Life Sciences 109 (2014) 116–126

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Proteome and phosphoproteome analysis of the serine/threonine protein kinase E mutant of *Mycobacterium tuberculosis*

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ARTICLE INFO

Article history:
Received 9 January 2014
Accepted 9 June 2014
Available online 24 June 2014

Keywords:
PknE
Mycobacterium tuberculosis
Proteome
Phosphoproteome
Middlebrook 7H9
Nitrate stress
2D gel electrophoresis
Western blotting
Sigma B

ABSTRACT

Aims: Serine/threonine protein kinases (STPKs) have prominent roles in the survival mechanisms of *Mycobacterium tuberculosis* (*M. tuberculosis*). Previous studies from our laboratory underscored the role of PknE, an STPK in virulence, adaptation and the suppression of host cell apoptosis. In this study, two-dimensional gel electrophoresis was used to study the proteome and phosphoproteome profiles of wild type *M. tuberculosis* and its isogenic *pknE* deletion mutant (Δ*pknE*) during growth in Middlebrook 7H9 and nitric oxide stress.
Main methods: Wild-type *M. tuberculosis* and its isogenic *pknE* deletion mutant strain were grown in Middlebrook 7H9 as well as subjected to nitric oxide stress using sodium nitroprusside. Whole cell lysates were prepared and analyzed by 2D-gel electrophoresis. Phosphoproteomes were analyzed using phospho serine and phospho threonine antibodies after subjecting the 2D-gels to western blotting. Proteins of interest were identified using mass spectrometry.
Key findings: Our analysis provides insights into the targets that impose pro-apoptotic as well as altered cellular phenotypes on Δ*pknE*, revealing novel substrates and functions for PknE.
Significance: For the first time, our proteome and phosphoproteome data decipher the function of PknE in cell division, virulence, dormancy, suppression of sigma factor B and its regulated genes, suppression of two-component systems and in the metabolic activity of *M. tuberculosis*.

Introduction

Disease prevalence of tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) remains high due to the emergence of drug resistant *M. tuberculosis* and HIV coinfection (Ahmad, 2010). The protective immunity of the only available TB vaccine, attenuated *M. bovis* Bacillus Calmette–Gue'rin (BCG) is restricted to TB in children (Dey et al., 2011). *M. tuberculosis* survives inside the hostile macrophage environment by preventing phagolysosome fusion and subsequent acidification of the phagosomes. *M. tuberculosis* resists the antimicrobial nitric oxide (NO) and oxidative stress initiated by the macrophages. Several genes were reported to play in these responses (Voskuil et al., 2011) including *nuoG* and *pknE* (Jayakumar et al., 2008; Velmurugan et al., 2007). Exclusion of iNOS involved in NO production by reducing scaffolding protein EBP50 (Davis et al., 2007; Miller et al., 2004), increasing Arg-1 (El Kasmi et al., 2008) are some of the mechanisms used by *M. tuberculosis* to evade NO stress of the host. Several studies have shown the significance of NO stress in the pathogenesis of *M. tuberculosis* using microarray (Schnappinger et al., 2003; Voskuil et al., 2003) and proteomics (Garbe et al., 1999) approaches. Global pursuits to identify newer drug targets and improved vaccines for TB warrant studies on the molecular pathogenesis and physiology of *M. tuberculosis*. Such studies will identify host biomarkers, bottlenecks in host–pathogen interplay, correlate of risk as well as protection against active disease and determine the responses to TB therapy (Lamichhane, 2011; Russell et al., 2010; Walzl et al., 2011).

Adaptations of *M. tuberculosis* in response to environmental cues are regulated by 11 serine/threonine protein kinases (STPKs) (Molle and Kremer, 2010). STPKs are druggable targets due to their diverse roles that include cell division (PknA, PknB and PknF) and intracellular survival (PknE, PknG, PknH, PknI and PknK) of *M. tuberculosis* (Deol et al., 2005; Gopalaswamy et al., 2008, 2009; Jayakumar et al., 2008; Kang et al., 2005; Kumari et al., 2012; Malhotra et al., 2010; Papavinasasundaram et al., 2005; Walburger et al., 2004).

Functional genomics of *M. tuberculosis* revealed *nuoG*, *SecA2*, *pknE*, Rv3654c, Rv3655c, *Zmp1*, Rv3364, and *eis* genes in the suppression of apoptosis, pyroptosis and autophagy respectively (Danelishvili et al., 2010, 2012; Hinchey et al., 2007; Jayakumar et al., 2008; Kumar and Narayanan, 2012; Master et al., 2008; Miller et al., 2010; Shin et al., 2010).

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http://dx.doi.org/10.1016/j.lfs.2014.06.013
0024-3205/

However, the impact of gene deletion on metabolism or the proteome that imposes altered phenotypic characteristics is not well defined except for *SecA2*, *nuoG*, *phoP* and *pknH* deletion mutants (Braunstein et al., 2003; Chao et al., 2010; Gonzalo-Asensio et al., 2008; Velmurugan et al., 2007). Proteomic studies using the deletion mutants of *pknH* and *phoP* that are involved in the signal transduction of *M. tuberculosis* revealed multiple functions that include dormancy and persistence emphasizing the regulatory role of kinases in the pathogenesis of *M. tuberculosis* (Chao et al., 2010; Gonzalo-Asensio et al., 2008).

Previous studies from our laboratory using the deletion mutant Δ*pknE* (an STPK *pknE* mutant of *M. tuberculosis*) highlighted the role of *pknE* in intracellular survival (Jayakumar et al., 2008), apoptotic suppression (Kumar and Narayanan, 2012), adaptive responses (Kumar et al., 2013) and HIV co-infection (Parandhaman et al., 2014). Deletion of *pknE* resulted in reduced intracellular survival and promoted apoptosis (Jayakumar et al., 2008). Subsequent studies using microarray showed Δ*pknE* to undergo macrophage cell death by genes involved in intrinsic pathway of apoptosis, *TP53* and *Arg2*. This cell death did not involve TNF-α, iNOS, Akt, Arg1, Erk1/2 or pro-inflammatory cytokines (Kumar and Narayanan, 2012). Analysis of in vitro phenotypes showed the mutant to have defective growth in pH7.0 and lysozyme (cell wall damaging agent) with better survival in pH5.5 (acidic stress), SDS (surfactant stress), and kanamycin (second-line anti-tuberculosis drug). Furthermore, the mutant had reduced size during growth in Middlebrook 7H9 and exhibited hypervirulence in a guinea pig model of infection (Kumar et al., 2013). Analysis of the intracellular signaling in macrophages showed Δ*pknE* to be impaired in MAPK signaling evidenced by reduced phosphorylation kinetics of MAPKs (p38MAPK, Erk½ and SAPK/JNK) and their transcription factors ATF-2 and c-JUN. In the presence of MAPK inhibitors Δ*pknE* exhibited crosstalks with Erk½ signaling influenced by SAPK/JNK and p38 pathways independently. Modulations in intra cellular signaling altered the expression of coreceptors CCR5 and CXCR4 in Δ*pknE* infected macrophages (Parandhaman et al., 2014). Collectively these phenotypes suggest a role for *pknE* in immune evasion and intracellular survival for *M. tuberculosis*. In the current investigation, comprehensive proteome and phosphoproteome of wild-type *M. tuberculosis* and its isogenic *pknE* deletion mutant (Δ*pknE*) were analyzed to unravel the substrates that were responsible for the phenotypes, suppression of host cell apoptosis and regulation of physiological responses in *M. tuberculosis*.

Our observations suggest that PknE has multiple roles such as metabolism, dormancy, suppression of sigma factor, suppression of other kinases etc., which could tightly control the regulation of adaption in *M. tuberculosis* to hostile environments.

Materials and methods

Mycobacterial strains, growth and NO stress

Wild-type *M. tuberculosis* H_{37}Rv and isogenic *pknE* deletion mutant Δ*pknE* were grown in complete Middlebrook 7H9 broth with 50 μg/ml of hygromycin when required (Kumar and Narayanan, 2012). NO stress experiments were performed as described previously (Florio et al., 2006). Briefly, cells were treated with 1 mM sodium nitroprusside (SNP) (Sigma, USA) for 24 h and harvested, washed with 0.9% saline and stored at −80 °C until used.

Preparation of mycobacterial cell extract

Mycobacterial cell extracts were prepared as reported earlier (Singhal et al., 2012). Briefly, *M. tuberculosis* cells were suspended in lysis buffer (50 mM Tris–HCl containing 10 mM $MgCl_2$, 0.1% sodium azide, 1 mM PMSF and 1 mM EGTA; pH 7.4) containing protease inhibitor cocktail (Sigma, USA) and sonicated intermittently for 15 min at 4 °C. The lysate was clarified by centrifugation at 12,000 g for 20 min at 4 °C and the supernatant was stored at −80 °C until used.

Protein precipitation

Cell lysates were treated with 1% SDS and then subjected to Trichloro acetic acid (TCA)–acetone precipitation procedure as reported earlier (Bisht et al., 2007). Proteins were precipitated at −20 °C using 10% TCA and collected by centrifugation at 18,000 g at 4 °C for 15 min. Pellets were washed twice using 100% ice cold acetone, air dried and suspended in appropriate volume of 2D-rehydration buffer (BIO-RAD, Hercules, CA, USA). The protein concentration was estimated using Bradford assay as reported earlier (Singhal et al., 2012).

Two-dimensional gel electrophoresis (2D-GE)

Isoelectric focusing (IEF) was carried out using "in-gel rehydration" method. 17 cm immobilized pH gradient (IPG) strips of pH 4–7 (BIO-RAD, Hercules, CA, USA) were rehydrated overnight at 20 °C with 500 μg protein. Strips were then focused on an IEF unit PROTEAN IEF Cell (BIO-RAD, Hercules, CA, USA) at 20 °C using the following four-step program: a) 0–250 V for 2 h in linear mode; b) 250 V constant for 2 h in rapid mode; c) 250–5000 V for 4 h in linear mode; and d) 5000 V constant until 35 kVh was reached. The current limit was set at 50 μA per strip. After IEF, strips were equilibrated for 15 min in equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris; pH 8.8, 20% glycerol) containing 130 mM DTT followed by equilibration buffer II containing 135 mM iodoacetamide instead of DTT for 15 min.

Proteins were separated in second dimension using 12% SDS-polyacrylamide gels (PAGE) in a vertical electrophoretic unit PROTEAN II XI (BIO-RAD, Hercules, CA, USA) at a constant voltage of 250 V for 5–6 h and gels were stained with Coomassie brilliant blue (CBB) R250 to visualize proteins.

Phosphoproteomics

Isoelectric focusing (IEF) was carried out using "in gel rehydration" method using 7 cm IPG strips of pH 4–7 (BIO-RAD,Hercules, CA, USA). After second dimension electrophoresis, separated proteins were blotted on PVDF membrane and blocked using 5% skim milk in Tris buffered saline (TBS) containing 0.1% Tween 20 (0.1% TBST) for 1 h at room temperature. After three washes in TBST, the membrane was incubated overnight at 4 °C with anti-phosphoserine mouse mAb (16B4) (Calbiochem,USA) and anti-phosphothreonine mouse mAb (14B3) (Calbiochem,USA) at 1:5000 dilution. After three washes in TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated sheep anti-mouse antibody (1: 10,000 in TBST) (Amersham Biosciences, USA). After three washes in TBST, bound antibodies were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) by exposure to Eastman Kodak x-ray film.

Spot selection

2D gel electrophoresis for comprehensive proteomic and phosphoproteomic analysis was performed in triplicates to overcome the technical variations. Gel images were acquired and analyzed by Chemidoc (BIO-RAD, Segrate [Milan], Italy) using Quantity One software (BIO-RAD). Differentially expressed proteins were shortlisted by Student *t*-test using PDQuest software (Bio-Rad, Hercules, CA, USA). Besides this, the spots of interest were visually confirmed by three independent readers before subjecting to mass spectroscopic analysis.

In-gel digestion with trypsin

Protein spots of interest were excised from 2D gels using spot picker 'Investigator ProPic' (Genomic Solutions, Huntingdon, UK), collected in 96 well PCR plate and processed as described earlier (Singhal et al.,