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Development and characterization of rabbit and mouse antibodies against ebolavirus envelope glycoproteins

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Ebolaviruses are the etiologic agents of severe viral hemorrhagic fevers in primates, including humans, and could be misused for the development of biological weapons. The ability to rapidly detect and differentiate these viruses is therefore crucial. Antibodies that can detect reliably the ebolavirus surface envelope glycoprotein $GP_{1,2}$ or a truncated variant that is secreted from infected cells (sGP) are required for advanced development of diagnostic assays such as sandwich ELISAs or Western blots (WB). We used a $GP_{1,2}$ peptide conserved among Bundibugyo, Ebola, Reston, Sudan, and Taï Forest viruses and a mucin-like domain-deleted Sudan virus $GP_{1,2}$ (SudanGPΔMuc) to immunize mice or rabbits, and developed a panel of antibodies that either cross-react or are virus-specific. These antibodies detected full-length $GP_{1,2}$ and sGP in different assays such as ELISA, FACS, or WB. In addition, some of the antibodies were shown to have potential clinical relevance, as they detected ebolavirus-infected cells by immunofluorescence assay and gave a specific increase in signal by sandwich ELISA against sera from mouse-adapted Ebola virus-infected mice over uninfected mouse sera. Rabbit anti-SudanGPΔMuc polyclonal antibody neutralized gammaretroviral particles pseudotyped with Sudan virus $GP_{1,2}$, but not particles pseudotyped with other ebolavirus $GP_{1,2}$. Together, our results suggest that this panel of antibodies may prove useful for both *in vitro* analyses of ebolavirus $GP_{1,2}$, as well as analysis of clinically relevant samples.

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1. Introduction

The family *Filoviridae* currently includes the two established genera, *Ebolavirus* and *Marburgvirus*, and one tentative genus, *Cuevavirus*. The *Ebolavirus* genus consists of five species, all of which have one virus member: *Bundibugyo ebolavirus* (Bundibugyo virus, BDBV), *Reston ebolavirus* (Reston virus, RESTV), *Sudan ebolavirus* (Sudan virus, SUDV), *Taï Forest ebolavirus* (Taï Forest virus, TAFV), and *Zaire ebolavirus* (Ebola virus, EBOV). The genus *Marburgvirus* contains one species (*Marburg marburgvirus*) with two member viruses, Marburg virus (MARV) and Ravn virus (RAVV) (Kuhn et al., 2010). With the exception of RESTV, ebolaviruses have been associated with naturally occurring outbreaks of severe viral hemorrhagic fever in humans. EBOV causes the most severe disease outbreaks with lethality reaching up to 90% (Feldmann et al., 2003; Towner et al., 2008).

Ebolaviruses are enveloped viruses and cell entry is mediated by the envelope glycoprotein ($GP_{1,2}$). Definitive identification of the ebolavirus receptor remains elusive, although several surface molecules have been shown to enhance infection (Dolnik et al., 2008). The full-length $GP_{1,2}$ on the surface of ebolavirions is translated from an mRNA that is the product of cotranscriptional editing and contains an additional non-template adenosine residue within a stretch of seven consecutive adenosine residues near the center of the coding region for the protein (Sanchez et al., 1996; Volchkov et al., 1995). Post-translational cleavage by a cellular protease yields the mature virion-associated form comprised of two protein subunits, the virion surface protein, GP_1, and the transmembrane protein, GP_2, linked together through a disulfide bond ($GP_{1,2}$). GP_1 contains the receptor binding site (RBS) (Kuhn et al., 2006) and a highly *N*- and *O*-glycosylated and diverse region at the C-terminus called the mucin-like domain. GP_2 is anchored on

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the virus envelope and cell surface through the transmembrane domain (TMD). The extracellular domain (ECD) of GP_2 contains the functional domains necessary for inducing the fusion of viral and cellular membranes upon GP_1 binding to the ebolavirus receptor. Translation from the unedited GP gene (~80% of the time) gives rise to a much shorter soluble GP (sGP). The N-terminal 295 amino acid residues of sGP are identical to those of GP_1. The C-terminus of sGP is unique and also highly diverse between sGP proteins from different ebolaviruses. One potential role of sGP is to serve as a decoy for ebolavirus-neutralizing antibodies (Feldmann et al., 1999; Volchkov, 1999; Volchkova et al., 1998).

There are no FDA-licensed therapies or vaccines for filoviruses. Neutralizing antibodies reacting with $GP_{1,2}$ may provide passive immunity (Gupta et al., 2001; Jahrling et al., 1996; Parren et al., 2002; Takada et al., 2007). $GP_{1,2}$-specific antibodies are also very useful reagents for research. For example, Lee et al. used an EBOV-neutralizing antibody, KZ52, to form a complex with ebolavirus $GP_{1,2}$ and elucidated the crystal structure of $GP_{1,2}$ (Lee et al., 2008). In addition, ebolavirus-specific antibodies are critical tools for detection and diagnosis of ebolavirus infection in the field and in the laboratory. Most anti-ebolavirus $GP_{1,2}$ monoclonal antibodies reported to date are ebolavirus-specific, and many target the highly variable mucin-like domain, presumably because it contains immuno-dominant epitopes. In addition, most of the reported monoclonal antibodies are of mouse origin only (Shahhosseini et al., 2007; Wilson et al., 2000), which provides less flexibility in the development of some assays, for example, sandwich ELISAs.

This report describes the development and characterization of a new panel of ebolavirus-specific antibodies. Some of these antibodies are broadly cross-reactive to $GP_{1,2}$ of different ebolaviruses. In addition, both mouse and rabbit monoclonal antibodies were generated that might allow for the development of complementary strategies in the establishment of ebolavirus-detection assays. Previous findings that the phenylalanine residue at position 88 (F88) of EBOV GP_1 plays an important role in viral entry, is conserved among all currently known ebola- and marburgviruses, and region flanking F88 are highly conserved among different ebolaviruses (Mpanju et al., 2006) suggested that antibodies raised against this highly conserved region would be broadly cross-reactive. A 38-mer peptide was synthesized corresponding to this region, designated as F88 peptide, and was used to develop rabbit polyclonal antibodies and mouse monoclonal antibodies. Additionally, SUDV $GP_{1,2}$ with a deletion of 190 amino acid residues comprising the mucin-like domain (referred to as GP-S) was also used to develop rabbit polyclonal and monoclonal antibodies. Since most type-specific neutralizing antibodies reported to date recognize the highly glycosylated mucin-like domain that is also the most variable region of $GP_{1,2}$, it is proposed that the use of mucin-like-domain deleted $GP_{1,2}$ would induce more broadly reactive antibodies. The mouse and rabbit polyclonal and monoclonal antibodies generated in this work will contribute to the development of detection assays for all the known ebolavirus species.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney (HEK) 293T (obtained from T. Dull, Cell Genesys, CA, now BioSante Pharmaceutical, Lincolnshire, IL), grivet kidney Vero E6 (ATCC, # CRL-1586, Manassas, VA) and grivet COS-7 cells (ATCC, #CRL-1651, Manassas, VA) were cultured at 37 °C in 5% CO_2 and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Lonza, Walkersville, MD). Culture conditions used for mouse and rabbit hybridoma cells are described below.

2.2. Plasmids

Expression plasmids encoding the $GP_{1,2}$ of EBOV (Mayinga variant), SUDV (Gulu variant), and TAFV (Côte d'Ivoire variant), pVR1012-ZaireGP, pVR1012-SudanGP and pVR1012-TaiForestGP, respectively, were provided by Dr. Gary Nabel (Vaccine Research Center, NIH, Bethesda, MD) and Dr. Anthony Sanchez (CDC, Atlanta, GA) (Yang et al., 1998). Each of the plasmids had been mutated at the *GP* gene editing site, resulting in predominant expression of full-length $GP_{1,2}$ rather than sGP. Plasmid pGP-S, which encodes SUDV (Gulu variant) $GP_{1,2}$ amino acid residues (aa) 1–315, aa 506–650, a short linker (GG) and a His6 tag (Fig. 2C), was obtained by over-lapping PCR using pVR1012-SudanGP as template. Primers used to amplify the region that encodes aa 1–315 were designated GP-S-#1 (5′-GATCTCGAGCTCGCCACCATGGAGGGTCTTAGCCTACTCC-3′) and GP-S-#2 (5′-ACCCGTGGCCCTCTCGTTGAGCGATAAAGTTTCGAA-3′). Primers used to amplify the region that encodes aa 506–650 were designated GP-S-#3 (5′0TCGCTCAACGAGAGGGCCACGGGTAAATGCAATCCC-3′) and GP-S-#4 (5′-CGGGCCCGCGGTTAGTGATGGTGATGGTGATGGCCACCCTGTCTCCAGCCCGTCCACCAATTATC-3′). The coding sequence for the His6 tag (underlined) is contained within primer GP-S-#4. After gel purification, these two DNA fragments were linked by overlapping PCR using primers GP-S-#1 and GP-S-#4 and then ligated into pIRES2-EGFP (Clontech, Mountain View, CA) that had been restriction digested with *Nhe* I and *Sac* II (New England BioLabs, Ipswich, MA). The sequence of the resulting plasmid pGP-S was confirmed using the ABI Prism 3100 Sequence Detection System using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). pBundiGP encodes the full-length BDBV (Bundibugyo variant) $GP_{1,2}$. The coding sequence was designed according to the deposited sequence information (GenBank Accession No. FJ217161), codon-optimized for optimal expression in mammalian cells, synthesized, and inserted into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) by a commercial gene-synthesizing company (DNA2.0, Menlo Park, CA, USA). pReston-sGP encodes the wild-type unedited gene for RESTV (Pennsylvania variant) $GP_{1,2}$ and expresses predominantly sGP (a gift from Michael Farzan, Harvard Medical School, Boston, MA, USA). Plasmid pMARV-Mus $GP_{1,2}$, encodes Marburg virus (MARV) $GP_{1,2}$ and a C9 epitope tag at its C-terminus, was described previously (Kuhn et al., 2006).

2.3. Peptide and protein immunogens used for antibody development

F88 peptide (Fig. 1B) includes 38 amino acid residues of EBOV $GP_{1,2}$, a short linker peptide of serine–glycine–serine residues (SGS) and biotin at the N-terminus. It was synthesized and purified to ≥95% homogeneity at PolyPeptide Laboratories (San Diego, CA). To increase its immunogenicity, F88 peptide was conjugated to Keyhole Limpet Hemacyanin (KLH) carrier protein using the Imject Maleimide Activated mcKLH kit from Pierce (Rockford, IL) and inoculated at Spring Valley Laboratories, Inc. (Woodline, MD). The chimeric fusion protein GP-S includes SUDV $GP_{1,2}$ aa 1–315, aa 506–650, a short linker (GG) and a His6 tag. The mucin-like domain, cleavage site between GP_1 and GP_2, transmembrane domain, and cytoplasmic tail of SUDV $GP_{1,2}$ were deleted in GP-S (Fig. 2C). The fusion protein was expressed and purified at Chesapeake PERL (Savage, MD) using the PERLXpress System, a baculovirus based system to express proteins in cabbage looper (*Trichoplusia ni*) larvae (Kovaleva et al., 2009). The purity of GP-S was