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Ebola virus glycoprotein Fc fusion protein confers protection against lethal challenge in vaccinated mice

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ABSTRACT

Ebola virus is a *Filoviridae* that causes hemorrhagic fever in humans and induces high morbidity and mortality rates. Filoviruses are classified as "Category A bioterrorism agents", and currently there are no licensed therapeutics or vaccines to treat and prevent infection. The Filovirus glycoprotein (GP) is sufficient to protect individuals against infection, and several vaccines based on GP are under development including recombinant adenovirus, parainfluenza virus, Venezuelan equine encephalitis virus, vesicular stomatitis virus (VSV) and virus-like particles. Here we describe the development of a GP Fc fusion protein as a vaccine candidate. We expressed the extracellular domain of the Zaire Ebola virus (ZEBOV) GP fused to the Fc fragment of human IgG1 (ZEBOVGP-Fc) in mammalian cells and showed that GP undergoes the complex furin cleavage and processing observed in the native membrane-bound GP. Mice immunized with ZEBOVGP-Fc developed T-cell immunity against ZEBOV GP and neutralizing antibodies against replication-competent VSV-G deleted recombinant VSV containing ZEBOV GP. The ZEBOVGP-Fc vaccinated mice were protected against challenge with a lethal dose of ZEBOV. These results show that vaccination with the ZEBOVGP-Fc fusion protein alone without the need of a viral vector or assembly into virus-like particles is sufficient to induce protective immunity against ZEBOV in mice. Our data suggested that Filovirus GP Fc fusion proteins could be developed as a simple, safe, efficacious, and cost effective vaccine against Filovirus infection for human use.

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1. Introduction

Ebola virus (EBOV) and Marburgvirus (MARV) are members of the *Filoviridae*, a family of viruses classified as "Category A" bioterrorism agents that cause severe hemorrhagic fever in humans and nonhuman primates with high morbidity and mortality rates up to 90% [1]. After a short incubation period of 4–10 days, Filovirus-infected individuals develop an abrupt onset of symptoms that include fever, chills, malaise, and myalgia that are common to many other viral infections. MARV is antigenically stable and exists in only one species, whereas EBOV is more variable and has five species. The Bundibugyo EBOV emerged recently in a late 2007 outbreak in Uganda [2], and is more related to the Ivory Coast than to the Zaire (ZEBOV), Sudan, or Reston EBOV species. ZEBOV is typically associated with the highest lethality. The increased number of outbreaks in Africa and the recent EBOV outbreak in pigs [3], which raised concerns that livestock could transmit the deadly disease to humans, highlighted the urgency for the development of vaccines and rapid diagnostic tests to contain outbreaks. Vaccines based on the Filovirus glycoprotein (GP) are in preclinical and clinical evaluation, and currently there are no licensed therapeutic agents to treat Filovirus infection. Since licensing of safe and effective Filovirus vaccine could take several more years, diagnosis and quarantine of infected individuals is currently the main tool to limit outbreaks.

Filovirus particles contain a negative-strand RNA genome of about 19 kb that encodes seven genes [4]. The envelope glycoprotein (GP) encoded in gene 4 is present as spikes on the virion surface and is responsible for receptor binding, viral entry, and immunity [5,6]. The Filovirus GP is a class 1 integral membrane glycoprotein derived from gene 4 that undergoes a complex processing involving furin cleavage and disulfide-bond formation between the N-terminus and the membrane proximal portion of the GP. The mature transmembrane GP present on the viral envelope and membrane of infected cells is formed by two subunits: the membrane anchored GP2 that is covalently linked via disulphide linkage to the N-terminus of GP1, which contains a highly O-glycosylated mucin-like domain [7,8]. A significant amount of GP1 is shed from the cells after release from the GP2 subunit. In addition, a nonstructural

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soluble glycoprotein (sGP) that shares the amino-terminal 295 amino acids with GP1, lacks a transmembrane anchor, and forms disulphide-linked homodimers, is produced by EBOV but not MARV infected cells [9].

Several Filovirus vaccine candidates containing GP are currently being developed based on recombinant adenovirus [10–12], parainfluenza virus [13], Venezuelan equine encephalitis virus [14], replication-competent [15,16] and -deficient [17] vesicular stomatitis virus, and virus-like particles [18,19]. Initial studies using baculovirus-expressed Filovirus GP induced partial protection, which could be attributed to the glycosylation and processing of GP in insect cells [20]. In this work, we analyzed the immunogenicity of a recombinant protein consisting of the extracellular domain of ZEBOV GP fused to the Fc fragment of human IgG1 (ZEBOVGP-Fc) expressed in mammalian cells. We hypothesized that the Fc tag in the ZEBOVGP-Fc would simplify purification of the fusion protein through protein A columns using mild conditions, increase protein stability of the fusion protein, and confer an adjuvant effect in non-human primates (NHP) and humans due to the interactions with Fcγ receptors on antigen presenting cells [21–23]. Here, we show that ZEBOVGP-Fc expressed in mammalian cells undergoes the complex posttranslational modification of the native GP, including the furin cleavage and homotrimer formation. Vaccination with the EBOVGP-Fc protected mice against challenge with a lethal dose of ZEBOV. Our results clearly indicate that the ZEBOVGP-Fc alone and without the need of a viral vector or assembly into virus-like particles is sufficient for inducing protection against ZEBOV infection and suggest that Filovirus GP Fc fusion proteins could be developed into a cost-effective safe and effective subunit vaccine against Filovirus infection.

2. Materials and methods

2.1. Cells lines

Chinese hamster ovary (CHO) cells deficient in the enzyme dihydrofolate reductase (dhfr$^-$) were obtained from the American Type Culture Collection and expanded in growth medium consisting of Iscove's medium containing 10% fetal bovine serum (FBS) [24]. Human embryonic kidney HEK293-H cells (Invitrogen) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. Vero E6 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% FBS. Baby hamster kidney cells (BHK-21) were maintained in DMEM medium supplemented with 5% FBS. The BSR-T7 cells, which are BHK-21 cells that express bacteriophage T7 RNA-polymerase [25], was kindly provided by Dr. K. Conzelmann (Pettenkoffer Institute, Munich, Germany) and maintained in DMEM medium supplemented with 5% FBS and 1 mg/ml geneticin (Invitrogen).

2.2. Mice

C57BL/6 and BALB/c mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). All mice were housed in micro-isolator cages and provided standard rodent feed and water ad libitum. Blood samples were obtained from the lateral tail vein. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where these researches were conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal protocols were approved by CBER-FDA or USAMRIID Institutional Animal Care and Use Committees (IACUC).

2.3. Cloning procedures

The cDNA of the Zaire Ebola virus (ZEBOV) glycoprotein (GP), Mayinga strain (GenBank accession no. AF272001), in pVR-1012-ZEBOV-GP was kindly provided by Dr. Gary Nabel, Vaccine Research Center, NIH, Bethesda, MD [26]. The glycoprotein gene has eight adenosine (A) residues at the RNA editing site needed to produce the full-length ZEBOV GP. The following plasmids were constructed using standard technique of genetic engineering:

2.3.1. pEF1-EBOV-GP

The GP region was excised from pVR-1012-ZEBOV-GP using NcoI and Asp718 (Roche Applied Science) restriction enzymes, filled in with DNA polymerase Klenow (New England Biolabs) enzyme to create blunt ends and subsequently cloned into the EcoRV site of the mammalian expression plasmid pEF1/Myc-His-B (Invitrogen). The resulting plasmid was termed pEF1-EBOV-GP.

2.3.2. pEF-ZEBOVGP-Fc

To construct a plasmid for the expression of an ZEBOV GP Fc fusion protein, a PCR fragment coding for amino acids 1–637 (GenBank accession no. U23187) of the ZEBOV GP ectodomain was amplified from pVR-1012-ZEBOV-GP using synthetic oligonucleotides GP/SalI (5′-GTCGACAGTATGGGCGTTACAGGAATATTGCAGTTA-3′), which contains SalI site before the sequence coding for the signal peptide GP, and GP/Flag/SpeI (5′-ACTAGTACTCACCTCCCTTGTCATC GTCGTCCTTGTAGTCTCCACCGCCGTCCGGAAGGGTTTTATCAACAAA-3′), which contains an SpeI site followed by an artificial splicing donor site, the coding sequence for the FLAG tag peptide DTKD-DDDK, and nucleotides 1887–1911 of GP. This PCR fragment was cloned into the SalI and SpeI sites of pEF-ICAM5(1–2) Fc replacing the ICAM 1 cDNA fragment and in-frame with the Fc fragment of human IgG1 [24]. Silent mutations (GTCGAC to GTAGAC, and CTAGTT to CTCGTT) were introduced into the GP coding sequence to eliminate internal SalI and SpeI restriction sites. The resulting plasmid was termed pEF-ZEBOVGP-Fc.

2.3.3. pEF-FLAG-Fc

To construct a plasmid for the expression of the Fc fragment of human IgG1, we replaced the ICAM 1 sequence in pEF-ICAM5(1-2)Fc with a cDNA fragment coding for the signal peptide of the ZEBOV GP and a FLAG tag. To do so, we amplified a PCR fragment coding for amino acids 1–32 of the ZEBOV GP signal peptide using pVR-1012-ZEBOV-GP as a template and synthetic oligonucleotides GP/SalI (see above) and SP/Flag/SpeI (5′-ACTAGTACTCACCTCCCTTGTCATCGTCGTCCTTGTAGTCTCCACCGCC-GGAAAATGTTCTTTGGAAAAGGAT-3′), which codes for a FLAG tag, nucleotides 73–96 of the GP cDNA, and an SpeI restriction site. The amplified PCR fragment was digested with SalI and SpeI restriction enzymes and cloned into pEF-ICAM5(1-2)Fc digested with the same enzymes. The resulting plasmid was termed pEF-FLAG-Fc.

2.3.4. pVSV-EBOVGP

To construct replication-competent VSV-G deleted recombinant Vesicular Stomatitis Virus (VSV) expressing the ZEBOV GP (rVSV-ZEBOVGP), a PCR fragment coding for GP of ZEBOV (amino acids 1–676) amplified from pVR-1012-ZEBOV-GP using oligonucleotides GP/NheI(+) (5′-ACTAGTAGTATGGGCGTTACAGGAATATTGCAGTTA-3′), which is identical to GP/SalI except for the SalI site that was substituted for an NheI restriction, and antisense primer GP/NheI (−) (5′-GCTAGCCTAAAAGACAAATTTGCATATACAGAA-3′), was cut with