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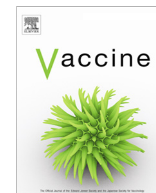

Development of a novel dual-domain nanoparticle antigen construct for universal influenza vaccine

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ABSTRACT

A highly effective antigen construct for presenting conserved antigen domains is essential to the development of a universal influenza vaccine. We have developed a novel dual-domain nanoparticle fusion protein (DDNFP) which allows independent presentation of two conserved domains. The conserved domains used were from two separate viral surface proteins, M2e of M2 and fusion peptide (FP) or long alpha helix (CD) of HA2. The carrier is a novel nanoparticle protein – the dodecameric DNA binding protein from starved cells (Dps) of bacteria or archaea. Dps was found to be uniquely capable of simultaneous fusion and surface presentation at both N- and C-termini while retaining the ability to form nanoparticles. Thus, DDNFPs with M2e and FP or CD fused at N- and C-termini of Dps from *E. coli* (EcDps) or other bacteria were first constructed based on the H1 subtype sequences along with corresponding single-domain nanoparticle fusion proteins (SDNFPs). They were expressed at high levels in bacteria and found to form nanoparticles of the expected size (~9 nm). They were stable against treatment at high temperatures. The DDNFPs (M2e-EcDps-FP and M2e-EcDps-CD) induced strong antibody responses against individual antigen domains and provided full protection against lethal challenge with PR8 virus (H1N1). Importantly, the protection by DDNFPs was synergistically enhanced as compared to SDNFPs. The M2e-EcDps-CD provided an even stronger protection than M2e-EcDps-FP and therefore appeared to be the superior construct. Together, with novel domain combination, enhanced protection and ease of production, this M2e/CD DDNFP could potentially be a highly effective antigen construct for the universal influenza vaccine.

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1. Introduction

Influenza viruses undergo constant genetic and antigenic changes. There are 18 influenza A subtypes which fall into five clades within two broad phylogenetic groups [1]. Current trivalent and quadrivalent inactivated vaccines (TIV and QIV; H1, H3, and one or two B strains) for seasonal influenza are strain-specific and not suited for controlling pandemic or variant epidemic viruses. Thus, a universal influenza vaccine which is effective against divergent influenza viruses is greatly needed to provide better control of seasonal epidemics and an effective counter measure against pandemics. The basic strategy for developing such a vaccine is to target the conserved domains so that immune responses generated against them can be cross-protective against divergent influenza viruses [2,3]. The conserved domains such as M2e and those from HA2 have been major targets for universal influenza vaccine candidates [1,3]. In addition, the HA stem region recognized by broadly neutralizing antibodies has also been targeted with headless HA or whole stem constructs [4–6].

M2e is the 24-aa extracellular domain of the minor envelope protein M2 which is highly conserved among all influenza A viruses, especially within the first nine amino acids [7,8]. M2 has a low copy number per virion, but is present abundantly on infected cells [9]. It is one of the most widely studied targets for the universal influenza vaccine and has been shown to provide strong protection through antibody-dependent cell-mediated cytotoxicity (ADCC) [1,8]. The HA2 makes up the bulk of HA stem and consists of distinct domains including FP, A, B, C, D, E, F, G and H [10]. It is highly conserved within each or closely related subtypes, but varies between subtypes from different phylogenetic groups [11,12]. FP consists of the first 38 aa of HA2 and is conserved among both influenza A and B viruses with substitution found only at two positions (2 and 12) among its first 14 amino acids (GLFGAIAGFIEGGW) [7,13]. The CD domain, also referred as the long alpha helix, consists of aa 76–130 of HA2 which forms the

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central part of the HA stem. Both have been shown to induce the protective immune responses [14–17].

Various approaches have been used to target these conserved domains [3,18]. Different carriers including nanoparticles or virus-like particles have been evaluated, including Hbc [19], papaya mosaic virus [20], influenza virus NP protein [21], tuftsin [22], flagellin [23], bacterial phage [24], adenovirus vector [25], and influenza virus-like particle [26]. However, these efforts have been primarily based on the single-domain approach.

We selected the DNA binding protein from starved cells (Dps) as the carrier protein to generate an antigen construct which can combine two separate domains for enhanced protection. Dps is a nanoparticle protein (~9 nm) present exclusively in bacteria or archaea [27,28]. Each Dps particle consists of 12 identical subunits (~20 kDa each). Through evaluation of various influenza virus domains and Dps from different bacteria, we found that Dps is uniquely capable of simultaneous and separate fusion and surface presentation of antigen domains at both N- and C-termini. The resulting DDNFP incorporating M2e and a HA2 domain (FP or CD) were successfully expressed at high levels in *E. coli*. Importantly, the DDNFP generated balanced immune responses against individual domains and provided synergistically enhanced protection against the lethal challenge. It could therefore be a highly effective antigen construct for development of the universal influenza vaccine.

2. Materials and methods

2.1. Cloning, expression, and purification of Dps fusion proteins

The M2e, FP and CD domains were fused individually or in combination to N- and/or C-termini of Dps as SDNFP or DDNFP (Fig. 1). The amino acid sequences of the domains (M2e, FP, and CD) used are listed in Supplementary Table 1. The M2e is the consensus sequence of human influenza A viruses. FP and CD sequences were the consensus sequences of the H1 subtype. Two Dps carriers from *E. coli* (EcDps) and the hyperthermopile *S. solfataricus* (SsDps) were evaluated (Supplementary Table 2). The fusion protein genes were generated by DNA synthesis (DNA 2.0, Menlo Park, CA and Genscript, Piscataway, NJ) or PCR using appropriate primers for linking the domain to either N- or C-terminus of the Dps carrier. Proteins were expressed at either 37 °C or room temperature (RT) in *E. coli* (BL21) using pET 11 or pJexpress (DNA 2.0, Menlo Park, CA) vector following induction with 1 mM IPTG. Bacterial cells were lysed by sonication in phosphate buffered saline (PBS). Purification was performed by ion exchange (Q-Sepharose) followed by the size exclusion chromatography (SEC, Sephacryl S300). Purified fusion proteins reached a purity of at least 90% by SDS-PAGE and densitometry analysis of the fusion protein and any host cell protein bands. Protein concentrations were determined by BCA assay.

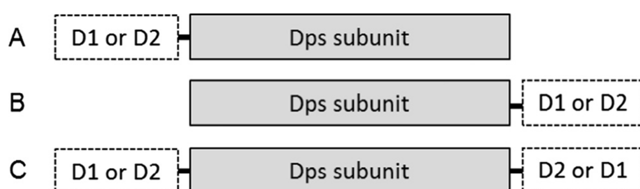

Fig. 1. A schematic representation of Dps fusion proteins with influenza virus antigen domains. A and B, single-domain fusion proteins; C, dual-domain fusion protein. D1 and D2 represents two different domains which can be M2e, FP or CD.

2.2. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed using the sodium phosphotungstate negative stain to examine the morphology of Dps and SDNFP and DDNFP at the Imaging Center of College of Veterinary Medicine and Biomedical Sciences, Texas A&M University.

2.3. Animals and challenge experiments

All animal studies were conducted with the approval of the Institutional Animal Use and Care Committee at Texas A&M University. The 6–8 week old Balb/c mice were obtained from Envigo (formerly Harlan Laboratories Inc). Groups of mice (n = 5) were immunized by intramuscular injection with fusion proteins at 10 μg/mouse in combination with Sigma adjuvant system (SAS) (Sigma Chemical Co., St. Louis, IL; formerly Ribi adjuvant) in 50 μl, twice four weeks apart. The SAS, a squalene oil-in-water emulsion containing MPL (Monophosphoryl lipid A) and trehalose dicorynomycolate, was used by mixing with antigen at 1:1 ratio per the instruction provided by the manufacturer. It was more effective than Alhydrogel (Invivogen, San Diego, CA; 0.5% (w/v) or 250 μg/mouse) for enhancing antibody responses as shown in a comparison experiment with a DDNFP (M2e/FP) (Supplementary Fig. 3). Serum samples were collected every two weeks by submandibular bleeding technique till the end of the experiment. Challenge was performed intranasally with the PR8 virus (A/Puerto Rico/8/34, H1N1) at a lethal dose of 5 or 10 LD_{50} in 30 μl. The PR8 virus was propagated in MDCK cells and diluted to appropriate dose (LD50) with PBS. Mice were briefly anesthetized with isoflurane for nasal administration.

2.4. ELISA and neutralization test (NT)

ELISA was performed with 96-well plates (Maxisorp, Nunc). The plates were coated with synthetic peptides (2 μg/ml), inactivated whole virus (5 μg/ml, A/New Caledonia/20/1999, H1N1), or recombinant HA (1 μg/ml, A/New Caledonia/20/1999, H1N1; BEI Resources) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. The inactivated whole virus antigen was prepared by purification from infected MDCK cells and inactivation with formaldehyde [29,30]. The synthetic peptides were M2e (24 aa), FP (38 aa), and C (29 aa) made by Genscript (Piscataway Township, NJ) or Peptide 2.0 (Chantilly, VA). The C peptide was used to measure the responses against CD. PBS-T buffer (20 mM phosphate, 150 mM NaCl, pH 7.4; 0.025% Tween 20) containing 3% BSA was used for blocking and sample dilution. The plates were blocked at RT for 2 h. Serum samples were serially 2-fold diluted and incubated at RT for 2 h. After washing, plates were incubated with anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co, St. Luis) at RT for 1 h, which was followed by washing and incubation with PNPP substrate (Thermo Scientific Pierce) for 30 min. The OD was measured at 405 nm. The antibody titer was determined as the highest dilution with an OD value 2-fold above the background.

NT was performed with MDCK cells in 96-well plates as described in the WHO manual on animal influenza diagnosis and surveillance [31]. Briefly, pooled serum samples from each group were inactivated at 56 °C for 30 min and serially diluted by 2-fold before mixing with 100 $TCID_{50}$ of PR8 virus in duplicate. After incubation at 37 °C for 1 h, the mixtures were transferred to MDCK cells and incubated at 37 °C for 1 h. The plates were then washed twice with PBS after removing the mixtures, and fresh serum free media containing 2 μg/ml trypsin was added. The plates were incubated at 37 °C for 72 h and then fixed in formalin and stained by crystal violet. The neutralization titer is the highest serum dilution with the intact cell monolayer.

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