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A novel immunogenic spore coat-associated protein in *Bacillus anthracis*: Characterization via proteomics approaches and a vector-based vaccine system

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Abstract

New generation anthrax vaccines have been actively explored with the aim of enhancing efficacies and decreasing undesirable side effects that could be caused by licensed vaccines. Targeting novel antigens and/or eliminating the requirements for multiple needle injections and adjuvants are major objectives in the development of new anthrax vaccines. Using proteomics approaches, we identified a spore coat-associated protein (SCAP) in *Bacillus anthracis*. An *Escherichia coli* vector-based vaccine system was used to determine the immunogenicity of SCAP. Mice generated detectable SCAP antibodies three weeks after intranasal immunization with an intact particle of ultraviolet (UV)-irradiated *E. coli* vector overproducing SCAP. The production of SCAP antibodies was detected via western blotting and SCAP-spotted antigen-arrays. The adjuvant effect of a UV-irradiated *E. coli* vector eliminates the necessity of boosting and the use of other immunomodulators which will foster the screening and manufacturing of new generation anthrax vaccines. More importantly, the immunogenic SCAP may potentially be a new candidate for the development of anthrax vaccines.
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Keywords: Immunogenic; Spore coat; *Bacillus anthracis*; Proteomics; Vector; Vaccine

Recent terrorist attacks have involved the use of *Bacillus anthracis* spores as weapons. The development of vaccines for anthrax prevention has been extensively explored. The US anthrax vaccine which was licensed in 1970 is made from the filtrate of a non-encapsulated attenuated strain [1]. However, this regimen has unknown efficacy and can trigger local pain and edema as well as other undesirable side effects [2]. Contemporary anti-anthrax remedies focus mainly on three-component toxins: protective antigen (PA), lethal factor (LF), and edema factor (EF) which are massively produced by vegetative cells during the late stages of *Bacillus anthracis* infection [3,4]. Although targeting PA has been shown to have varying degrees of success against *Bacillus anthracis* [5], the degree of protection provided by PA targeting methods against inhalational anthrax remains unknown. The recombinant PA products are currently undergoing human clinical trials, but they would still require multiple, needle-based dosing, and the inclusion of the adjuvant aluminum [6]. Thus next generation anthrax vaccines targeting new antigens may be needed to enhance efficacies and eliminate side effects.

Using proteomics approaches, we identified a spore coat-associated protein (SCAP) in the dormant spores of *Bacillus anthracis* Sterne strain. To know if the SCAP is an immunogenic protein, we utilized a vector-based vaccine system to immunize mice. SCAP was expressed in an

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Escherichia coli vector. After irradiation with UV, an intact particle of irradiated *E. coli* vector overproducing SCAP was intranasally administered to the mice for immunization. Without adding extra adjuvants and boosting, immunized mice produced detectable antibodies against SCAP after a three-week administration, suggesting that SCAP is an immunogenic protein and the irradiated *E. coli* vector exhibits an adjuvant effect. Thus, SCAP can potentially be a new candidate for development of next generation anthrax vaccines. In addition, the irradiated *E. coli* vector overproducing an exogenous immunogen may facilitate the screening of new anthrax vaccine candidates and scale-up the production of next generation vaccines that can be manufactured rapidly and administered non-invasively in a wide variety of disease settings.

Materials and methods

Two-dimensional gel electrophoresis (2-DE)

Dormant spores of *Bacillus anthracis* Sterne strain were lyzed in lysis buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, followed by adding dithiothreitol (DTT) to 0.1% and tricholoroacetic acid (TCA) to 10%]. TCA-precipitated proteins were separated by 2-DE via an IPGphor (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) according to protocols in our laboratory [7]. The 11 cm linear gradient Immobiline Dry-Strips (pH4-7) were used. Proteins in Dry-Strips were subsequently separated by 12.5% polyacrylamide gels in a Hoefer SE600 system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Gels were visualized via silver nitrate staining [8]. In-gel digestion with trypsin and protein identification via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) analysis were performed essentially as described previously [9,10].

MALDI-TOF MS

Peptides in the tryptic digests were eluted from ZipTips with 75% acetonitrile/0.1% trifluoroacetic acid and air-dried. Peptide fragments were then mixed with a matrix solution containing α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile/0.1% trifluoroacetic acid and analyzed with a PerSeptive Voyager-DE MALDI-TOF MS (PerSeptive Biosystems, Framingham, MA) [9]. Peptides were laser-evaporated at 337 nm, and each spectrum was the cumuative average of 50–100 laser pulses. All peptides were measured as mono-isotopic masses, and a trypsin autolytic peak at 2164.04 m/z was chosen for internal calibration. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. This procedure resulted in mass accuracies of 100 ppm. Peptide mass spectra above 5% of full scale were analyzed, interpreted, and matched to SWISS-PROT database using Mascot, a searching algorithm available at the Matrix Science Homepage, http://www.matrixscience.com. Matches were computed using a probability-based Mowse score defined as $-10\times \log p$, where p is the probability that the observed match was a random event [9]. Mowse scores greater than 70 were considered significant ($p \leqslant 0.05$).

Q-TOF MS/MS sequencing and database searching

ZipTips-eluted samples were introduced into a nano reverse-phase column (75 μm × 15 cm, with Jupitor 4 μm Proteo bead packed in our laboratory), and gradient eluted into a Q-TOF 2 quadrupole time-of-flight tandem mass spectrometer (Waters, Milford, MA) through an electrospray interface for tandem mass spectral analyses. Liquid chromatography was conducted using a LC Packings Ultimate LC, Switchos microcolumn switching unit, and Famos autosampler (LC Packings, San Francisco, CA). Spectral analyses were performed in automatic switching mode whereby multiply-charged ions were subjected to MS/MS if their intensities rose above 6 counts. The MassLynx 3.5 software (Waters, Milford, MA) [10] was utilized for instrument operation, data acquisition, and analysis. The search for amino acid sequence similarity was performed using BLAST and/or Scanps available from ExPASy internet server at http://www.expasy.ch.

Plasmid construction and recombinant SCAP expression and purification

SCAP was amplified via polymerase chain reaction (PCR) by using Platinum Pfx polymerase (Invitrogen, Carlsbad, CA) with primers 5′-AGTCTGAAAAAG AAATTAGGT ATGG and 5′-GGAGCTCGAGTTATTTTCTTCT CCAGCTTCTTGG. The PCR product was digested with XhoI and inserted into pIVEX-MBP vector (Roche, Nutley, NJ) through StuI and XhoI in the multiple cloning sites. This insertion creates an in frame fusion of 6× His and maltose binding protein (MBP) tags followed by factor Xa protease cleavage sequence at the N terminus of the cloned SCAP. The insert was verified by DNA sequence analysis (data not shown). In order to achieve high levels of protein expression and tight regulation in *E. coli*, the 6× His-MBP-SCAP DNA fragment was cleaved and ligated into pET15b vector (EMD Biosciences, Inc., San Diego CA) through XbaI and XhoI sites and transformed into BL21(DE3) (EMD Biosciences, Inc., San Diego, CA). Thus, the *E. coli* vector-based vaccine (*E. coli BL21 (DE3) T7/lacO SCAP*) was constructed in the pET15b vector which contains a *T7/LacO* promoter to control protein expression. Protein expression was induced by 1 mM of isopropyl b-D-thiogalactopyranoside (IPTG) when the bacteria culture was in logarithmic growth phase (OD_{595} at 0.4–0.5). After 3 h IPTG induction at 37 °C, the cells were sonicated and lysed with 50 mg/ml lysozyme and 0.5% Triton-X 100 in phosphate buffer saline (PBS) for 30 min. The fusion protein (approximately 1 mg)