shows that 200 ng/ml, and 400 ng/ml gave us a good expression of PDI as well as less affects on the cells phenotype. For the further experiments, 200 ng/ml DNA concentration of the PDI plasmid will be used.

Figure 3.5: Expression and characterization of HA-PDI. A, CHO cells were transfected with HA- PDI at concentration 100 ng/ml, 200 ng/ml, 400 ng/ml, 800 ng/ml, 2 µg/ml, respectively. At 24 h post-transfection, the whole cell lysates were subjected to SDS-PAGE followed by Western blot using anti-HA antibody. B, CHO cells were co-transfected with HA- PDI at concentration 100 ng/ml, 200 ng/ml, 400 ng/ml, 800 ng/ml, 2 µg/ml, respectively. At 24 h post-transfection, the percentage of cell rounding was calculated under the fluorescent microscope (percentage of cell rounding = rounding transfected cells/total transfected cells).
3.5 PDI localization

CHO cells were transiently transfected with pcDNA-HA-PDI, and fixed in 4% paraformaldehyde in the PBS buffer, and then incubated with the mouse anti-HA antibody. This was followed by reaction with a secondary antibody, goat anti mouse conjugated with Alexa Fluor 488 (green). Fluorescence was localized in the nuclear and in the cytoplasm, but we were not able to see the membrane localization of PDI (Fig 2.9B). Specificity was shown by using non-transfected CHO cells; no staining was observed (Fig 2.9A). The expression and cytosolic localization of PDI was further confirmed in fractionation analysis using Anti-HA antibody (Figure 2.9 C). PDI was found in the cytoplasmic and membrane fractions as well.

Figure 3.6: PDI localization. A and B, immunofluorescence of paraformaldehyde-fixed CHO cells showing cytoplasmic localization. A non-transfected CHO cells, left panel DAPI, middle panel Alexa Fluor 488 (green), right panel merged. B, CHO cells were transfected with HA-PDI, left panel DAPI, middle panel Alexa Fluor 488 (green), right panel merged. C, pCDNA-HA- PDI was transfected into CHO cells. At 24 h post-transfection, the cells were lysed and fractionated into post-nuclear supernatant (P), membrane (M), and cytosol (C), which was followed by ECL Western blotting using HA antibody as the primary antibodies.
3.6 Protein preparation for anthrax toxicity assay

3.6.1 Expression and purification of LF\(_{N}\)-DTA

LF\(_{N}\)-DTA is a fusion protein composed of the catalytic chain of the diphtheria toxin fused to the C-terminus of the PA recognition domain of LF (residues 1-255), which binds to the PA\(_{63}\) heptamer and enters cells by the same pathway as EF and LF. pET-15b-LF\(_{N}\)-DTA (Addgene) has His6- tag at the N terminus. LF\(_{N}\)DTA was shown to be expressed as a soluble product of \(\sim 50\) kDa, after a four hour induction, in Escherichia coli BL21 DE3 (Figure 2.9). LF\(_{N}\)-DTA protein was further purified by using size exclusion chromatography.

![Figure 3.7: Induction and purification of LF\(_{N}\)-DTA protein in BL21 (DE3).](image)

(A) First lane molecular weight marker, lane 2 is whole cell lysate before induction (WCB), lane 3 is whole cell lysate after induction (WCA), lane 4 is the flow through, lanes 5-10 are different fractions.

(B) LF\(_{N}\) DTA final purification step (gel filtration) in a 12% SDS-PAGE. First lane is molecular weight marker, lanes 1-4 different fractions.
3.6.2 PA$_{83}$ purification

Recombinant wild-type PA$_{83}$ was over-expressed in the periplasm of the Escherichia coli strain, BL21 (DE3). The 83-kDa PA monomer was purified from the periplasm using anion exchange chromatography.

Figure 3.8: Expression and purification of PA$_{83}$ protein in BL21 (DE3). A), AKTA chromatography showing A280 in blue, fractionation aliquots in red, and buffer conductance Brown. (B), lanes 1-8 are different fractionations of the purification process in a 12% SDS-PAGE, last lane (M) is molecular weight marker.
PA$_{83}$ protein was further clarified by gel filtration using a Superdex 200 column at flow rate 0.5 ml/min in the buffer gel filtration buffer (20 mM Tris-HCl, 150 mM NaCl, at pH 8.0).

**Figure 3.9:** Size Exclusion Chromatography of PA$_{83}$. (A), AKTA chromatography showing A280 in blue and fractionation aliquots in red. (B), first lane (M) is molecular weight marker, lanes B6 – C5 are fractionations of the purification process in a 12% SDS-PAGE.
PA$_{83}$ was digested using the protease trypsin at a final trypsin:PA ratio of 1:1000 (w:w), and incubated the digested reaction at room temperature for 1 hr. then, will use a 5mL HiTrap (GE Healthcare) column for Anion Exchange Chromatography (Figure 3.10 A). Fractions containing PA were determined by SDS-PAGE (Figure 3.10 B).

**Figure 3.10:** PA$_{83}$ digestion with trypsin. A, AKTA chromatography showing $A_{280}$ in blue, fractionation aliquots in red, eluant gradient concentration in green, and buffer conductance in brown. B, SDS-PAGE analysis of digested PA$_{83}$, Lane 1, M, molecular weight markers (in kDa); lane 2, PA$_{83}$; lane 3, flowthrough; lane 4, elutes of PA$_{83}$ digested with trypsin.
3.7 Anthrax toxicity assay

After we confirmed the expression of the recombinant PDI, we tested the effect of the anthrax toxin on CHO cells by inhibiting the PA-mediating translocation of the lethal factor LF$_N$-DTA. LF$_N$-DTA, is a fusion between diphtheria toxin A-chain (DTA) to the c-terminus of LF$_N$, which binds to PA$_{63}$ heptemer and enters the cells by the same pathway as LF/EF. After entry into the cytosol, DTA catalyzes the ADP-ribosylation of elongation factor-2, which inactivates the elongation factor, inhibiting protein synthesis and ultimately causing cell death. Cell viability was estimated using the MTS assay, which measures the reduction of a tetrazolium salt to formazan by metabolically active cells. We first searched for incubation conditions that would lead to reduce metabolic activity in CHO K1 cells by LF$_N$-DTA and PA$_{63}$. Three trials of the toxicity assay were performed, and we found that exposure of the CHO cells for 4 hours to a mixture of $10^{-6}$ M LF$_N$-DTA and $10^{-7}$ M PA$_{63}$ was sufficient to reduce metabolic activity by 40% (Figure 2.15).

![Figure 3.11: Effects of LF$_N$-DTA on cell viability and cytotoxicity in CHO cells. MTS colorimetric assay was performed to measure the survival rate of CHO cells after treatment with PA, and different concentrations of LF$_N$-DTA. Data points represent the means ± SEM of experiments performed in triplicates.](image)
3.8 Discussion

Evidence has shown that cellular redox factors play critical roles in pathogen and toxin entry into the endocytic pathways, particularly through modulating the thiol-dithiol states of pathogen- and/or host-factors. For instance, cellular entry of diphtheria toxin to the cytosol requires host cellular thioredoxin reductase cell factor, PDI, which has been found to mediate the reduction of diphtheria toxin interchain. Also, cholera toxin, botulinum neurotoxins, anthrax toxin are apparently dependent on the redox states (reduced or oxidized) of the specific disulfides of either the toxin compounds, or the host receptors. In this study we attempted to study the role of the redox regulator factor, PDI, which may affect anthrax toxin-mediated cytotoxicity through regulating the redox states of anthrax toxin receptors. The question this project addresses is: does PDI involve redox regulation of ANTXR2. Our first goal was to characterize the role of PDI in renaturation of the extracellular domain of ANTXR2 in vitro, in which we investigated if PDI can modulate the refolding of the extracellular domain of ANTXR2 protein in vitro through catalyzing the formation of the mis-matched disulfide bonds. We found that purified PDI partially can convert the oligomeric receptor to the monomeric protein. We also found that PDI catalyzes the oxidative refolding of the ANTXR2 ectodomain in a concentration-dependent manner. From these results we concluded that ANTXR2 is a specific substrate for PDI enzyme in vitro.

Our second goal was to test the effects of over-expression of PDI on the receptor-mediated anthrax toxin action. PDI is essential for cell viability, therefore gene deletion approaches can’t be used to studying its role in anthrax toxin action. However, we can up-regulate PDI by over-expression of a recombinant PDI, and then measure the LF₅-DTA membrane translocation in the CHO wide type cells. Here, we subcloned the entire PDI gene into pCDNA3.1(+) vector with a generation of a C–terminus HA tag to monitor the expression
using anti-HA antibody. We successfully transiently transfected PDI into CHO cells with a good expression level. We found that over-expression of PDI in CHO cells affected the phenotype of the cell. We performed a DNA concentration titration to optimize PDI expression and monitor the potential effects on the phenotype. A concentration of a 200 ng/ml of the PDI plasmid was found to be the optimal DNA concentration to give us a good expression with less numbers of cell rounding.

To study the Effect of PDI over-expression on the toxic activity of LF_N-DTA, our first step was to prepare the PA_63, and LF_N-DTA proteins. Successful purifications for both proteins were achieved with a high yield and about 90% purity. LF_N-DTA, a fusion between diphtheria toxin A-chain (DTA) to the C-terminus of LF_N, will bind to PA_63 heptemer and enters the cells by the same pathway as LF/EF. After entry into the cytosol, DTA catalyzes the ADP-ribosylation of elongation factor-2, which inactivates the elongation factor, inhibiting protein synthesis and ultimately causing cell death. Different LF_N-DTA protein molar concentrations (10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M, and 10^{-11} M) in a combination with a constant concentration of (PA_63)^7 were added to CHO cells seeded 24 hours before the assay in the 96-well plate. Cell viability was estimated using MTS assay. We found that adding a mixture of 10^{-6} M LF_N-DTA and 10^{-7} M PA_63 for 4 hours was sufficient to reduce metabolic activity by 40% of the CHO cells. 4 hours exposure is enough to cause this metabolic activity reduction.

3.9 Future directions

Studying the Effect of PDI over-expression on the toxic activity of LF_N-DTA will be the next step of this study. Expression of PDI will be induced for 24 hours in CHO cells, following by incubation with the toxin mixture for 4 hours, and estimation of the cell viability using MTS. A more accurate method for measuring the LF_N –DTA translocation into the cytosol is to measure the [^3]H-leucine incorporation. In this method, after adding the toxin mixture to the
PDI transfected CHO cells, and incubating them for 4 hours in the cells incubator, the medium will be replaced with leucine-free F-12 medium supplemented with $[^3\text{H}]$ leucine and incubated at $37^\circ\text{C}$ for 1 hour. Incorporation of $^3\text{H}$-Leucine into the cells will be used as an indicator of protein synthesis.
Chapter 4: Role of Human gamma interferon-inducible thiol reductase (GILT) in the Redox Regulation of ANTXR2

4.1 Gamma interferon-inducible thiol reductase (GILT)

GILT was first discovered as a novel IFN-γ-inducible protein in the human monocytic cell line U937. Later, its function as a thiol reductase was described by Arunachalam et al, where they used $^{125}$I-F(ab’)² as a substrate. The reduction of F(ab’)² into Fab heavy and light chains was analyzed by non-reducing SDS-PAGE followed by autoradiography. The reduction of F(ab’)² with GILT was maximal between pH 4.0 and pH 5.0, whereas the spontaneous reduction of F(ab’)² by DTT in the absence of GILT, was seen at pH 7.0 (28).

GILT is a unique member of the thioredoxin (thiol reductase) family, because the reduction of disulfide bonds by the mature form of GILT in vitro proved to be optimal at low pH (4.0-5.5) and it is the only thiol reductase known to be active at low pH. The majority of thioredoxin family enzymes catalyze, at a neutral pH, dithiol oxidation, disulfide bond reduction, and disulfide bond isomerization. In addition, it’s catalytic active site CXXC, does not have the common active site motif WCGH/PCK (29,30).

GILT is expressed in antigen-presenting cells. It is synthesized as a 35-kDa soluble glycoprotein. Following transport to MIICs via the mannose-6-phosphate receptor, it is processed to the 30 kDa mature form by proteolytic cleavage of its N- and C-terminal propeptides (27, 28). The mature protein is delivered by M-6-P receptors through the endocytic pathway to late endosomes and lysosomes. At residues 46-49 there is a CXXC motif similar to the WCGH/PCK motif of proteins in the thioredoxin family (31).

It has been shown that GILT is a critical host factor that facilitates *Listeria monocytogenes* infection. *L.monocytogenes* is actively phagocytosed by macrophages,
internalized into the phagosome, and secretes haemolysin listeriolysin O (LLO). Since reduction is required to activate the lytic activity of LLO in vitro, it was found that GILT is responsible for the reduction and activation of LLO which form pores on the endosomal membranes that facilitate bacterial escape from the phagosomes to the cytosol. Mice lacking GILT are resistant to *L. monocytogenes* infection, suggesting that GILT activates LLO within the phagosome by the thiol reductase mechanism (32).

4.2 Objectives

Previous work has shown that the intact disulfides in the Ig-like domain of ANTXR2 are important for the anthrax toxin action. Upon disruption of these disulfide bonds, PA pore formation and translocation of LF, EF are significantly inhibited, suggesting that disulfides in the Ig-like domain of ANTXR may regulate anthrax toxin action. In this project we will investigate the role of the redox regulator factor, GILT, which may affect anthrax toxin-mediated cytotoxicity through regulating the redox states of anthrax toxin receptors.

Gamma Interferon-Inducible Thiol Reductase (GILT) is localized in the endosomes and lysosomes, and shows optimal activity at pH 4.0-5.5, thus it is of interest to know whether or not GILT interact with anthrax receptors in vivo during intoxication. To test the effects of over-expression of GILT on anthrax toxin action through redox regulation of ANTXR2, we will first over-express GILT in COS-1 cells. GILT gene will be cloned into pcDNA3.1(+) to generate C-terminus HA-tagged protein, and then we will transiently transfect COS-1 cells with the generated construct. The expression will be monitored by western blotting using HA antibody, and similarly, the effect of GILT over-expression on anthrax toxin action will be measured with the cytotoxicity assay, in which PA translocates the model substrate, LF_{N}-DTA, into the cytosol, where LF_{N}-DTA inhibits protein synthesis.
4.3 Materials and methods

4.3.1 Construction of HA-tagged GILT

The cDNA GILT sequence encodes the mature form of GILT was cloned into pcDNA3.1(+) with a generation of HA tag at both the C- and N-terminus to monitor the expression by HA antibody.

4.3.2 Cell Culture and Media

(Cos-1 cell line) (ATCC) was grown in DMEM medium supplemented with 10% calf serum, 2 mM L-glutamine, 500 units/ml penicillin, 500 units/ml streptomycin sulfate under a humidified atmosphere with 5% CO2.

4.3.3 Transient transfection:

The day before the assay, COS-1 cells were seeded in a 6-well plate to 90% confluency at the time of transfection. 10μl of Lipofectamine® 2000 Reagent was diluted in 250μl DMEM free serum medium, and combined with GILT plasmids (N-terminus HA-GILT, and C-terminus HA-GILT). 72 hours post transfection; cells were collected using cell scraper, centrifuged at 1200 rpm for 5 min. After determining the concentration of the whole protein, the SDS loading sample buffer was added to the lysate, and Boiled for 10 min. Samples were applied to SDS-PAGE, followed by western blotting using HA antibody (Santa Cruz Biotechnology, INC) to monitor the expression of GILT.

4.3.4 Western blotting:

The proteins were transferred onto PVDF membrane in the transfer buffer (25mM Trizma Base, 192mM Glycine, 3.5mM SDS, 20% Methanol) for 30 min at 100 volts. The membrane was incubated with the primary antibody (Anti-HA antibody, Santa Cruz Biotechnology, INC) in 2% milk solution overnight. After being washed three times with 0.5% Tween20 TBS buffer (20mM Tris, 150mM NaCl, 0.5% Tween,) the membrane was incubated
with secondary antibody, goat anti mouse antibody (Santa Cruz Biotechnology, INC), and then washed five times with TBST buffer. The bands were visualized using supersignal west pico chemiluminescent substrate.

4.4 Results and discussion

Precursor GILT is processed to the 30 kDa mature form by proteolytic cleavage of its N- and C-terminal propeptides, thus, we could not check the expression levels of the mature protein using wild type GILT with a tag to either N- or C- terminus. Therefore, we generated two constructs to express the mature form of GILT with N- and C- terminus HA-tag to monitor the expression using anti HA antibody. The reason we made two constructs with C- and N-terminus tag was because we thought that the tag position may affect the expression and the binding to M-6-P receptors, therefore affecting the localization of GILT.

COS-1 cells were grown in a 6-well plate, and transiently transfected with the GILT constructs using Lipofectamide reagent. 72 hr post-transfection, cells were collected, lysed, and analyzed by SDS-PAGE followed by western blot using HA antibody. As shown in Figure 4.1, and as expected, the mature form of GILT was detected in the cells transfected with N-terminal HA-GILT and C-terminal HA-GILT as well. Also, over-expression of GILT showed no affect on the cells phenotype even after 72 hours of transfection. We concluded that expression of the mature form of GILT is not affected by the tag position (N- or C- terminus).

![Figure 4.1](image.png)

**Figure 4.1:** Over-expression of mature GILT in COS-1 cells.
4.5 Future studies

Gamma interferon Inducible Lysosomal Thiol reductase (GILT) is an enzyme that facilitates protein unfolding, which makes the endocytosed protein accessible to further enzymatic processing by lysosomal/endosomal proteases. In order to study the role of GILT in the redox regulation of anthrax receptor mediated toxin action, the mature form of GILT was successfully over-expressed in COS-1 cell line. Further experiments need to be done to check the localization of GILT. For instance, immunofluorescence using HA antibody to check if GILT is localized and delivered to the endosomes and lysosomes using endosomal and lysosomal protein markers.

The effect of the GILT over-expression on the toxic activity of anthrax toxin will be performed using a combination of the protective antigen and LF_N-DTA. LF_N-DTA is a fusion protein that contains the N-terminus of LF and the catalytic subunit of diphtheria toxin. As LF, LF_N-DTA binds to PA(63)7 heptamers, and it requires acidic pH to translocate from an endosome into the cytosol, where it leads quickly to inhibition of protein synthesis. Inhibition of cellular protein synthesis is thus a straightforward measure of LF_N-DTA entry and translocation.

Previous data generated using GILT-deficient mice strongly suggests that the role of GILT is to reduce disulfide bonds of endocytosed protein, which leads to protein unfolding. Anthrax toxin translocation requires an oxidative environment that favors the receptor disulfide bond formation, GILT may interact with the receptor and reduce receptor disulfide bonds resulting in inhibition of the toxin translocation. This project will provide us with insights into the mechanisms of anthrax pathogenesis and may lead to development of effective drugs against anthrax.
Chapter 5: Cloning, Expression, and Purification of Mycobacterium tuberculosis

5.1 Introduction

*Mycobacterium tuberculosis*, the bacterium that causes tuberculosis, still remains a major global health problem with an estimated 9.4 million new cases and 2-3 million TB related deaths annually. TB is transmitted by particulates containing Mtb, released from the lungs of an infected individual through coughing, sneezing, and spitting (33).

*M. tuberculosis* encodes 23 Esx proteins, EsxA-W, which are generally characterized by their small size (about 100 residues), the presence of a central WXG motif and their organisation in pairs within the genome (34). ESAT-6, a 6 kDa early secreted antigenic target, has received great attention in recent year. ESAT-6 is secreted by a secretion system called the ESAT-6 system-1 (ESX-1) or type VII secretion system in a 1:1 heterodimeric complex with 10 kDa culture filtrate protein (CFP-10) (35). The system is encoded by the region of difference 1 (RD1) of the mycobacterial genome, and is conserved in *M. tuberculosis* and closely related mycobacteria, such as *M. marinum* and *M. bovis*. However, it is absent in the genome of the attenuated vaccine stain *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). ESAT-6 is thought to play a role in the spread of Mtb from one macrophage to another (36).

ESX-1 and ESX-5 are thought to play a role in mycobacterial virulence and have been linked to the bacteria escape from phagosomes, while ESX-3, which encodes the proteins EsxG and EsxH, is required for optimal in vitro growth of *M. tuberculosis* and has been associated with iron and zinc acquisition (34).
EsxG and EsxH have been shown to form a stable 1:1 heterodimeric complex. The high resolution structure of the *M. tuberculosis* EsxG and EsxH complex has been reported (Figure 5.1). The structure reveals some striking differences in surface features, including a potential protein interaction site on the surface of the EsxG•EsxH complex. EsxG•EsxH was also found to contain a specific Zn$^{2+}$ binding site formed from a cluster of histidine residues on EsxH. This site may reflect an essential role in zinc ion acquisition, or point to Zn$^{2+}$-dependent regulation of its interaction with functional partner proteins. The surface features of both the EsxG•EsxH and EsxA•EsxB complexes suggest functions mediated via interactions with one or more target protein partners (34).

![Ribbon representations of the backbone topology of the EsxG•EsxH complex](image)

**Figure 5.1:** Ribbon representations of the backbone topology of the EsxG•EsxH complex, EsxG shown in red and EsxH in blue (34).

It has been shown that the protein products of EsxG/EsxH form a 1:1 tight complexe, which is likely to be the functional form of these proteins (34). In order to study the functional form of EsxG/EsxH proteins (heterocomplex), we need to purify both proteins and mix them to do further studies on the heterodimer. EsxG and EsxH are expressed as inclusion bodies in *E. coli* with a poor expression. Callahan at, el. (2010) utilized a fusion approach to improve the
expression of WXG100 proteins which resulted in high yields of soluble, recombinant protein. This fusion approach should dramatically simplify structural and biochemical studies of related protein pairs from the WXG family (37). Here we used the same approach, in which the EsxG and EsxH genes are fused together into a single open reading frame by a linker, thrombin-cleavage site – 9 amino acids.

5.2 Aims

Aim 1:

Purify recombinant Mycobacterium tuberculosis EsxG and EsxH as a fusion protein expressed in Escherichia coli.

Aim 2:

Investigate the membrane activities of EsxG and EsxH heterocomplex using the K⁺ release assay on liposomes.

5.3. Materials and methods

5.3.1 Construction of pET-22b- EsxG and EsxH

EsxG and EsxH genes were first amplified separately by PCR using the primers listed in Table 1, P1 and P2 to amplify EsxG, P3 and P4 to amplify EsxH. For overlap extension, the PCR products of EsxG and EsxH were mixed and then used as a template for overlap extension using P1 and P2 primers, in which the two open reading frames were joined into a single gene; the DNA sequences of the resulting clones were confirmed. The 27-bp linker used for overlap extension encoded a thrombin-cleavage site – 9 amino acids, GLVPRGSTG. Fusion products were cloned into pET22b in frame with the vector’s C-terminal 6-His tag, as an NdeI/Xhol fragment.
Table 5.1: Primers used for EsxG/H cloning

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
</tr>
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<tbody>
<tr>
<td>TB_GH_P1</td>
<td>5’ atgccatatatgagctttttgatgtca 3’</td>
</tr>
<tr>
<td>TB_GH_P2</td>
<td>5’ gccgtgtgcgcggcgagcaccaggccgaaccggtataggtcgac 3’</td>
</tr>
<tr>
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<td>5’ cgccggcgcagcacgggcatgtcgcaaatcatgtacaact 3’</td>
</tr>
<tr>
<td>TB_GH_P4</td>
<td>5’ catcctcgagcccgccccatgtggccg 3’</td>
</tr>
</tbody>
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EsxG, and EsxH PCR products are about 300 base pair (Figure 5.2 left panel). The overlap extension PCR resulted in about 600 bp band (Figure 5.2 middle panel). Fusion products were subcloned into pET-22b an NdeI/XhoI fragment (Figure 5.2 right panel).

Figure 5.2: Construction of EsxG/EsxH. left panel, electrophoresis result of EsxG and EsxH amplification (from left to right: DNA marker, 1–4: PCR production of EsxG and EsxH, respectively. Middle panel, the electrophoresis result of EsxG and EsxH fusion gene (from left to right: 1: DNA marker, 1, 4: PCR production of EsxG and EsxH). Right panel, enzymatic result of pET-22b - EsxG/H, NdeI and XhoI digestion.
5.3.2 Purification of EsxG and EsxH fusion protein

BL21 (DE3) cells were transformed with pET-22B- EsxG/ EsxH were grown in LB media containing 100 μg/mL carbenicillin. Culture was induced with IPTG (1mM) at OD600 of 0.8 and incubated for 4 hours at 37°C. Cells were harvested by centrifuging at 10,000 rpm for 15 min at 4°C and the pellet was suspended into (20 mM Tris-HCl, 300 mM NaCl, 20 mM Imidazole, pH 8.0 (buffer A), and disrupted with an ultrasonic cell disrupter. Cells were then centrifuged at 19,000 rpm for 40 mins at 4°C. Supernatants were loaded on an immobilized metal affinity column precharged with Ni2+ and equilibrated with buffer A. The purity of eluted protein fractions was analyzed on Coomassie Brilliant Blue stained SDS-PAGE. Purified protein was then quantified using BCA Protein Assay Kit (Thermo Scientific Pierce).

5.3.3 Thrombin digestion

Fusion protein was cleaved site-specifically within the linker by digesting 1 mg of fusion protein with 1 µg of thrombin overnight at room temperature.

5.3.4 K⁺ release assay

20mg of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were resuspended in 1mL of Chloroform and dried in a vaccum overnight. The dried lipid film was rehydrated in a 10mM Hepes, pH 7.4, 150mM KCl and then subjected to six freeze/thaw cycles using ethanol and dry ice. The solution was passed 20 times through a 200-nm pore size polycarbonate filter using a mini extruder. Then the liposomes were desalted using a 5mL HiTrap Desalting Column in the desalting buffer (20mM Tris HCl pH 8.0, 100mM NaCl). EsxG/EsxH heterocomplex, were added to a beaker containing 5 ml reaction buffer (10mM Hepes pH 4.0 and 1M NaAc) and 100 µl of prepared liposomes with constant stirring. Release of K⁺ ions from liposomes was monitored using a potassium selective electrode and recorded using DATAQ software.
5.4. Results and discussion

5.4.1 Expression and purification of EsxG and EsxH fusion protein in E.coli

EsxG and EsxH, both are expressed in Escherichia coli as an insoluble protein. To check the solubility of the fusion protein, a small-scale preparation of E. coli BL21 (DE3) cells expressing the recombinant fusion protein (pET22b-EsxG/EsxH) was grown in 50 ml of LB media and induced with 1mM IPTG for 4 hrs. The bacterial cell pellet was resuspended in 300 µl of lysis buffer. Samples were boiled for 10 min and the recombinant protein was then analyzed by SDS-PAGE. As shown in (Figure 4.2 A) the fusion protein was expressed as a soluble protein. The recombinant fusion protein was purified based on the presence of the 6× His-tag at the C-terminus using Ni-NTA affinity columns. To purify the protein, a large-scale preparation of BL21 (DE3) cells were transformed with pET-22B- EsxG/ EsxH were grown in LB media and induced with 1 mm IPTG. The final bacterial cell pellet was lysed and loaded on the Ni-NTA column. The recombinant fusion protein was eluted and then evaluated by SDS-PAGE (Figure 5.3 B). With the Gene fusion approach we were successful in producing a strong over-expression of the EsxG and EsxH proteins. We got high yields of the soluble, recombinant protein (more than 40 mg protein / 1 liter of cells) with a purity of >90%.
Figure 5.3: Induction and expression of EsxG and EsxH fusion protein. A, Lanes 1-3 are whole cell lysate (WCL), soluble fraction, and insoluble fraction, respectively. Lanes 4-6 are samples which have been induced with IPTG, showing WCL, soluble fraction, and insoluble fraction, respectively. B, purification of EsxG and EsxH fusion protein, lane 1 molecular weight marker, lane 2 WCL after induction, lane 3 flowthrough, lane 4 wash, lanes 5-7 are elutes.

The purified EsxG and EsxH fusion protein were incubated with thrombin (1 unit/mg of protein) at RT for 14 hrs. The extent of digestion was >95%, as assessed by SDS PAGE (figure 5.3).

Figure 5.4: Proteolytic cleavage of purified EsxGH into EsxG and EsxH. Lane 1 shows molecular weight marker, lanes 2-4 are different amount of the digested protein.
5.4.2 Disruption of liposomal membranes at pH 4.0

To study the protein-membrane interaction we used a KCl rich liposomes in which upon liposomal membrane disruption a K⁺ release could be read using a K⁺ ion selective electrode. Different amount of EsxG and EsxH heterocomplex were used (10 µg, 20 µg, 30 µg, 100 µg). The complex appeared to disrupt the liposomal membrane by the detection of the K⁺ (Figure 5.4). Previous works in our lab used the same approach to study the ability of mycobacterium tuberculosis secreted proteins, ESAT 6 and CFP-10 to cause membrane disruption. They found that the ESAT 6 and CFP-10 heterocomplex induced little release of K⁺ from the liposomes, while ESAT 6 alone induced a higher release of K⁺ ions from the liposomes at pH 4.0 (38). Here our results showed that EsxG and EsxH heterocomplex was shown to generate a K⁺ reading similar to that of ESAT-6 and CFP-10 heterodimer at pH 4.0.

![Graph showing K⁺ release over time](image)

**Figure 5.5:** EsxG and EsxH heterocomplex disrupts liposomes by measuring the release of K⁺.
References


Naima Alshrif was born in khoms, Libya. She is the youngest daughter of her parents, Mohamed Alshrif and Hawa Ali. Naima graduated from Alhafer High School, Khoms, Libya, in 1999 and entered Almergib University to pursue a Bachelors of Science in Microbiology. She worked as a teaching assistant at the Department of Microbiology, College of Science, Almergab University during her graduate work until graduating with a MS in Microbiology in the Summer of 2008. In 2010 she joined the University of Texas at El Paso to pursue a Master's Degree in the Biological Science Department. During this time she taught undergraduate lab courses in microbiology. Naima's research focused on Anthrax Toxin under the mentorship of Dr. Jianjun Sun.

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