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A highly immunogenic fragment derived from Zaire Ebola virus glycoprotein elicits effective neutralizing antibody

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ABSTRACT

In order to produce polyvalent vaccines based on single rVSV vector, we investigated the immunogenicity, antibody neutralizing activity, and antigenic determinant domain of Zaire Ebola's fragment MFL (aa 393–556) that contains furin site and internal fusion loop. Both the recombinant protein and the recombinant plasmid of fragment MFL elicited high levels of antibody, similar to those of Zaire Ebola GP (ZGP). The MFL fragment of ZGP also elicited high levels of neutralizing antibody and induced moderate cellular immune response in mice, as revealed by the proliferation and cytokine secretion of splenocytes. Through the analysis of the induction of neutralizing antibody by pVAX1-based recombinant plasmids that expressed truncated fragments of MFL, we found that the domain containing the internal fusion loop and the furin site was the major contributor of fragment MFL's immunogenicity. Furthermore, the rVSV-based bivalent vaccine expressing Sudan Ebola GP (SGP) and MFL fragment elicited efficient cross-immunity against ZGP and SGP with high levels of neutralizing antibody. Our results indicate that fragment MFL is an effective and novel antigen for the production of neutralizing antibody and polyvalent vaccines of Ebola virus.

1. Introduction

Ebola is an enveloped, non-segmented, negative-sense RNA virus that belongs to the family Filoviridae; it causes severe hemorrhagic fever in humans and nonhuman primates with a high fatality of 90% (Beer et al., 1999; Feldmann and Geisbert, 2011). The Ebola epidemic occurs primarily in central and western Africa and Philippines (Peterson et al., 2004). A total of five Ebola virus subtypes have been discovered, which are Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SUDV), Taï Forest ebolavirus (TAFV), Reston ebolavirus (RESTV), and Bundibugyo ebolavirus (BDBV) (Kuhn et al., 2010, 2011). Among these subtypes, Zaire ebolavirus and Sudan ebolavirus are the most toxic subtypes with high pathogenicity and infectiousness. Currently, no effective treatments and licensed vaccines are available for EBOV infection.

As the sole membrane protein encoded by the Ebola virus, the glycoprotein (GP) plays a critical role in the entry and pathogenicity of Ebola virus (Elliott et al., 1985; Gene et al., 2009). Viral vector-based Ebola vaccines expressing GP, such as recombinant adenovirus vector vaccine (rAD5-GP) and recombinant vesicular stomatitis virus vector vaccine (rVSV-GP), have demonstrated effective protection against the lethal challenge of Ebola virus in nonhuman primates (Feldmann et al., 2007; Hensley et al., 2010; Jones et al., 2005; Richardson et al., 2009; Geisbert et al., 2009). Although GP may cause endothelial cell damage and abnormal cellular adhesion and immune surveillance (Chan et al., 2000; Francica et al., 2009; Simmons et al., 2002; Sullivan et al., 2005; Yang et al., 2000), the safety of rVSV-based Ebola vaccines has been demonstrated in NHP (Geisbert et al., 2008; Mire et al., 2012).

However, it is difficult to prepare polyvalent vaccines that express several glycoproteins using one single rVSV vector (Haglund et al., 2000). In order to prepare bivalent VSV vaccines, we aimed to find a fragment from Zaire ebolavirus glycoprotein (designated as ZGP) that could replace GP to produce effective vaccines. Some epitopes in the linkage region (aa 393–556) of GP1 and GP2 have been reported to induce neutralizing antibodies (Wilson et al., 2000; Takada et al., 2003; Dowling et al., 2007; Lee et al., 2008a, 2008b; Qiu et al., 2011), and this linkage region also contains furin site and internal fusion loop that both carry important functions (Lee and Saphire, 2009). Therefore, we speculated that this fragment, designated as fragment MFL, may induce strong immune response and neutralizing antibodies, which as a result effectively inhibit Ebola infection. Using indirect ELISA and neutralization assay, we found that fragment MFL effectively induced humoral immune response and neutralizing antibody as ZGP did.

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Similar levels of neutralizing antibody induced by pVAX1-based recombinant plasmids that express the MFL-truncated fragments indicated that the internal fusion loop and the sequence around furin site were the main contributors of the immunogenicity of fragment MFL. In addition, the rVSV-based bivalent vaccine that expresses Sudan Ebola-GP (SGP) and MFL fragment elicited efficient cross-immunity against ZGP and SGP with high levels of neutralizing antibody.

2. Materials and methods

2.1. Expression and purification of recombinant ZGP protein and MFL-fragment

The ZGP gene was synthesized and cloned into pCAGGS vector by GENEray Biotechnology (Shanghai, China). The coding regions of ZGP and MFL were amplified using Prime Star DNA polymerase (TAKARA, Japan) with the recombinant plasmid as the template. The PCR products were inserted into the cloning sites of pET24a(+) vector (Novagen, USA), and a His-tag was added at the C-terminus of the target protein. All expression plasmids were verified by sequencing analysis.

The expression and purification of recombinant proteins were performed as previously described with some modifications (Zhang et al., 2012; Berlec et al., 2013). Overnight culture of *E. coli* Rosetta(DE3) transformed with pET24a(+)-ZGP or pET24a(+)-MFL was diluted at 1:100 in 2 L fresh LB medium with 50 μg/mL kanamycin, and 1 mM IPTG was added after OD600 reached 0.6 at 37 °C. After additional culture for 5–6 h at 30 °C, the bacteria were centrifuged at 6000 × *g* for 5 min. The pellets were resuspended in 150 mL NTA-0 buffer (20 mM Tris–Cl, 500 mM NaCl, 10% glycerol, pH7.9) and lysed with 30 min sonication. The cell lysate was centrifuged at 11,000 × *g* for 25 min. For the recombinant fragment MFL, the supernatants were purified by using Ni^{2+}-NTA agarose (Institute of Process Engineering, Chinese Academy Of Sciences) with imidazole elution (NTA-500 buffer, 20 mM Tris–Cl, 500 mM NaCl, 10% glycerol, 500 mM imidazole, pH7.9). For the recombinant ZGP, the inclusion bodies were dissolved in UNTA-0 buffer (20 mM Tris–Cl, 500 mM NaCl, 10% glycerol, 8 M urea, pH7.9) and purified by using Ni^{2+}-NTA agarose with imidazole elution (UNTA-500 buffer, 20 mM Tris–Cl, 500 mM NaCl, 10% glycerol, 8 M urea, 500 mM imidazole, pH 7.9). The soluble ZGP was obtained by desalination using a 50 kDa ultrafiltration tube (Millipore, USA). Finally, the purified proteins were checked by SDS-PAGE and Western blot, and the concentrations of purified proteins were determined by using BCA™ protein assay kit (Thermo Scientific, USA).

2.2. Preparation and verification of recombinant plasmids

The coding regions of ZGP and its fragments (MFL, MFLA, MFLM and MFLB) were amplified from pCAGGS-ZGP (Fig. 1). The PCR fragments were inserted into the cloning sites of pVAX1 vector (Invitrogen, USA), and Flag-tag was added at the C-terminus of the targets. These recombinant plasmids were individually verified by sequencing analysis and designated as pVAX1-ZGP, pVAX1-MFL, pVAX1-MFLA, pVAX1-MFLM, and pVAX1-MFLB, respectively.

The plasmids were purified by using Endofree maxi plasmid kit (Tiangen Biotech, China) and dissolved in saline at 1 mg/mL. HEK-293FT cells were cultured to 90% confluence in 6-well plates at 37 °C with 5% CO_2. pVAX1-ZGP, pVAX1-MFL, pVAX1-MFLA, pVAX1-MFLM, pVAX1-MFLB, or pVAX1 was transformed into 293FT cells by using Lipofectamine2000 following the manufacturer's instructions (Invitrogen, USA). After 48 h, cells were lysed by RIPA lysis buffer, and supernatants were detected by Western blot analysis.

2.3. Production of rVSVΔG-based vaccines

The plasmid rVSVΔG-GFP was digested to produce the rVSVΔG vector fragment for the expression of the antigen gene. The coding regions of ZGP and MFL were amplified from pCAGGS-ZGP, and the coding region of SGP was amplified from pDC316-SGP. Then the gene fragments of ZGP and SGP were inserted into the rVSVΔG vector to construct recombinant plasmids, rVSVΔG-ZGP and rVSVΔG-SGP, respectively. The gene fragments SGP and MFL were linked by 2A-peptide self-cleavage sequence (Carey et al., 2009; Tang et al., 2009) to construct a recombinant plasmid, rVSVΔG-SGP-2A-MFL.

The recovery of rVSVΔG-based vaccines was carried out as previously described with some modifications (Whitt, 2010; Garbutt et al., 2004). Briefly, BHK21 cells were grown to 90% confluence in 6-well plates. Then the cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3, ATCC) at an MOI = 5. One hour later, the vTF7-3 inoculum was removed and the cells were transfected with the support plasmids expressing viral ribonucleoprotein constituents (3 μg of pBS-VSV N, 5 μg of pBS-VSV P, and 1 μg of pBS-VSV L) and the recombinant plasmid (5 μg) described above using Lipofectamine2000 (Invitrogen, USA) following the manufacturer's instructions. After 48 h at 37 °C, the supernatants were filtered using a 0.22 μm syringe filter (PALL, USA) and transferred to fresh BHK21 cells (90% confluent) to confirm the recovery of infectious vaccines by scanning BHK21 monolayers for cytopathic effect. Rescued recombinant vaccines were passaged on BHK21 cells to obtain a vaccine stock. The vaccine stock was titrated on BHK21 cells using $TCID_{50}$.

The control virus rVSVΔG-GFP were produced and titrated as previously described (Whitt, 2010).

2.4. Immunization of mice

All animal experiments were handled in accordance with the guidelines of the Beijing Institutes for Biological Science Animal Research Advisory Committee and conformed to the European Community Directives for the care and use of laboratory animals. One hundred 6–8 weeks old female BALB/c mice were randomly divided into 10 groups (10 mice/group) and vaccinated (vaccination regimen shown in Fig. 2). Forty-eight hours after i.m. injection of 0.25% procaine hydrochloride, 100 μL 1 mg/mL pVAX1, pVAX1-ZGP, pVAX1-MFL, pVAX1-MFLA, pVAX1-MFLM, or pVAX1-MFLB was injected into mice (i.m.), respectively. Three immunizations were performed for the same plasmid with 2-week intervals (days 0, 14, 28). For protein immunization, saline, ZGP, and fragment MFL were injected subcutaneously at 35 μg/mouse on days 0 and 14. The first dose contained 50% Freund's complete adjuvant (Sigma, USA), and the second dose contained Freund's incomplete adjuvant (Sigma, USA). For the immunization of rVSVΔG-based vaccines, 1×10^4 PFU of rVSVΔG-SGP-2A-MFL, rVSVΔG-ZGP, rVSVΔG-SGP, rVSVΔG-ZGP + rVSVΔG-SGP, or rVSVΔG-GFP was injected into mice (i.p.).

2.5. Analysis of antibody response and IgG2a/IgG1 ratio

For ZGP or SGP specific antibody test, ELISA 96-well plates (Nunc, USA) were coated with purified ZGP or SGP (0.5 μg/well) in carbonate–bicarbonate buffer (CBB) (pH 9.6) and incubated overnight at 4 °C. The plates were washed 4 times with PBST buffer (0.05% Tween 20 in PBS, pH 7.4) and blocked with 100 μL 5% non-fat milk at 37 °C for 2 h. Serial dilutions of serum were added for 1 h incubation at 37 °C. After washing 4 times with PBST, the HRP-conjugated goat anti-mouse IgG antibody (CWBIO, China) (1:10,000 diluted in 1% BSA in PBST) was added for 1 h incubation at 37 °C. Plates were then washed 5 times in PBST, and the reaction was